ASBMR 2014 Annual Meeting

Meet-the-Professor

Handout Booklet

September 12 – 15, 2014

George R. Brown Convention Center

Houston, Texas, USA

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Epigentic Regulators

Jane Lian, Ph.D. and

Jonathan Gordon, Ph.D.

2014 ASBMR Meet-the-Professor Session: Epigenetic Regulators

Friday, September 12, 10:00am to 11:00am

Presented by Jonathan Gordon[1] and Jane Lian[1]
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Significance

Most skeletal elements in the body are derived from mesoderm cells that undergo a programmed commitment, first to mesenchymal progenitors and ultimately chondrocytes and osteoblasts that will define mature bone. This developmental process is defined by a coordinated gene expression in a spatial-temporal and lineage specific manner. Among the predominating genes required for normal bone formation, turnover are transcripts encoding growth factors, hormones, extracellular signaling mediators and transcriptional regulators. Ultimately, minor alterations in gene expression in response to physiological and environmental signals can translate to significant changes in heritable gene expression patterns.

Epigenetic control refers to mechanisms that alter gene expression in a heritable manner that is distinct from the underlying hard-coded, genetic information defined by DNA sequence. This epigenetic regulation can involve several different mechanisms including biochemical modifications of DNA structure, reversible post-translational modification of histone proteins, modification of nuclear architecture and post-transcriptional modulation of RNA transcript levels. Recent advances have characterized these processes has helped define distinct roles for epigenetic modulators in normal biology and more importantly, disease states.

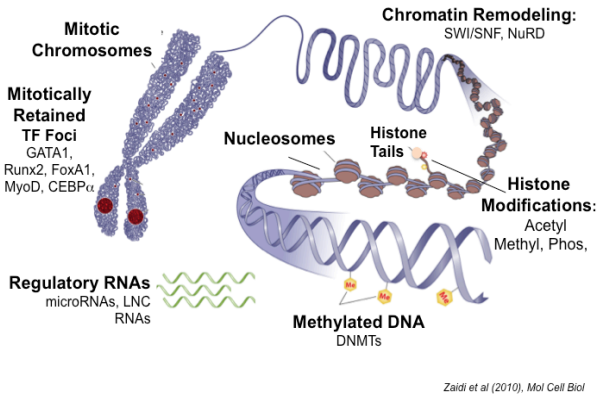

Figure 1: Multiple Levels of Epigenetic Control

In the nearly 5 decades since the discovery of the DNA code, transcriptional control by transmissible DNA regulatory elements in gene promoters was established as the fundamental determinate of gene regulation and tissue-specific commitment and progression of cells to a differentiated phenotype. This dogma of gene regulation changed with the characterization of several reversible, post-translation modifications of chromatin that were able to orchestrate heritable changes in gene expression provided a new level of understanding for regulation of gene expression that continues to expand. The variation in gene regulation, referred to "trait-associated DNA" is important to understand biological variation not transmitted through DNA sequence (Figure 1). Currently, epigenetic pathways are being recognized for their associations with diseases, including diabetes (1), osteoporosis (2) and osteoarthritis (3) in addition to variations induced by drugs, pharmaceuticals, diet, aging, environmental factors and chemicals. In this seminar we will address several topics dealing with the biological relevance, analysis and clinical and therapeutic concerns relating to these epigenetic mechanisms.

Learning Objectives

As a result of participating in this session, attendees should be able to gain a level of knowledge in:

- The different levels of epigenetic control regulating gene expression.
- The basic strategies for discovery of epigenetic regulators (e.g. miRNAs, lncRNAs, histone modifcations/modifying–enzymes and classical transcriptional mediators) in bone-related models.
- Identify the challenges in using epigenetic regulators as biomarkers or direct targets for therapeutic intervention in bone pathologies.

Modes of Epigenetic Control

Non-coding RNAs

There are several categories of non-protein encoding RNAs that serve various purposes to control gene transcription and/or translation.

microRNAs (miRNAs) and small interfering RNAs (siRNAs, shRNAs):

miRNAs are small (~22 nt) polynucleotides that can bind to the UTR's of targeted mRNAs resulting in decreased protein translation. Precursor miRNAs are short single-stranded RNAs that can fold and form secondary structures which are recognized and processed by enzymes of the Drosha/Dicer family. miRNAs regulate many biological processes and pathways because a single miRNA sequence may target several different genes. Analogous to the endogeneous miRNA, the synthetic research tool, small-interferring- (siRNA) or small hairpin- (shRNA) can function to repress protein translation or mRNA levels. In the case of shRNA, precursor shRNAs are processed by Dicer from long double stranded RNAs very similar to the processing of endogenous miRNAs.

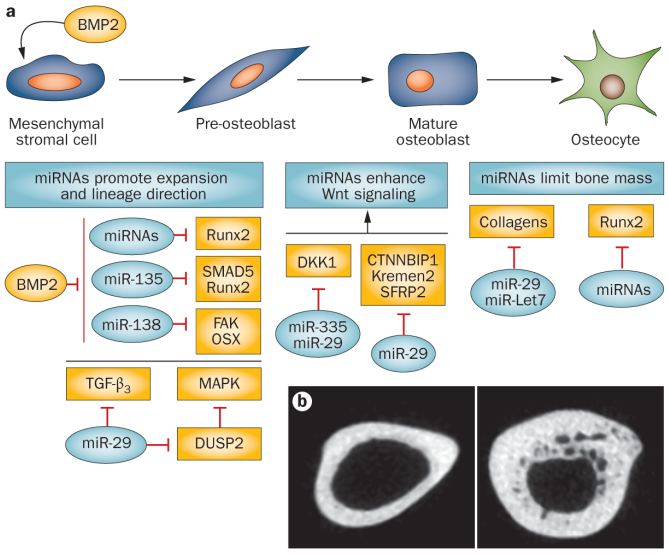

Figure 2: Effect of microRNAs (miRNAs) on osteoblast differentiation. a) Selected miRNAs in relation to their targets influencing each stage to regulate progression of differentiation are indicated. b) The consequence of *Dicer* deletion in osteoblasts and osteocytes (left panel; Dicer-C/C) driven by osteocalcin (OC-Cre) (right panel; $Dicer^{\Delta OC/\Delta OC}$).

Several hundred papers have been published on miRNAs regulation of MSCs, osteoblasts, osteoclasts as well as many excellent reviews (4–6). Our understanding of miRNA function during osteogenesis has been greatly expanded and some relevant highlights include:

- Conditional ablation of the Dicer gene in osteoblasts demonstrated that miRNA processing is necessary for normal skeletal development and limiting bone mass in the adult skeleton (7) (Figure 2)
- During osteogenesis, the same miRNA can target different genes dependent on the stage of osteoblasts differentiation.
- miRNA suppression of lineage–specific transcription factors is a mechanism by which cells can maintain multi- or pluripotency.
- miRNAs may serve complex functions during lineage determination. For example: Runx2 is downregulated by more than 15 miRNAs (when considering conserved and non-conserved miRNAs in mice, rat and human) targeting Runx2. These Runx2-targeting miRNAs are selectively and highly expressed in
- BMP2 induces the osteogenic phenotype in part by downregulating multiple miRNAs that inhibit a large majority of regulatory factors needed for commitment and differentiation of osteoblasts.
- Within bone tissue, miRNAs can be secreted from one cells via exosomes, circulate and affect other cells. Direct miRNA effects from one cell to another can occur through Gap junctions to affect neighboring cells.

Long non-coding RNAs:

Currently it is known that a large number of lncRNA transcripts exist in mammalian transcriptomes. Increased resolution from transcriptome sequencing has allowed for their detection, however the function and activity of lncRNAs are not clear. One emerging function is their regulating of miRNAs (8) Although several lncRNAs have been implicated with specific diseases, these lncRNAs are at their infancy in the scope of osteogenesis.

DNA Methylation

The methylation of cytosine bases in DNA is usually a repressive modification that results in repressed gene expression. Large stretches of cytosine and guanine dinucleotides (CpG islands) around genes and gene promoters are primary targets for the DNA methyltransferases (DNMTs). Many studies have examined the role of DNA methylation in directly regulating bone-related genes (e.g osteocalcin) (9, 10). Recent studies have demonstrated DNA methylation linked to osteoporosis and osteoarthritis (11), methylation levels predicting vitamin D responsiveness (12) and the effects of dexamethasone on promoter methylation favoring adipogenesis over osteoblastogenesis.

Histone Post-translational Modification

The four, core histone proteins that comprise the nucleosome (in addition to coiled DNA, Fig 1) can be post-translationally modified through enzyme activity. These modifications include acetylation, methylation, phosphorylation and several other chemical modifications that may result in altered gene expression. These modifications can influence the binding of chromo- and bromo- domain containing proteins and/or affect nucleosome positioning and stability. In addition, specific modifications on histones may regulate displacement of nucleosomes through activity of the SWI/SNF nucleosome remodeling complex inducing structural changes that promote gene transcription(8).

Several studies have highlighted the importance of lysine acetylation (e.g. H3K9Ac, H3K27ac) and specifically the actions of the lysine deacetylases (HDACs) in regulating osteogenesis (13–15). Regulation of methylation on lysine residues on histones (specifically H3K9me3 and H3K27me3) by specific KDMs have also been demonstrated to be important in MSC commitment to osteoblasts (16). In addition several other histone modifying enzymes including WDR5 (17), NO66 (18) and others (19) have been demonstrated to play a role in osteogenesis and/or bone formation.

Mitotic Bookmarking.

Phenotype stability is maintained during cell division through a process known as “mitotic bookmarking”. During mitosis the majority of phenotypic genes are not transcribed; rather genes are poised for transcription immediately after completion of cell division. This suggests that the cell has a “memory” of the chromatin state prior to cell division (hence “bookmarked”). This assures that genes required to maintain a lineage specific phenotype or induce a differentiation program are rapidly transcribed in the post-mitotic cells. In the case of Runx2 expressing cells, Runx2 remains bound to genes on acrocentric chromosomes and ribosomal genes in the nucleolus during mitosis. Apparently, only the prototypical tissue–specific transcription factors have this bookmarking ability. For example, C/EBPα was observed to be associated with mitotic chromosomes pre-adipocyte stability during proliferation and not PPARγ (which follows C/EBPα expression). Several reviews have described and expanded the concept of epigenetic bookmarking (20, 21).

Functional Analysis of Epigenetic Mechanisms in Bone

MicroRNA profiling

Like other forms of RNA analysis, sample processing and RNA extraction methods can have a substantial impact on the results of miRNA profiling. It is possible to extract high-quality miRNA from a wide range of sources, including cell lines, fresh and formalin-fixed tissues, plasma, serum and other body fluids but each has special concerns and considerations (22, 23). After isolation it is important to characterize RNA quality using a capillary electrophoresis-based technique (e.g. Bioanalyzer) before embarking on expensive and time-consuming analysis.

As mature miRNA transcripts are very small in size (~22nt) and relatively low abundance compared to mRNA transcript (~0.01% of cellular RNA) it is difficult to quantify miRNA by conventional RT-qPCR. Many commercial kits are now available that increase both ease and sensitivity when analyzing miRNAs. However, analysis of the miRNA transcriptome is frequently done by array-based or next-generation sequencing (NGS) technologies.

For data analysis, several platforms can be used to evaluate microarray or NGS data and many free or low cost versions are available (e.g. R-based Bioconductor packages (24) and the web-based Galaxy

(25)). After establishing relative or differential expression specific miRNAs can be interrogated for functionality using a variety of databases including miRBASE (26), Targetscan (27) and Ingenuity (IPA) (28).

Chromatin modification profiling

Analysis of chromatin modifications (i.e. histone post-translational modifications) is performed by chromatin immunoprecipitation (ChIP). As with miRNA analysis, ChIP can be done on a wide range of tissues and depending on the proficiency of the individual and quality of reagents, can recover interpretable results from as low as 100-1000 cells (29). Isolated DNA can be analyzed on a gene-by-gene basis using conventional qPCR, however this is very time consuming and analysis by genome-scale NGS is more efficient.

Data analysis for NGS involves alignment of reads to a reference genome (i.e. human or mouse) and evaluating areas of enrichment that correlate to the genomic location of a modified histone. Sequences are aligned using alignment tools BWA or Bowtie, available through Galaxy (30). Sites of histone modification or transcription factor enrichment are identified using model-based peak callers MACS, SPP or similar programs (31, 32). After enrichment profiles are generated, tracks can be displayed on UCSC genome browser or IGV to interrogate regions of enrichment on a gene-by-gene basis (33) (Figure 3). After initial alignment and enrichment profiling, further in-depth analysis may include genome-wide segmentation analysis (34), interrogation of specific genomic regions (using tools such as NGS plots (35), Figure 3) or combined gene-based pathway analysis.

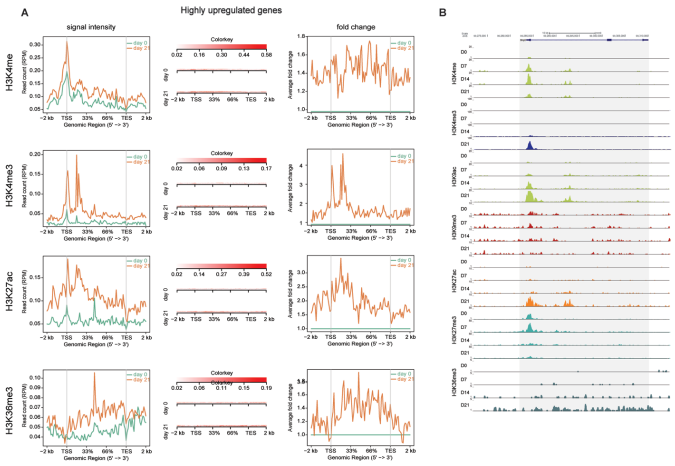

Figure 3: Analysis of histone marks in genes upregulated during osteogenesis. A) NGS plot generated signal intensities at upregulated genes during MSC differentiation. B) UCSC view of histone marks at the Bmp3 gene

Technical Challenges and the Management of Big Datasets

To effectively evaluate epigenetic profiles of tissues or cell lines using NGS strategies, many components must be assessed and careful thought must be given to the experimental design. A frequently underestimated parameter that should be addressed before undertaking a NGS based strategy is the pipeline in your institution for building libraries, sequencing and most importantly, bioinformatic analyses of data sets. Effective communication with a bioinformatician is a crucial step to answer biological questions using complex datasets.

Clinical significance

The levels of epigenetic control illustrated in figure 1 have far reaching effects and exhibit significant cross-regulation. However this complexity is a reason for consideration of intervention in pathological disorders by targeting epigenetic pathways. Using epigenetic profiling, expression of bone or cartilage genes that are deregulated in disease states, could be reversed by targeting the enzyme class that generates the histone modifications. Identifying an epigenetic fingerprint of multiple histone marks for expression of specific genes has implications for tissue regeneration. For cancer and skeletal diseases, drugs that target DNA and chromatin modifying enzymes are currently in clinical use or clinical trials and include:

- DNMT inhibtors: 5-azacitidine and Decitabine
- HDAC inhibitors: Vorinostat/SAHA and Romidepsin
- HMT and HDM inhibitors: CPI-169, GSK343 (EZH2) and SGC0946 (DOT1L)

MicroRNAs that circulate and reflect the activities of skeletal lineage cells are being explored as biomarkers of disease progression for osteoporosis, osteoarthritis and other aging disorders. Although the study groups are

a small size, the most recent of these studies highlights the potential of miRNAs as biomarkers (36, 37) or patient population-specific signatures (38). A few *in vivo* miRNA studies have been published demonstrating the effectiveness of a single miRNA for protection against or intervention of OA, osteoporosis in either transgenic mouse models or by systemic delivery. A signature of miRNAs was identified in osteosarcoma tumors, a pressing clinical situation due to the poor survival outcome of children. These miRNAs, could predict contributing factors as metastasis, recurrence, resistance to chemotherapy and hence be useful in monitoring course of the disease at the onset of detection or be of therapeutic value for miRNA intervention (39).

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Exome Sequencing and How to Identify a Disease Gene

Ingrid Holm, M.D., MPH and
Catherine Brownstein, Ph.D., MPH

EXOME SEQUENCING AND HOW TO IDENTIFY A DISEASE GENE
ASBMR Meet the Professor Session
Friday September 12, 2014

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Boston Children's Hospital
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Significance of the Topic

For genetic bone conditions, like XLH and tumoral calcinosis, next generation sequencing (NGS) has resulted in the discovery of new causative genes and pathways. Here, we go through three case studies that resulted in gene discovery, and illustrate the tools and techniques necessary to make them happen.

Learning Objectives

As a result of participating in this session, attendees should be able to:

1. Appreciate the opportunities and complexities of using NGS for gene discovery.
2. Have a better understanding of tools to aid in gene discovery.
3. Understand techniques to differentiate causative mutations from non-causative rare variants.

Points of Interest/Clinical Pearls

1. Don't overlook the gifts – what is obvious?
2. Finding new genes: What is related? What are the great candidates? What genetic changes are predicted to be damaging?

Background

<u>Human genomic variation</u>

- ~3 billion bases / human genome
- Any two human individuals are remarkably similar, and share 99.9% of their DNA
- The remaining 0.1% is variable and responsible for diversity within the human population
- 0.1% variability is still a lot: 3 billion base pairs x 0.1% = ~3 million sites of variation ("variants") between any two individuals
- The vast majority of this variability is inherited from one's parents

<u>NGS analysis</u>

- In principle a two-step process
  1. Millions/billions of reads are mapped en masse to a reference genome
  2. Variants are detected when enough reads disagree with reference

- Complicating factors
  1) Mapping can be tricky
  2) Sequencing coverage is biased & may result in gaps in coverage (insufficient breadth) or just inadequate coverage (insufficient depth)
  3) Not all variant calls are created equal
     - We do quite well with SNPs (i.e., single base substitutions) – Calls are reliable: >99% concordance with chip-based SNP genotyping
     - But indels (small insertions or deletions) are significantly harder – It is computationally hard to map a 100bp read to the genome if you allow for gaps
  4) Beyond SNPs and small indels – Algorithms for other variant classes are coming, but still largely investigational:
     - CNVs and structural variants
     - Larger insertions (>20bp) or deletions (>50bp)
     - Repeat expansions/contractions
     - Transposable elements

Figure 1. Variant calling summary

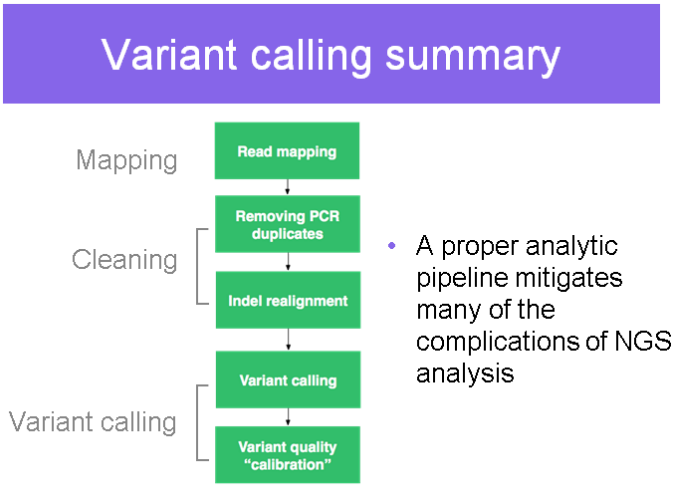


Figure 2. Identifying novel disease mutations

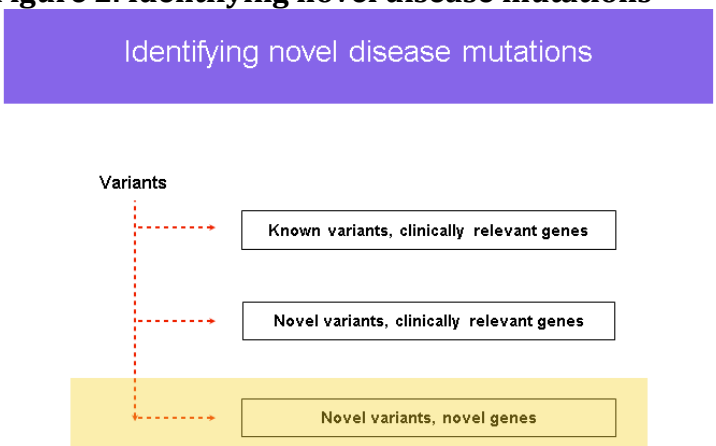
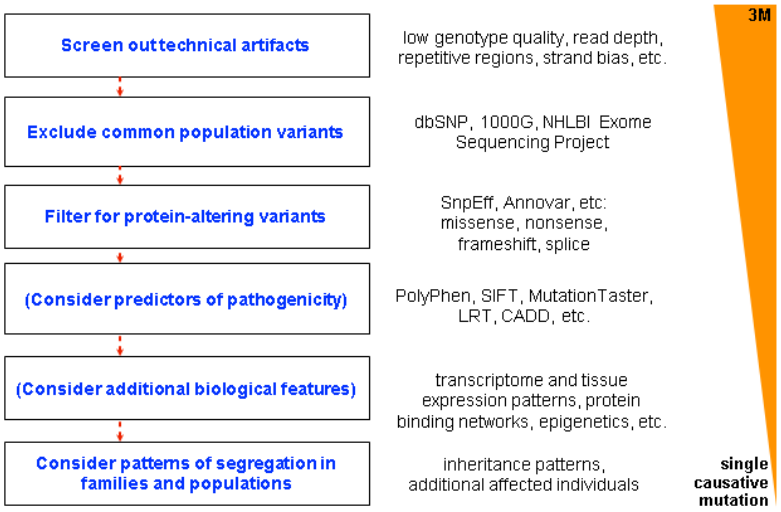


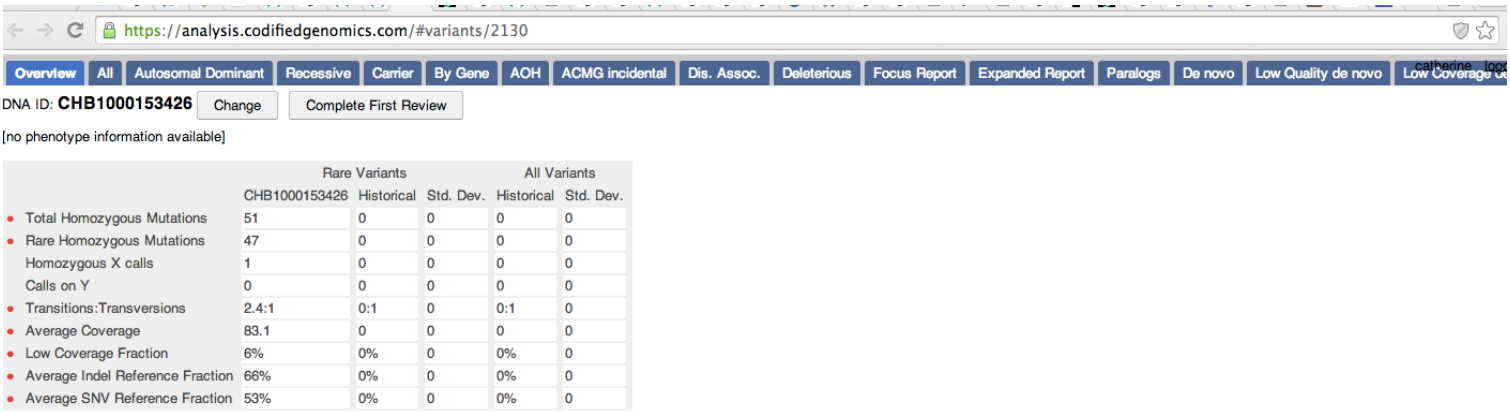
Figure 3. Finding mutations from 3 million variants



What do you get back and what do you analyze?

1. Types of files
    a. Fastq
    b. Bam files
    c. Variant call files (vcf)
    d. Annotation file (often XLS or SQL or proprietary formats, see Figure 4)

Figure 4. Screenshot of an example annotation file viewer



	Rare Variants			All Variants	
	CHB1000153426	Historical	Std. Dev.	Historical	Std. Dev.
• Total Homozygous Mutations	51	0	0	0	0
• Rare Homozygous Mutations	47	0	0	0	0
Homozygous X calls	1	0	0	0	0
Calls on Y	0	0	0	0	0
• Transitions:Transversions	2.4:1	0:1	0	0:1	0
• Average Coverage	83.1	0	0	0	0
• Low Coverage Fraction	6%	0%	0	0%	0
• Average Indel Reference Fraction	66%	0%	0	0%	0
• Average SNV Reference Fraction	53%	0%	0	0%	0

Cases

Case 1. Cantu syndrome with no mutation in ABCC9

Case Report: Patient with Cantú syndrome, or hypertrichotic osteochondrodysplasia (hypertrichosis, macrosomia, osteochondrodysplasia, and cardiomegaly). Recently, the KATP gene ABCC9 has been associated with Cantú syndrome. The patient tested negative for mutations in ABCC9. The patient was enrolled in the *Manton Center for Orphan Disease Research*, a rare diseases program at Boston Children's Hospital. Whole exome sequencing revealed a *de novo* nonsynonymous KCNJ8 SNV (p.V65M) (Figure 5).

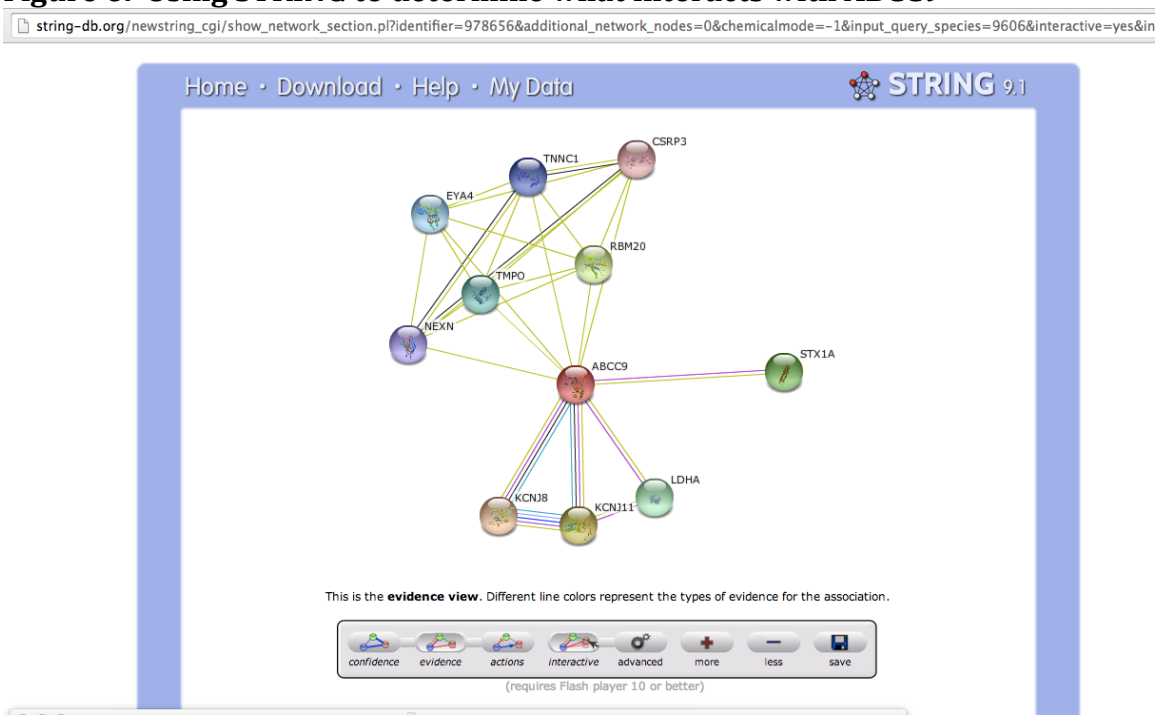
Discovery Method:

A. *de novos*, recessives
B. *EOMES* – doesn't fit phenotype
C. What's related to ABCC9?
   STRING - Known and Predicted Protein-Protein Interactions http://string-db.org/ (see Figure 6)
D. How do they interact? Appropriate expression pattern? Appropriate function?

Figure 5. Mutation in KCNJ8!

19149	12	21791272	LDHB	T	0	C	52	0	C	60	0	C	48	intronic			
19150	12	21791455	LDHB	A	0	T	11	0	T	4	0	T	4	intronic			
19151	12	21794865	LDHB	A	0	T	40	0	T	33	0	T	26	intronic			
19152	12	21796814	LDHB	G	0	A	14	0	A	15	0	A	20	intronic			
19153	12	21797029	LDHB	A	0	G	82	0	G	73	0	G	72	exonic	NM_002300	c.T261C	p.T87T
19154	12	21799999	LDHB	G	0	C	45	0	C	37	0	C	37	intronic			
19155	12	21807432	LDHB	T	0	C	44	0	C	44	0	C	34	intronic			
19156	12	21807640	LDHB	G	0	A	61	0	A	54	0	A	47	intronic			
19157	12	21807687	LDHB	C	10	G	10	11	G	8	12	G	7	intronic			
19158	12	21810646	LDHB	C	12	T	10	10	T	17	12	T	16	UTR5			
19159	12	21918199	KCNJ8	A	60	C	64	41	C	36				UTR3			
19160	12	21926358	KCNJ8	C	25	T	20							exonic	NM_004982	c.G193A	p.V65M
19161	12	22005003	ABCC9	T	30	G	16	30	G	22	19	G	26	intronic			
19162	12	22005510	ABCC9	A	0	G	24	0	G	22	0	G	23	intronic			
19163	12	22017422	ABCC9	A	0	G	96	0	G	91	0	G	71	intronic			
19164	12	22017486	ABCC9	C	0	G	14	0	G	13	0	G	10	intronic			
19165	12	22035873	ABCC9	C	4	T	7	0	T	32	3	T	6	intronic			
19166	12	22035883	ABCC9	T	5	G	3							intronic			
19167	12	22047151	ABCC9	G	0	T	22	0	T	18	0	T	23	intronic			

Figure 6. Using STRING to determine what interacts with ABCC9



Questions:

1. Why wasn't it EOMES with an expansion of phenotype?
2. What qualities do you look for in a candidate variant or gene?

Take home clinical message: A careful screening of the KATP genes should be performed in all individuals diagnosed with Cantú syndrome and no mutation in ABCC9.

Case 2: XLH with no mutations in PHEX

Case Report: A child with classic hypophosphatemic rickets and no family history. Clinical sequencing of the PHEX gene showed no known mutations in PHEX or other candidate genes including FGF23 and ENPP1. Patient was enrolled in the *Manton Center for Orphan Disease Research*.

Discovery Method:

A. Look at known genes (PHEX) – Again.
B. Recessives, *de novos,* etc.

Figure 7. It was PHEX all along (PHEX L748R)

G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
SwissPro	Zygosity	Inherita	CHROM	POS	REF	ALT	QUAL	FILTER	FORMAT	Pileup_c	Mutatio	Mutatio	Gene_n	Proximal_gene_information_(for_noncoding_	ESP5400
X	Het	.	1	1.14E+08	A	C	60	PASS	0/1:42:41:83:.	C,,Cc,,CCc,	nonsynony	nonsynony	PHTF1	PHTF1:NM_006608:exon8:c.T897G:p.S299R,,PHTF	.
X	Het	Complex	8	27362511	G	A	60	PASS	0/1:25:25:50:.	.,,A,.AAAA,	nonsynony	nonsynony	EPHX2	EPHX2:NM_001256482:exon4:c.G226A:p.D76N,EI	.
X	Het	.	16	71682818	G	A	60	PASS	0/1:20:15:35:.	..AAA.A,,AA	nonsynony	nonsynony	PHLPP2	PHLPP2:NM_015020:exon18:c.C3947T:p.P1316L,,	1
X	Het	.	17	47302377	G	C	60	PASS	0/1:15:14:29:.	,.,,c,c,cc,C..	nonsynony	nonsynony	PHOSPHO1	PHOSPHO1:NM_001143804:exon3:c.C110G:p.P37	3
X	Het	.	19	48495302	G	A	60	PASS	0/1:49:48:97:.	.AA.A.AAA,	stopgain_S	stopgain_S	BSPH1	BSPH1:NM_001128326:exon1:c.C37T:p.R13X,,BSF	.
Defects in F	Het	X-linked	X	18966927	T	C	60	PASS	0/1:60:73:133:.	,ScC.C,CC,,(	nonsynony	nonsynony	PHKA2	PHKA2:NM_000292:exon5:c.A472G:p.T158A,,PHK	24
Defects in F	Het	X-linked	X	22266063	T	G	60	PASS	0/1:37:50:87:.	.GG...Gg,gg	nonsynony	nonsynony	PHEX	PHEX:NM_000444:exon22:c.T2243G:p.L748R,,PHI	.
X	Het	.	2	97017678	C	T	60	PASS	0/1:37:43:80:.	...,TTT,T....,	nonsynony	nonsynony	NCAPH	NCAPH:NM_015341:exon7:c.C830T:p.P277L,,NCA	2

Question: How was the PHEX mutation missed? The patient was clinically sequenced!

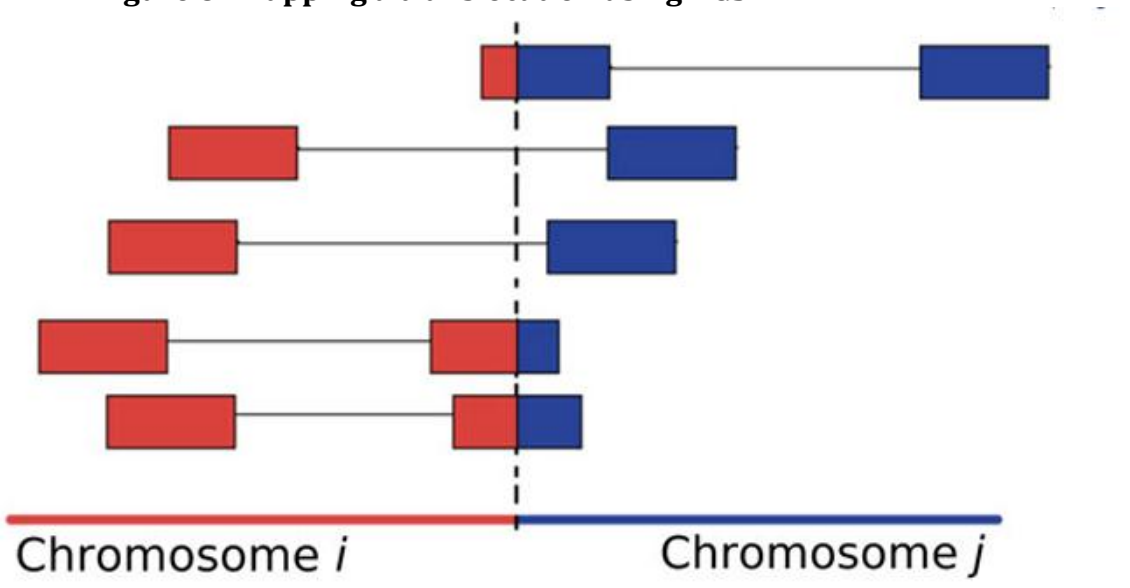
Case 4. t9;13 with hypophosphatemic rickets

Case report: 13-month old girl evaluated for poor linear growth and increasing head size. There was no history of intestinal malabsorption. Physical examination revealed a pleasant and alert infant who refused to stand. A prominent forehead, large open anterior fontanel, and knobby appearance of the wrists were present. The legs were moderately bowed, and hypertrophic physes were evident along the anterior ribs (rachitic rosary). Radiographs of the knees and wrists demonstrated florid rachitic changes of the growth plates.

Discovery Method:

a. Cytogenetic analysis revealed a balanced translocation between chromosomes 9 and 13: t(9,13)(q21.13;q13.1). Normal karyotypes were present in both parents. Southern blotting and long range PCR further refined the translocation interval to 50Kb 5' of Klotho.
b. Whole Genome sequencing, with longer inserts, paired ends, mate pair libraries (Figure 8).

Figure 8. Mapping a translocation using NGS



The formation of soft-clipped reads. Soft-clipped reads span the translocation boundary between chromosomes *i* and *j*. As a result, these reads may align partially to chromosome *i* and partially to chromosome *j*.

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Questions:

1. Why does it benefit you to know the exact breakpoint?
2. What could make this process difficult?

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Fibrous Dysplasia

Michael Collins, M.D.

Meet the Professor – 2014 ASBMR Houston, Texas

Fibrous Dysplasia

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Significance of the Problem

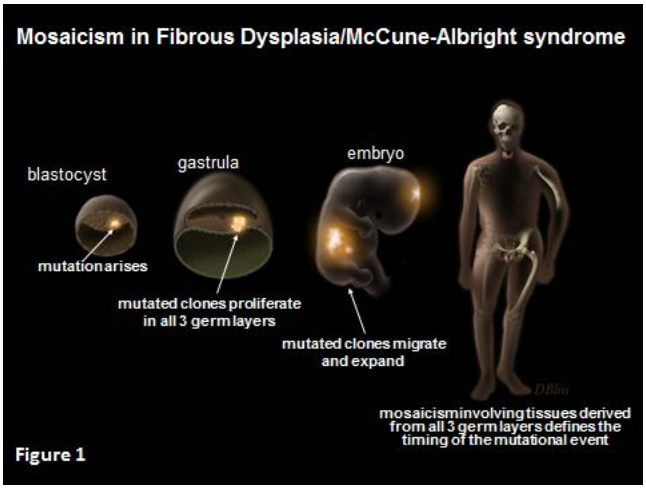
Fibrous dysplasia of bone (FD) is a skeletal disease frequently encountered by clinicians specializing in skeletal disorders. Disease severity is highly variable. Further complicating the diagnosis and treatment of FD is the fact it can be accompanied by a wide array of extraskeletal manifestations. When extraskeletal manifestations are present, the disease is referred to as the McCune-Albright syndrome (MAS). The high degree of phenotypic variability makes the diagnosis and management of FD/MAS challenging. An understanding of the physiologic consequences of the underlying molecular and developmental biology can make the evaluation and treatment of FD/MAS relatively straightforward.

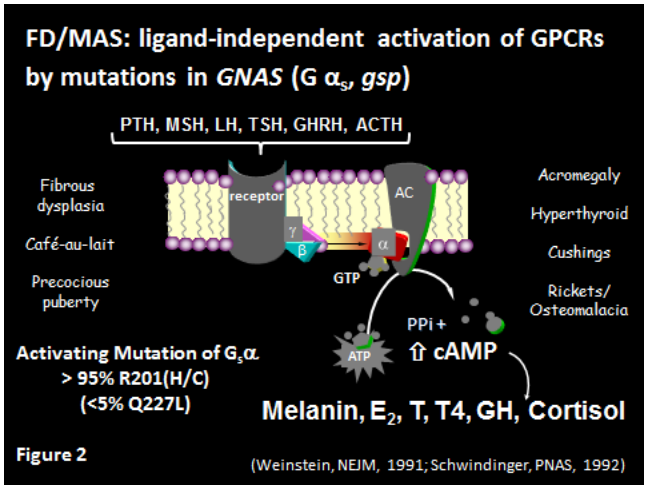
Learning Objectives

As a result of participating in this session, attendees will:
1) Understand the biology that underlies the often complex phenotype of FD/MAS.
2) Understand what is involved in the diagnosis and staging of FD/MAS
3) Know how to treat the various aspects of the disease

Fibrous Dysplasia/McCune-Albright Syndrome

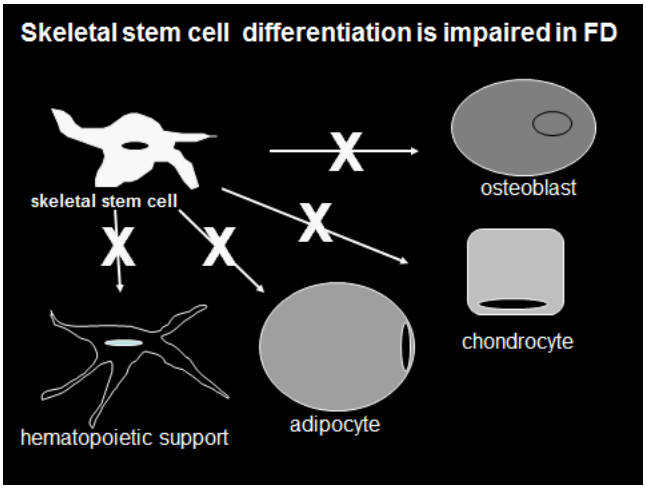
Developmental and Molecular Etiology: FD/MAS is caused by somatic, activating mutations in the ubiquitously-expressed G-protein coupled receptor associated protein $G_s\alpha$ (*gsp*). As a result of the widespread expression of $G_s\alpha$, FD can be accompanied by a number of extraskeletal manifestations, including café-au-lait macules, precocious puberty, hyperthyroidism, growth hormone excess, and others. For tissues derived from all germ layers to be involved (e.g., ectoderm – café-au-lait macules, mesoderm – FD, endoderm – thyroid), the mutation must have arisen very early in development, prior to gastrulation (Fig. 1). An understanding of the role of activated $G_s\alpha$ in a given tissue allows for an understanding of the phenotype exhibited by mutation-bearing cells (Fig. 2).

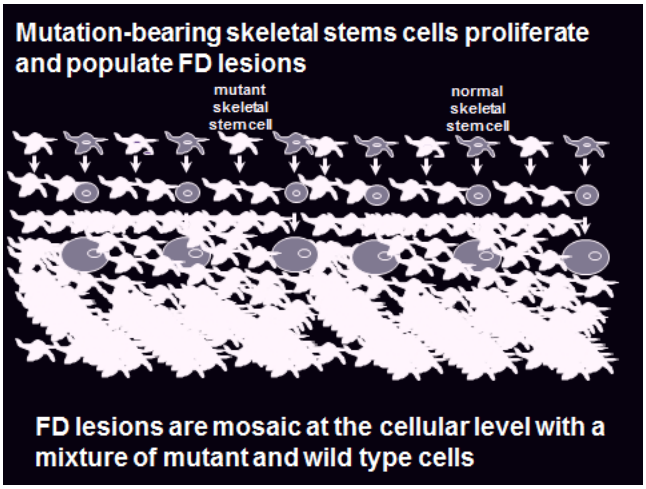




Fibrous Dysplasia:

FD is a skeletal stem cell disease that results from the inability of *gsp*-bearing stem cells to differentiate into the progeny of skeletal stem cells. Instead of differentiation down a given developmental pathway, cells proliferate, giving rise to sheets of fibroblast-like immature osteogenic cells that fill the marrow space and cause marrow fibrosis characteristic of FD (Figs. 3&4).

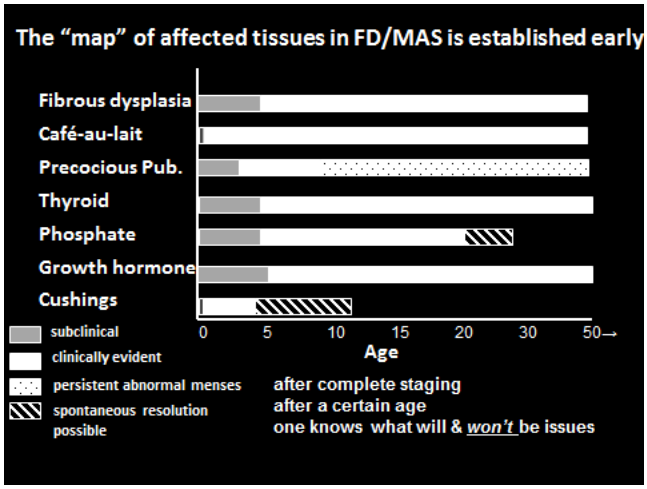




Any or all bones can be involved with FD. When a single bone is involved, it is termed monostotic FD, many bones polyostotic, and all bones panostotic. Limp, pain, and fracture are the most common presenting features. Presentation before the age of 5 usually heralds severe disease likely to result in significant morbidity.

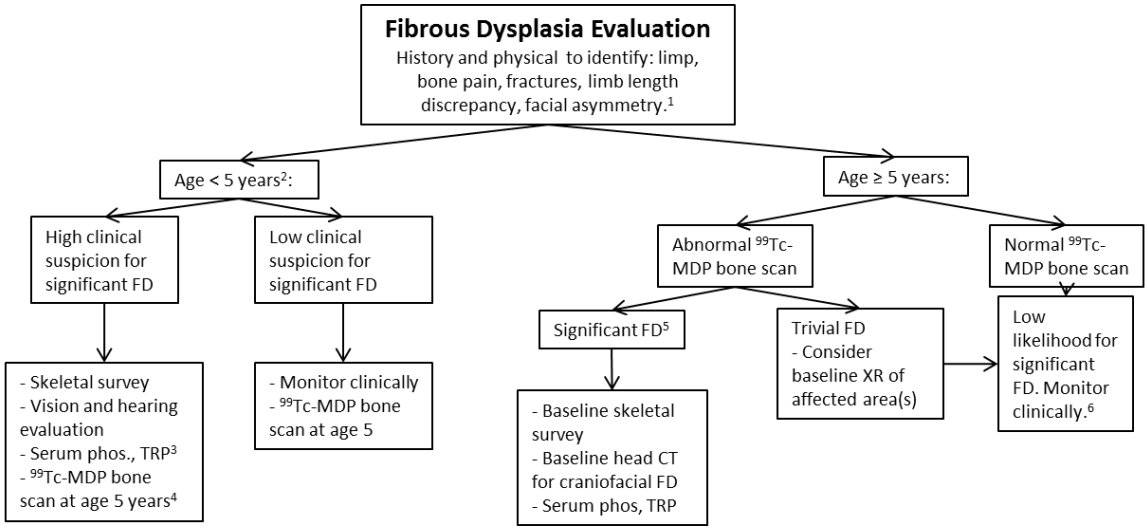
Diagnosis is usually made on clinical grounds based upon the radiographic appearance and location. The skull base and proximal femur are the bones most commonly involved. On radiographs, lesions in the long bones typically display a homogenous "ground glass" appearance. Craniofacial FD has a sclerotic appearance on x-rays, but on CT a ground glass appearance. There are age-related changes in the radiographic appearance of FD. Long bone disease tends to become more sclerotic as the disease quiets with age and craniofacial FD lesions become more inhomogeneous and lytic on CT. Diagnosis can be supported by histological examination of bioptic material and/or mutation testing. Mutation testing requires affected tissue due to the somatic nature of the disease.

It is prudent to identify or exclude extraskeletal manifestations at the time of presentation. Certain extraskeletal manifestations can significantly worsen the clinical course of FD, e.g. hypophosphatemia and/or hyperthyroidism. Because the "map" of affected tissues is essentially established in utero, thorough phenotyping at presentation not only allows for identification of all affected tissues, importantly, it also allows the clinician to exclude the involvement of many tissues and thus reassure the patients and families (Fig. 5).

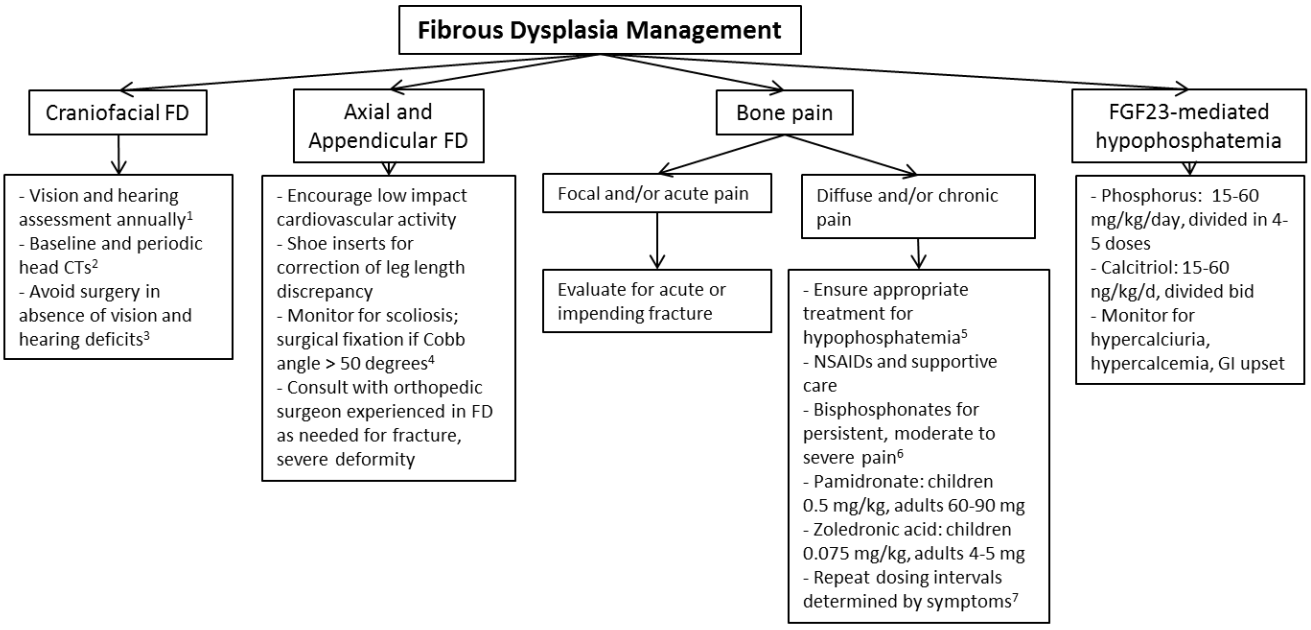


Diagnosis and Treatment of FD/MAS

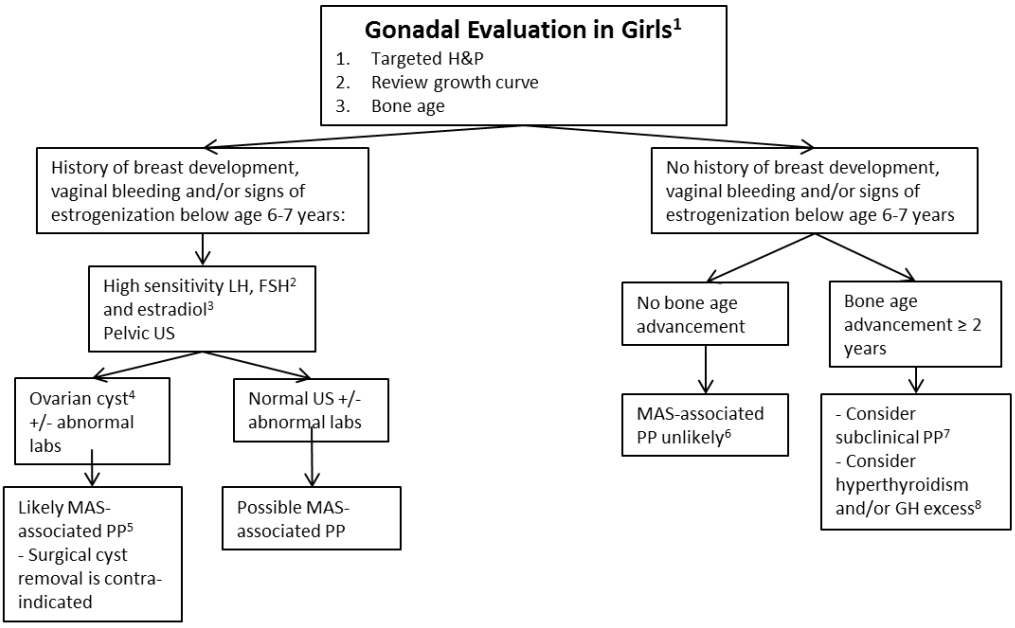
The following figures are algorithms for the diagnosis and treatment of the most common aspects of FD/MAS (Figs. 6-17).



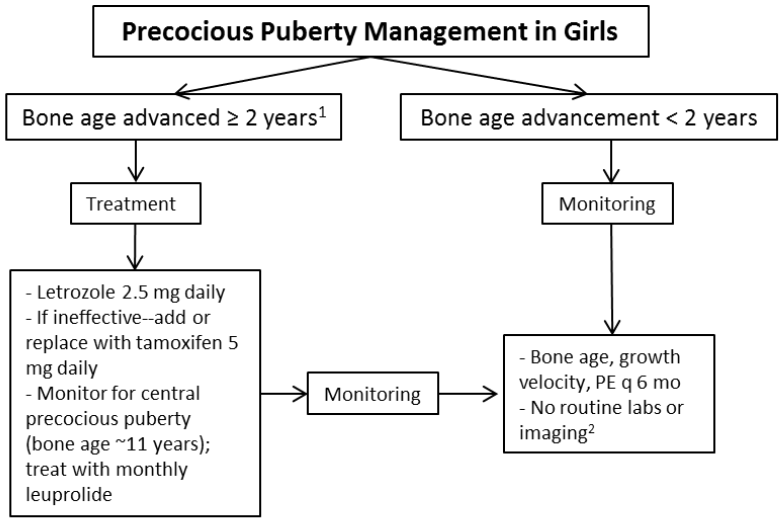
[1]Performed at initial presentation in all patients suspected of having MAS. [2]Areas of clinically significant FD will be apparent on bone scan by age 5 years. Prior to age 5, a normal Tc99 does not rule out the possibility of significant FD. [3]TRP = tubular reabsorption of phosphate = 1 – [(Uphos/Sphos)*(Scr/(Ucr*1000))]. Calculated from a spot urine collection. FGF23-mediated phosphate wasting is associated with high FD burden; may improve or resolve in adolescence or adulthood as FD becomes less active. [4]Consider performing ^{99}Tc-MDP bone scan in children < 5 years regardless of clinical suspicion for bone disease in instances where establishing the diagnosis of MAS may alter management – i.e. patients for whom diagnostic surgery is being considered, such as oophorectomy or skeletal biopsy. [5]Significance of FD is determined by both the amount and location of affected bone. Clinically significant disease is frequently associated with the craniofacial area, proximal femurs and spine. [6]A normal ^{99}Tc-MDP bone scan at age 5 years or older effectively rules out clinically significant FD, and no further radiologic monitoring is required.



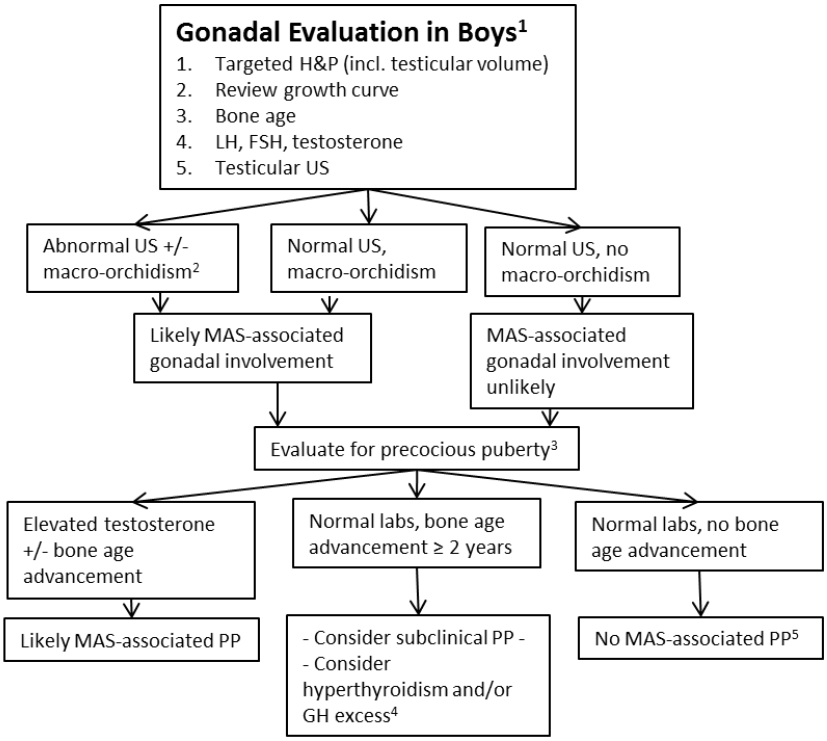
[1]Patients should be evaluated yearly by a neuro-ophthalmologist, those with evidence of optic neuropathy referred to an experienced craniofacial surgeon. [2]Repeat head CT approximately every 5 years, or sooner if vision or hearing deficits develop. [3]Optic nerve encasement is common and usually asymptomatic. Prophylactic optic nerve decompression in the absence of optic neuropathy is contraindicated . [4]Scoliosis may be progressive and potentially fatal in severe cases. All patients with scoliosis should be followed regularly by an orthopedic surgeon. [5]Inadequately treated hypophosphatemia may significantly worsen bone pain, and must be addressed before considering bisphosphonates. [6]Bisphosphonates have not been shown to affect disease progression, and use should be limited to treatment of FD-related bone pain. [7]Doses should be repeated as needed when pain returns rather than on a set dosing schedule.



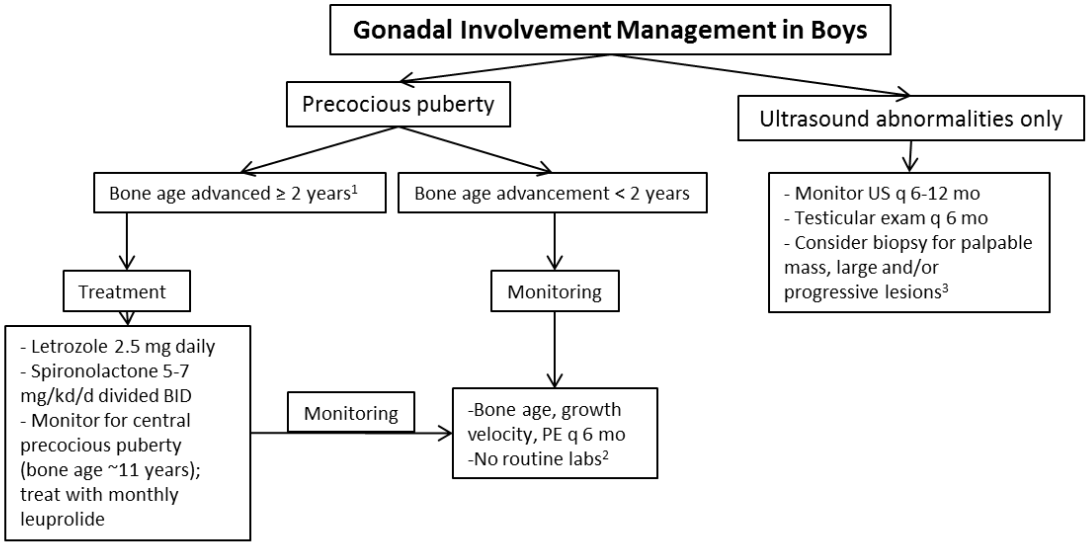
[1]To be performed at initial presentation in all girls suspected of having MAS, regardless of clinical symptoms. [2]Gonadotropins should be suppressed in MAS-associated PP, unless autonomous estrogen production has induced central PP (typically occurs when bone age reaches approximately 11 years). [3]Estrogen production in MAS-associated PP is intermittent, and undetectable levels do not rule out disease. [4]Ovarian cysts are suggestive of MAS-associated PP, however absence of cysts does not rule out disease. [5]In girls presenting with isolated peripheral PP, the differential also includes estrogen-producing tumors. Work-up for additional features of MAS may establish the diagnosis. [6]Unlike other features of MAS, autonomous ovarian activity may present at any time during childhood. Girls should continue to be monitored clinically for signs of peripheral PP, however routine labwork and imaging is not recommended. [7]Patients may rarely present with intermittent ovarian activity with only subtle signs of estrogenization (mild intermittent breast development without vaginal bleeding), which may not be appreciated by families and practitioners. [8]Hyperthyroidism and GH excess may present with bone age advancement.



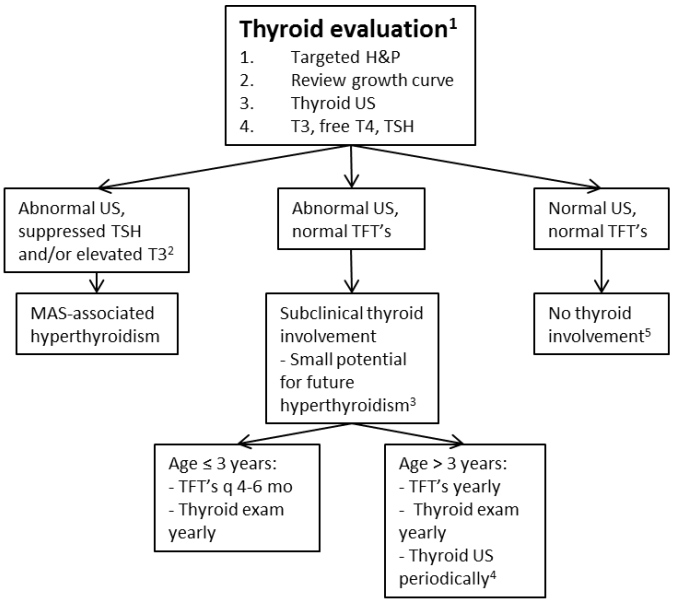
[1]The primary indication for treatment is to prevent impairment of adult height. Vaginal bleeding in the absence of bone age advancement does not typically warrant treatment. Exceptions may be made for very young children with frequent bleeding episodes deemed likely to lead to bone age advancement. [2]The primary endpoint for treatment efficacy is prevention of bone age advancement, which is assessed by growth velocity and bone age examination. Routine laboratory testing and ultrasound are unlikely to change management, and are not recommended.



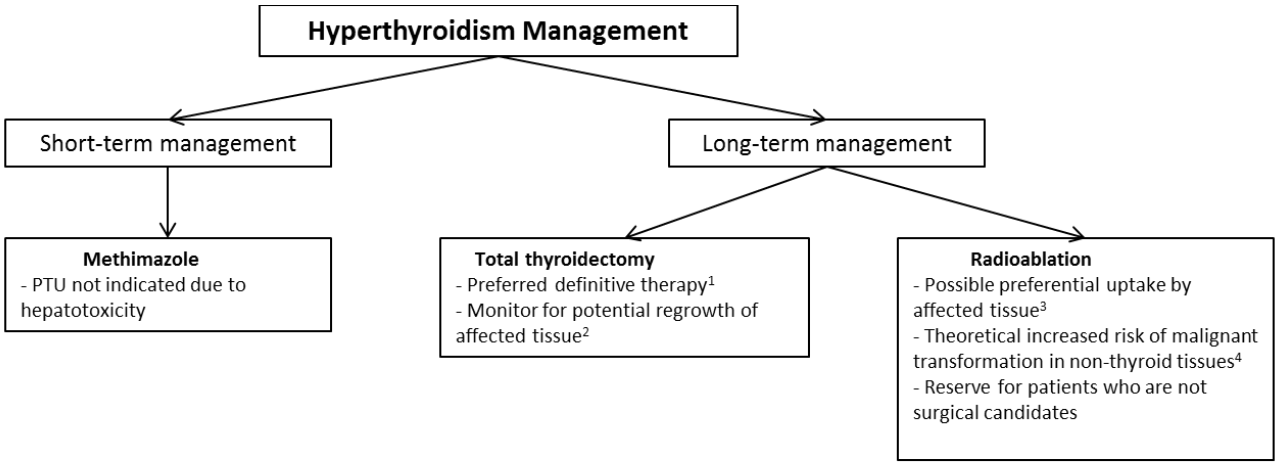
[1]Performed at initial presentation in all boys suspected of having MAS, regardless of clinical symptoms. [2]Typical MAS-associated macro-orchidism presents with uniform, unilateral or bilateral testicular enlargement without discrete masses. [3]Precocious puberty is less likely to occur in patients without testicular involvement on ultrasound. [4]Hyperthyroidism and GH excess may present with bone age advancement. [5]Autonomous testicular activity may present at any time during childhood. Boys should continue to be monitored clinically for signs of peripheral PP, however routine labwork and imaging is not recommended.



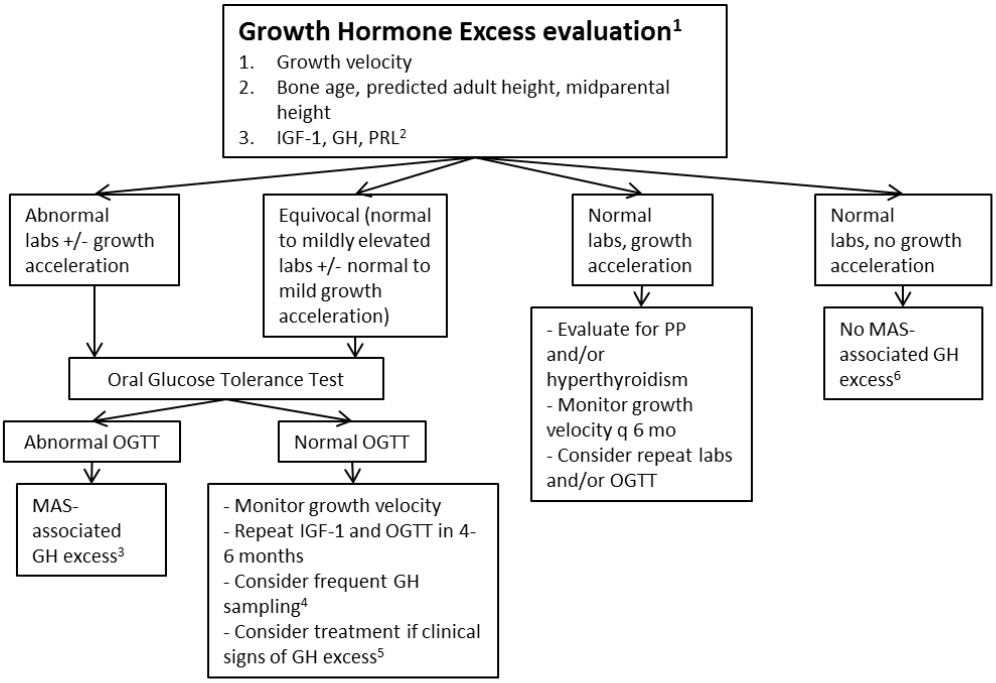
[1]The primary indication for treatment is to prevent impairment of adult height. Elevated testosterone levels in the absence of bone age advancement does not warrant treatment. Exceptions may be made for boys with testosterone-induced behavioral changes or progressive masculinization of the genitalia. [2]Routine labwork is unlikely to change management and is not recommended. [3]Typical MAS-associated testicular involvement is associated with Sertoli and/or Leydig cell hyperplasia, which likely carry a small theoretical risk of malignant transformation. In addition, MAS-associated testicular involvement is likely associated with a slight increased risk of testicular germ cell tumors. Biopsy of affected testes is not recommended. Lesions should be followed with serial exam and ultrasound. Consider biopsy for lesions with atypical features such as a palpable mass, or for lesions that are large and/or progressive.



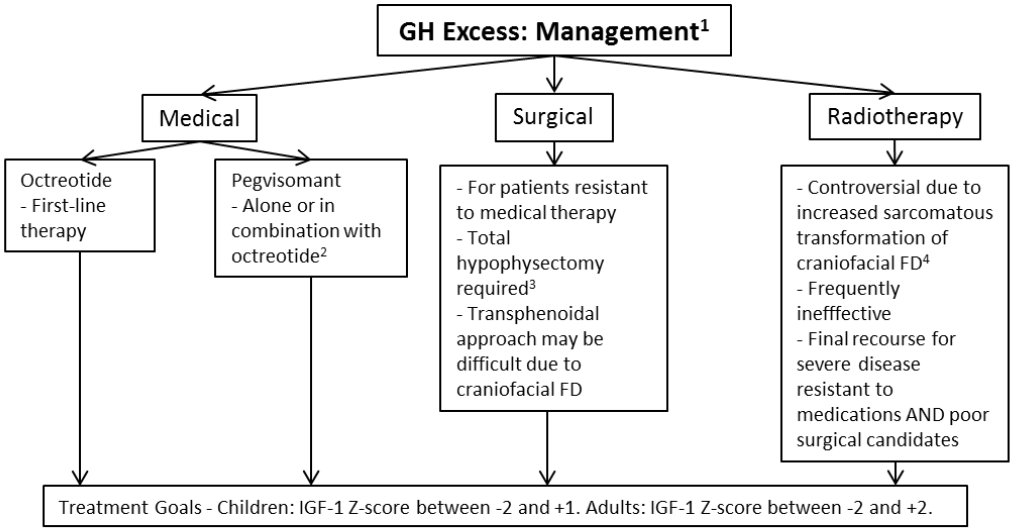
[1]To be performed at initial presentation in all patients suspected of having MAS, regardless of clinical symptoms. [2]The primary biochemical abnormality in MAS-associated hyperthyroidism is elevated T3 production, which may occur in the setting of normal thyroxine and free T4. In the absence of frank hyperthyroidism, an elevated T3/T4 ratio is suggestive of autonomous T3 production in a patient suspected of having MAS . [3]A small percentage of patients with radiologic disease and normal TFT's will go on to develop hyperthyroidism at some point during childhood. [4]MAS-associated thyroid disease is correlated with a slight increased risk of thyroid cancer. Patients with radiologic disease should be monitored with yearly physical exam and thyroid US every 2-5 years. [5]Absence of biochemical or radiologic thyroid abnormalities after age 2 years effectively rules out MAS-associated thyroid disease, and no further monitoring is required.



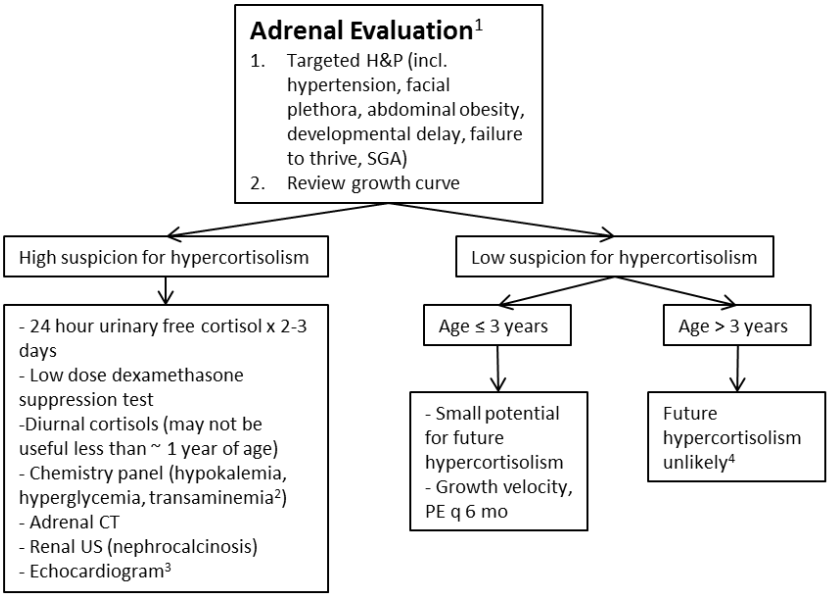
[1]Total thyroidectomy is preferred over subtotal as any remaining abnormal tissue has the potential to regrow, with recurrence of hyperthyroidism. Accordingly, radioactive iodine uptake scan will not alter management and is not part of routine pre-operative care. [2]After thyroidectomy patients should continue to be monitored with yearly physical exam and thyroid US. [3]Preferential uptake of radioactive iodine by diseased tissue may lead to a theoretical increased risk of thyroid cancer in the remaining unaffected tissue. [4]$G_s\alpha$ mutations carry a slight increased risk of malignant transformation in both thyroid and non-thyroidal tissues, which may be increased by radiation exposure.



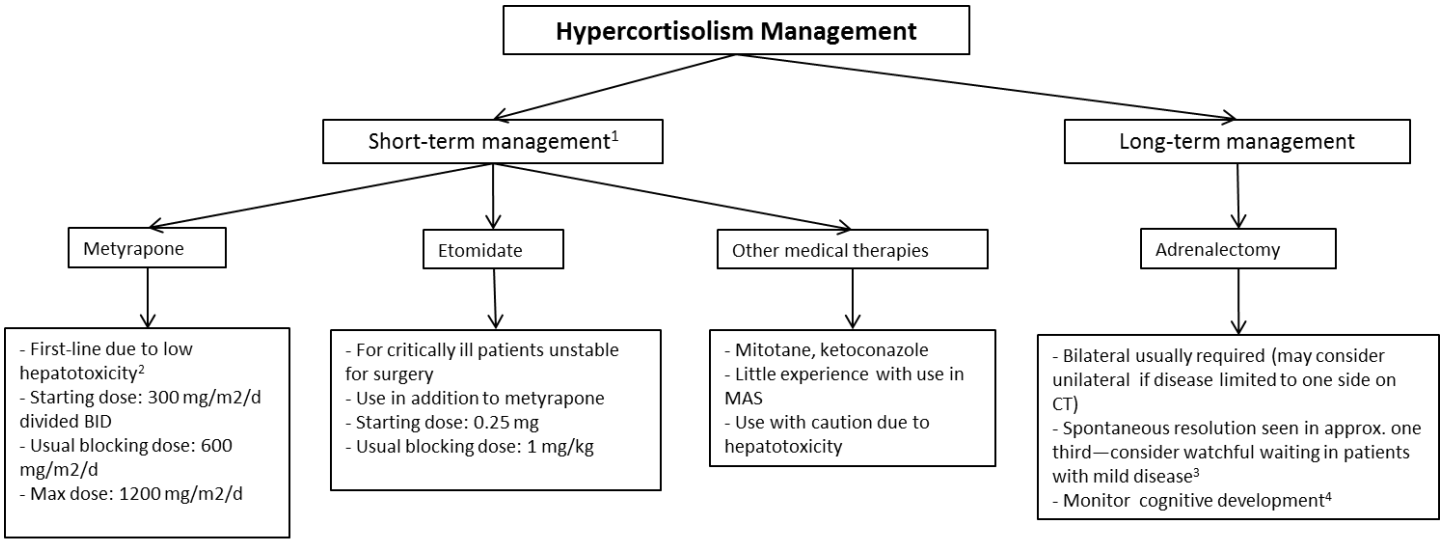
[1]To be performed at initial presentation in all patients suspected of having MAS, regardless of clinical symptoms. [2]The majority of patients with MAS-associated GH excess will have prolactin co-secretion. [3]Practitioners may consider pituitary MRI in patients suspected of having MAS-associated GH excess, however findings may be non-specific and rarely change management. [4]There are a variety of techniques for frequent GH sampling. Ours involves collecting GH samples every 20 minutes for 12 hours from 8 PM to 8 AM, with a lack of nadir below 1.0 ng/mL considered consistent with GH excess. [5]In patients with craniofacial FD it is prudent to have a low threshold for initiating treatment, as uncontrolled GH excess is associated with increased craniofacial morbidity. [6]If no clinical or biochemical evidence of GH excess is evident by age 5 years, MAS-associated GH excess is effectively ruled out.



[1]Hyperprolactinemia accompanies GH excess in approximately 90% of the patients with MAS. It usually only requires treatment if levels are very high and/or hyperprolactinemia is interfering with pubertal progression, menses, or sexual function. [2]Our practice is to add pegvisomant after reaching a maximal dose of 30 mg/mo of octreotide. [3]Due to characteristic diffuse somatolactotroph hyperplasia of the pituitary, total hypophysectomy is required for successful surgical treatment. [4]FD of the skull base is nearly universal in patients with MAS-associated GH excess. There are reports of fatal skull base osteosarcomas arising after pituitary irradiation for treatment of MAS-associated GH excess.



[1]To be performed at initial presentation in all patients suspected of having MAS, regardless of clinical symptoms. [2]Liver disease is highly correlated with MAS-associated hypercortisolism. [3]Prognosis of hypercortisolism is negatively correlated with the presence of comorbid heart disease. [4]Hypercortisolism in MAS results from autonomous activity of the adrenal fetal zone, which involutes rapidly after birth and is typically gone by age 1 year. MAS-associated hypercortisolism is unlikely after age 1 and effectively ruled out after age 3.



[1]Patients are often critically ill at presentation, which may impact treatment options. [2]Hepatotoxicity is an important consideration due to frequent comorbid liver disease. [3]Spontaneous resolution may occur due to involution of the adrenal fetal zone, which is the source of hypercortisolism in MAS. [4]Children with a current or remote history of MAS-associated hypercortisolism are at increased risk for neurodevelopmental delays, and should be considered for early interventional services.

Uncommon manifestations of FD/MAS

Liver: Hepatitis may be pronounced in infants, and generally wanes with age to a mild persistent form. Hepatic adenomas have also been reported. There are no reports of liver failure or functional defects associated with FD/MAS.
Gastrointestinal: Gastroesophageal reflux manifests in childhood, and may be severe. Gastrointestinal polyps have also been observed.
Pancreas: Pancreatic complications have been reported, including pancreatitis and intraductal papillary mucinous neoplasms. There have been no reports of pancreatic cancer associated with FD/MAS.
Myxomas: Intramuscular myxomas in association FD/MAS has been termed Mazabraud syndrome. These are benign, usually asymptomatic, and often found incidentally.
Malignancies: Cancers reported in association with FD/MAS include bone, thyroid, testicular, and breast. Support for the activating mutations that cause FD/MAS as etiologic is supported by the fact that the mutation is found in the cancer tissue but not in adjacent normal tissue. Likewise, the FD/MAS *GNAS* mutations are seen in nonsyndromic benign and malignant tumors. Other features of the disease such as precocious puberty and GH excess may also contribute to an increased risk of cancer. While a strong association between *gsp* mutations and cancer in FD/MAS is lacking, it is prudent to minimize additional risk factors, such as radiation exposure, and encourage vigilance and monitoring.
Health-related quality of life. Social and emotional functioning was found to be normal in one large series, with individuals reporting high levels of self-esteem and social function.
Prognosis. The prognosis for individuals with FD/MAS is based on disease location and severity. Medical therapies can ameliorate or control endocrine disease in most individuals. FD is progressive throughout childhood and adolescence, and typically plateaus in middle and late adulthood. Small amounts of FD may cause few or no symptoms, however patients with extensive bone disease may suffer significant sequelae including loss of mobility, progressive scoliosis, facial deformity, and loss of vision or hearing.

Cases – From the Attendees

References: Fibrous Dysplasia (1-4) Extraskeletal Manifestations (5-10)

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How Long Should We Treat Osteoporosis?

Dennis Black, Ph.D.

How Long Should We Treat Osteoporosis?
ASBMR 2014, Meet the Professor
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Background

Osteoporosis medications have been shown to be highly effective in reducing fracture risk over 3 to 5 years. Reductions in the first 3 to 5 years of treatment for vertebral fractures range from 40% to 75% and for hip fractures up to 50%. Reductions in fracture risk beyond 5 years of treatment for bisphosphonates and other antiresorptive treatments are less clear, although the evidence base is small. Importantly, concerns about side effects, particularly atypical femur fractures, which may be associated with longer term treatment, have raised concerns about risks vs. benefits for osteoporosis treatment in general but are particularly important to the question about continuing therapy beyond 5 years given more limited proven benefits during this time period.

Learning objectives:

In this session, we will discuss the evidence for benefits and for harm with long term osteoporosis treatment and review current recommendations. Specific objectives for participants:

- Discuss the evidence for long-term efficacy in terms of both BMD gains and fracture reductions
- Understand current evidence about the relationship of antiresorptive use and atypical fractures. Discuss strengths, limitations and variation in current studies of bisphosphonate use and atypical fractures
- Be able to compare the efficacy after discontinuation for various osteoporosis medications
- Compare benefits and risks for continuing therapy long term

Outline: An interactive session centered around 3 topics:

1. Long term efficacy of antiresorptive medications
2. Long term safety (emphasizing atypical femur fractures) of antiresorptive medications
3. Balance of benefits vs. risks for short term and long term treatment

Some selected references

Overviews of AFF and impact on long-term use of bisphosphonates (Recent ASBMR update on atypical fractures, IOF position paper and a recent meta analysis of AFF)

Shane E, Burr D, Abrahamsen B, et al. American Society for Bone and Mineral Research 2013 Atypical subtrochanteric and diaphyseal femoral fractures: Second report of a task force of the American Society for Bone and Mineral Research. J Bone Miner Res

IOF position paper on long term bisphosphonate use. www.iofbonehealth.org/atypical-fractures-and-long-term-bisphosphonate-use

New York Times article about 2012 FDA review: www.well.blogs.nytimes.com/2012/05/09/new-cautions-about-long-term-use-of-bone-drugs/?_r=0

Gedmintas L, Solomon DH, Kim SC 2013 Bisphosphonates and risk of subtrochanteric, femoral shaft, and atypical femur fracture: A systematic review and meta-analysis. J Bone Miner Res

Two key randomized trials of long term bisphosphonate continuation/discontinuation

Black DM, Schwartz AV, Ensrud KE, et al 2006 Effects of continuing or stopping alendronate after 5 years of treatment: the Fracture Intervention Trial Long-term Extension (FLEX): a randomized trial. JAMA 296:2927-2938

Black DM, Reid IR, Cauley JA, et al 2012 The Effect of 3 Versus 6 Years of Zoledronic Acid Treatment in Osteoporosis: a Randomized Extension to the HORIZON-Pivotal Fracture Trial (PFT). J Bone Miner Res 27:243 – 254

A recent analysis looking BMD and BTM's use in clinical decision-making about long-term use of BP's

Bauer D et al. 2014. Fracture Prediction After Discontinuation of 4 to 5 years of Alendronate Therapy: the FLEX study. JAMA Int Medicine, May, 2014.

Some of the larger and more interesting epidemiologic studies of subtrochanteric/atypical femur fractures

Schilcher J, Michaelsson K, Aspenberg P 2011 Bisphosphonate use and atypical fractures of the femoral shaft. N Engl J Med 364:1728-1737

Park-Wyllie LY, Mamdani MM, Juurlink DN, et al 2011 Bisphosphonate Use and the Risk of Subtrochanteric or Femoral Shaft Fractures in Older Women. JAMA 305:783-789

Feldstein AC, Black DM, Perrin N, et al 2012 Incidence and demography of femur fractures with and without atypical features. J Bone Miner Res

Dell RM, Adams AL, Greene DF, Funahashi TT, Silverman SL, Eisemon EO, Zhou H, Burchette RJ, Ott SM 2012 Incidence of atypical nontraumatic diaphyseal fractures of the femur. J Bone Miner Res

Black DM, Kelly MP, Genant HK, et al 2010 Bisphosphonates and fractures of the subtrochanteric or diaphyseal femur. N Engl J Med 362:1761-1771

FDA view on continuing long term treatment and recommendations for continuation based on FLEX

Whitaker M, Guo J, Kehoe T, Benson G 2012 Bisphosphonates for Osteoporosis - Where Do We Go from Here? N Engl J Med:2048-2051

Black DM, Bauer DC, Schwartz AV, Cummings SR, Rosen CJ 2012 Continuing Bisphosphonate Treatment for Osteoporosis - For Whom and for How Long? N Engl J Med

Two studies about longer term effects of non-bisphosphonates

McClung MR, Lewiecki EM, Geller ML, et al 2013 Effect of denosumab on bone mineral density and biochemical markers of bone turnover: 8-year results of a phase 2 clinical trial. Osteoporos Int 24:227-235

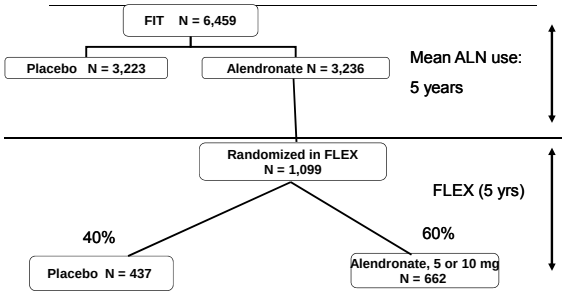
Black DM, Bilezikian JP, Ensrud KE et al 2005 One year of alendronate after one year of parathyroid hormone (1-84) for osteoporosis. N Engl J Med 353:555-565

Some Studies of Long Term Antiresorptive Use

Study	Drug	Design	N	Follow-up years
FIT Long-Term Extension (FLEX)*	Alendronate (5 & 10 mg/day)	Randomized, blinded trial	1099	5+5=10
HORIZON-PFT Extension**	Zoledronic acid (5 mg/year)	Randomized blinded trial	1233	3+3=6
Risedronate^	Risedronate daily	Observational study	164	3+3+3=9
Denosumab^^	Denosumab q 6 months	Observational	88 (long term d'mab)	8 years
Denosumab (abstract 2013 asbmr)	Denosumab q 6 months	Observational	1382	8 years

* Black, JAMA, 2006; **Black, JBMR 2012; ^Watts 2008; ^^McClung, OI, 2013

Design of the FIT Long-Term Extension (to 10 years) of Alendronate (FLEX)*



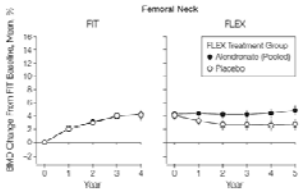
BMD: Primary endpoint

Fractures: Exploratory endpoint

*** Black, et al, JAMA 12/2006**

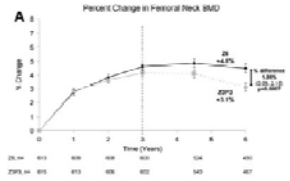
FLEX (ALN)
-5 years/5 years

~ 2% Hip BMD Difference

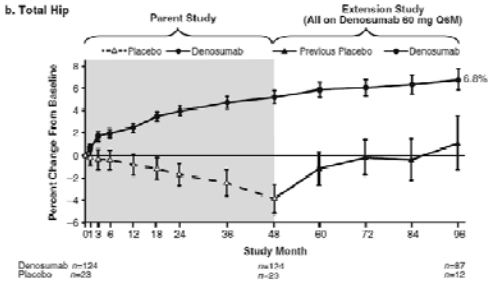


HORIZON (ZOL)
-3 years/3 years

~ 1.5% Hip BMD Difference



Long Term Treatment with Denosumab
(also Papapoulos ASBMR 2013)



McClung, et. al. OI 2013

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FLEX: Alendronate

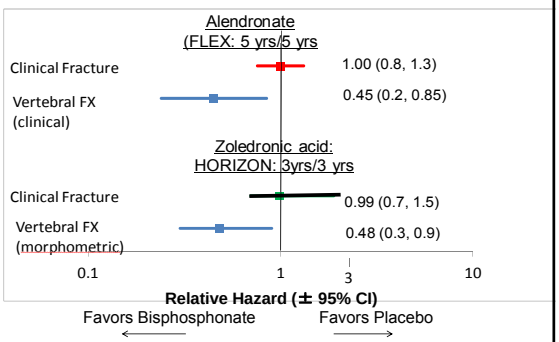
Randomized, Double-blind Treatment
5 years of ALN followed by 5 more years or PBO

FLEX: Incidence of Fracture by Treatment Group

Fractures	Placebo, No. (%) (n=437)	Pooled Alendronate, No. (%) (n=662)	Relative Risk (95% Confidence Interval)*
Vertebral			
Clinical	23 (5.3)	16 (2.4)	0.45 (0.24–0.85)
. Morphometric	46 (11.3)	60 (9.8)	0.86 (0.60–1.22)
Clinical			
Nonspine	83 (19.0)	125 (18.9)	1.00 (0.76–1.32)
Hip	13 (3.0)	20 (3.0)	1.02 (0.51–2.10)

Black DM, et al. *JAMA*. 2006;296:2927–2938.

Reductions (RR) for fractures for continuing bisphosphonates: Alendronate and ZOL



Black JAMA 2006;Black et a. JBMR 2012

FLEX vertebral fracture benefit:

Who to continue?

Femoral Neck BMD T-Score (start FLEX)	5 Yr risk (%) Clinical Vert. Fx. In PBO	Number Needed to Treat
All women in study		
All BMD values	5.5	34
≤ -2.5	9.3	21
-2.5 to -2	5.8	33
≥ -2	2.3	81
No prevalent vert. fracture (start of FLEX)		
≤ -2.5	8.0	24
-2.5 to -2	3.0	63
≥ -2	1.8	102
Prevalent vertebral fracture (start of FLEX)		
≤ -2.5	11.1	17
-2.5 to -2	11.1	17
≥ -2	3.7	51

*** Black, et al. NEJM: 5/9/12**

ONJ and oral Bisphosphonates: American Dental Assn (ADA) 2011*

- **Benefits for fracture prevention outweigh ONJ risks**
- **Recommendations**
  - **Regular dental visits**
  - **Good oral hygiene**
- **No diagnostic techniques available to identify increased risk (including bone turnover markers)**
- **No evidence that temporary discontinuation of bisphosphonates decreases ONJ risk**

*** Hellstein, et. al. ADA recommendations 2011**

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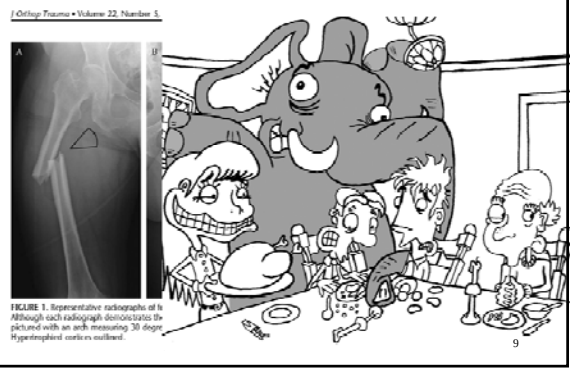
Atypical Femur Fractures: The Elephant in the Room



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Meta Analysis of Bisphosphonates & AFF

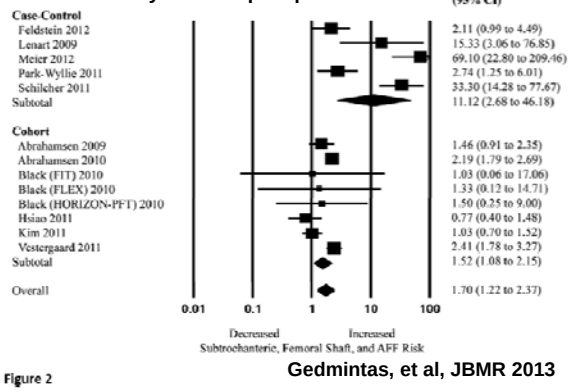

Figure 2

Gedmintas, et al, JBMR 2013

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3 Epidemiologic studies of Atypical Femur Fracture

1. Schilcher (Sweden) (radiographs)
2. Park-Wylie (Ontario, Can) (ICD codes)
3. Feldstein (Kaiser NW, US) (radiographs)

Swedish study: How many with AFF?

- ~12,777 femur fractures in 2008
  - 1.5 million Swedish women > age 55
- 1271 with subtroch/FS by ICD codes
- 322 met criteria for subtrochanteric/FS
  - 263 "control" frx (w/o atypical features)
  - 59 atypical
  - Odds ratio for bisphosphonate use=33 (!)

Schilcher et al, NEJM 5/11

Bisphosphonate use in Swedish study

- Odd results:
  - High OR
  - < 1 year of use increased risk
  - Risk resolves 4 months after stopping use

Schilcher et al, NEJM 5/11

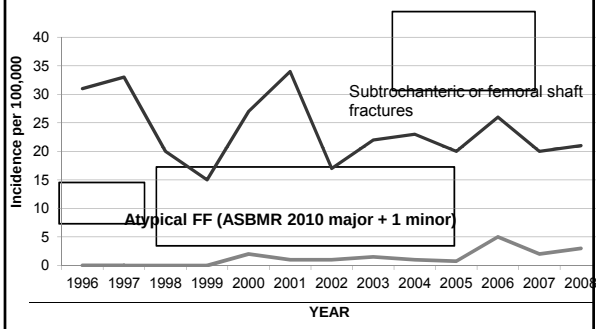
Duration of Therapy and RR for Subtrochanteric/Diaphyseal Fractures (Ontario)

- Nested case-control study. ICD code
- Cases: 716 subtroch/shaft, 9723 "hip" fracture

Fracture Type	RR for DURATION OF BISPHOSPHONATE USE			
	Transient < 100 d	100 d to 3 yrs	3-5 yrs	> 5 years
Subtroch/shaft	1.0	0.9	1.6 (ns)	2.7 (p<.01)
Fn or Intertroch	1.0	0.9	0.9	0.76

Park-Wylie, et al. JAMA 2/11

Subtrochanteric or Femoral Shaft Fractures over time (Kaiser, NW US)*



Feldstein, et al. JBMR 2012

Benefits vs. Risk, 10,000 women treated 3 years

	Fractures prevented	RR for AFF	AFF caused
Hip	112		
Spine	545		
Non-vertebral	164		
	822		
		1.7	1
		19 (worst case)	**11**

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Persistence of Effect After Discontinuation

Drug	
Zoledronic acid	
Alendronate	
Risedronate	
Ibandronate	**???**
D'mab, PTH, SERMS, HT	

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Throw the baby out with the bath water?????

Monoclonal Gammopathies and Bone Health

G. David Roodman, M.D., Ph.D.

Monoclonal Gammopathies and Bone Health

G. David Roodman, MD, PhD, Indiana University Department of Medicine, Hematology/Oncology

Significance: Myeloma is the most frequent cancer to involve the skeleton and over 80% of myeloma patients have bone disease. Myeloma Bone Disease (MMBD) has a tremendous impact on the patient's quality of life, and can result in severe bone pain, pathologic fractures, hypercalcemia, and increased mortality. Almost 20% of myeloma patients will present with a pathologic fracture and almost 60% of patients will sustain a pathologic fracture over their disease course. Patients with pathologic fractures have a 20% increase in mortality compared to patients without pathologic fractures, and the cost of myeloma bone disease adds at least $50,000 to the care costs for each patient compared to myeloma patients without bone disease. Further, MMBD can continue to progress even when patients are in complete remission from their tumor. Importantly, myeloma bone lesions rarely if ever heal even when the patients are in long term complete remission. Importantly, patients with the precursor conditions, monoclonal gammopathy of undetermined significance (MGUS) and smoldering myeloma (SMM), also have increased bone loss even though they do not have active myeloma. In this session, the mechanisms responsible for MMBD and bone loss in MGUS and SMM, and potential therapeutic approaches based on these mechanisms will be discussed.

Learning objectives: As a result of participating in this session, attendees should be able to:

1) State the differences between MGUS, SMM and active myeloma.

2) Identify three factors produced by myeloma cells or induced by myeloma cells of the marrow microenvironment that simulate bone destruction or inhibit osteoblast differentiation.

3) State current therapies for bone disease in patients with monoclonal gammopathies and be aware of potential new therapeutic approaches for bone disease in these patients.

Outline: Classifications of plasma cell dyscrasias:

Patient Criteria	MGUS[1,2]	Smoldering Myeloma[1]	Active Myeloma
M protein	< 3 g/dL spike	≥ 3 g/dL spike and/or	In serum and/or urine[2]
Monoclonal plasma cells in bone marrow, %	< 10	≥ 10	≥ 10[2]
End-organ damage	None	None	≥ 1 CRAB* feature[3]
Bone Disease	Increased fracture Risk	Increased fracture Risk	Lytic bone lesions

1. IMWG. Br J Haematol. 2003;121:749-757. 2. Kyle RA, et al. N Engl J Med. 2002;346:564-569.
3. Durie BG. et al. Hematol J. 2003:4:379-398.

MGUS:

1. MGUS occurs in 2% of the population over the age of 50 years and increases to 8% in patients >80 years.

2. Only 1% of MGUS patients per year progress to myeloma, although all MM patients initially had MGUS.

3. Cytogenetic changes present in plasma cells from active MM patients are already present in almost all MGUS patients regardless of whether they progress to MM or not. Thus, extrinsic changes such as alterations in the bone marrow microenvironment that previously controlled tumor growth may contribute to progression to MM.

4. MGUS patients have increased bone loss and axial fractures than age-matched controls. There is no association between the concentration of the monoclonal protein and the risk of fractures in MGUS patients.

5. Drake and colleagues found that serum levels of the Wnt inhibitor DKK1and the osteoclast activating factor MIP-1 alpha were significantly elevated in MGUS patients, and that MGUS patients also have increased cortical porosity and a lower apparent modulus in the distal radius suggestive of decreased bone strength. This was despite larger radial bone size.

6. Imaging studies using either MRI or FDG-PET have not shown any abnormalities in patients with MGUS in contrast to patients with SMM and active myeloma.

7. Therapeutic recommendations for bone loss in patients with MGUS are similar to those with osteoporosis, including oral alendronate, as well as zoledronic acid given every six months at 4 mg per dose. Both improve bone mineral density and decrease bone loss.

SMM:

- No symptoms; no related organ/tissue impairment. Have increased fracture risk.
- 10% to 20% of newly diagnosed myeloma [1]
- Can remain indolent for yrs
- Progression rate: ~ 50% at 5yrs [2]
  - Progression rate in high-risk subgroup: 50% at 2 yrs[3]

1. Kyle RA. ASCO Connection. 2012. 2. Kyle RA, et al. Br J Haematol 2007;139:730-743.
3. Mateos MV, et al. N Engl J Med. 2013;369:438-447. 4. Mateos MV, et al. Curr Hematol Malig Rep. 2013;8:270-276

1. High-risk SMM: progress within 2 yrs and have >5% circulating PCs, > 10% PCs on BM, > 3 g/dL IgG or 2 g/dL IgA M protein, immunoparesis, and abnormal κ/λ ratio.

2. Phase III trial of Zoledronate 4mg q month vs zoledronate and thalidomide showed decreased skeletal related events for both treatments, but only zol/thal combination slowed progression to active MM.

3. 86% of patients with SMM had normal MRIs and pet scans.

Multiple myeloma bone disease:

1. Myeloma is currently incurable and MMBD remains a major contributor to the morbidity and mortality of myeloma patients. Patients with fractures have a 20% increase in mortality.

2. MMBD is characterized by purely osteolytic bone destruction with markedly increased osteoclast activity adjacent to the tumor cells and little or no osteoblast activity.

3. Because there is little or no new bone formation in response to the bone destruction, bone scans can severely underestimate the extent of MMBD.

4. The increased bone destruction is mediated by the osteoclast and not tumor cells themselves, although tumor cells can directly stimulate osteoclast formation and suppress osteoblast differentiation.

5. Myeloma cells also induce cells in the marrow microenvironment to produce factors that drive osteoclast formation and suppress osteoblast formation.

6. Immune cells also contribute to the bone destructive process through production of cytokines and adhesion molecules that increase myeloma cell growth, enhance myeloma cell chemoresistance, increase osteoclastogenesis (in part, by driving dendritic cell and tumor-associated macrophages

towards the osteoclast lineage), suppress osteoblastogenesis, and polarize T cell subsets from predominantly Th1 to Th17.

Factors Stimulating osteoclast formation in MM:

1. Early studies of MMBD identifed Osteoclast Activating Factor activity (OAFs) in conditioned media from myeloma cell lines that stimulated bone resorption in bone organ culture systems.

2. Multiple factors have since been identified as important OAFs in myeloma, including RANKL, MIP-1α, TNF-α, Interleukin-3 (IL-3), and IL-6.

3. Several of these factors (IL-3,TNFα, MIP-1α)also suppress osteoblast formation and/or support myeloma cell growth/survival directly, indicating that they have multiple roles in MMBD.

4. RANKL is produced both by MM cells and is induced in bone marrow stromal cells (BMSC) by adhesive interactions between MM cells and BMSC via VCAM1 on BMSC and α4β1 integrin on myeloma cells. 1,25D3 can increase RANKL levels in MM cells and BMSC in MM as well enhance adhesive interactions between MM cells and BMSC to increase tumor cell growth.

5. OPG is markedly decreased in MMBD and the RANKL/OPG ratio in serum impacts survival of MM patients. Patients with high RANKL/OPG ratio have shortened survival.

6. In addition to resorbing bone and releasing growth factors from matrix, osteoclasts in MM also secrete several factors that support myeloma cells, including IL-6 (the most important growth factor for MM cells), TNFα, annexin II, BAFF and APRIL.

7. MM cells produce TNFα which induces RANKL and XBP1 in BMSC. XBP1s overexpression in BMSCs increases gene and protein expression of VCAM-1, IL-6, and RANKL, enhancing BMSC support of MM cell growth and osteoclast formation in vitro and in vivo.

8. MM cells also produce MIP-1α (CCL3), a potent osteoclast inducing chemokine that, like TNF-α, can both directly stimulate human osteoclast formation and potentiate the effects of RANKL. Small molecule antagonists to CCR1, a MIP-1α receptor, have been studied in models of myeloma and shown to block both tumor growth and bone destruction.

9. IL-3 levels are increased in the marrow of approximately 70% of myeloma patients and IL-3 is produced by myeloma cells and T cells in the myeloma microenvironment. IL-3 can both stimulate osteoclastogenesis and inhibit osteoblast formation. The effects of IL-3 appear to be indirect. IL-3 stimulates marrow macrophages in the myeloma microenvironment to produce activin A which increases osteoclast formation and suppresses osteoblast differentiation.

10. IL-6 is another potent inducer of human osteoclast formation produced in the myeloma microenvironment in response to myeloma cells and by myeloma cells themselves. IL-6 can directly induce human osteoclast formation and induce RANKL production as well as prevent MM cell apoptosis.

11. T cells in the MM microenvironment are predominantly Th17 rather than Th1 cells. IL-17 enhances the effects of RANKL on osteoclast formation.

Osteoblast Suppression in MMBD:

1. Osteoblast differentiation is severally inhibited in patients with myeloma and remains suppressed even after the tumor cells are eradicated so that bone lesions rarely heal.

2. Adiponectin, an adipocyte-derived factor is decreased in both mouse myeloma models and human bone marrow from MM patients and reduced levels of Adiponectin are permissive for myeloma growth.

3. Multiple osteoblast inhibitors are produced by MM cells or induced in BMSC by MM cells including DKK1, IL-3/ActA, sclerostin, IL-7 and HGF. HGF is a negative regulator of BMP-induced OBL differentiation. However, none of these can completely explain the long term suppression of OBL differentiation since once tumor cells are irradicated, they should not persist.

4. GFI-1, a transcriptional repressor of RUNX2 is also upregulated in BMSC from MM patients. Recent studies have reported that GFI-1 binds directly to the Runx2 promoter, that Gfi1 can recruit histone modifying enzymes to the Runx2 promoter, which may explain long term suppression of osteoblast differentiation in patients with myeloma.

Emerging role of the osteocyte in MMBD:

1. Recently, we and others have shown that myeloma cells induce increased osteocyte apoptosis and that this may be a critical contributor to MM induced bone disease.

2. In preliminary studies we found that interactions between osteocytes with MM cells have a profound effect on osteocytic gene expression, increasing Sost and RANKL transcripts and decreasing OPG.

3. Sclerostin levels are increased in MM patients and correlate with the extent of bone disease.

4. Direct interactions of osteocytes with MM cells induce caspase3-dependent osteocyte apoptosis, triggered by rapid activation of Notch signaling through cell-cell contact that is then maintained by accumulation of MM-derived TNFα.

Current and potential novel therapies for MMBD:

Current Treatment of MMBD:

- Bisphosphonates
  - Pamidronate
  - Zoledronic acid
- Surgical procedures
  - Vertebroplasty
  - Balloon kyphoplasty
- Radiotherapy
- RANKL inhibitor denosumab (investigational in this setting)

Potential Therapies:

Target	Potential Therapy
DKK1/sFRP-2	Anti-DKK1, Bortezomib
IL-3/Activin A	ACE-011
RANKL	Denosumab
MIP-1 alpha antagonist	CCR1 Receptor
GFI-1	GFI-1 anti-sense

References:

1. Melton, L.J., 3rd, et al., Fracture risk in monoclonal gammopathy of undetermined significance. J Bone Miner Res, 2004. 19(1): p. 25-30.

2. Kristinsson, S.Y., et al., Bone disease in multiple myeloma and precursor disease: novel diagnostic approaches and implications on clinical management. Expert Rev Mol Diagn, 2011. 11(6): p. 593- 603.

3. Terpos E, Berenson J, Cook RJ, Lipton A, Coleman RE. Prognostic variables for survival and skeletal complications in patients with multiple myeloma osteolytic bone disease. Leukemia. 2010;24(5): 1043-1049.

4. Farr, J.N., et al., Altered cortical microarchitecture in patients with monoclonal gammopathy of undetermined significance. Blood, 2014. 123(5): p. 647-9.

5. Ng, A C., et al., Bone microstructural changes revealed by high-resolution peripheral quantitative computed tomography imaging and elevated DKK1 and MIP-1alpha levels in patients with MGUS. Blood, 2011. 118(25): p. 6529-34.

6. Witzig, T.E., et al., A phase Ill randomized trial of thalidomide plus zoledronic acid versus zoledronic acid alone in patients with asymptomatic multiple myeloma. Leukemia, 2013. 27(1): p. 220-5.

7. Landgren 0, Kyle RA, Pfeiffer RM, et al. Monoclonal gammopathy of undetermined significance (MGUS) consistently precedes multiple myeloma: a prospective study. Blood. 2009; 113(22):5412-5417.

8. Garrett IR, Durie BG, Nedwin GE, et al. Production of lymphotoxin, a bone-resorbing cytokine, by cultured human myeloma cells. N Engl J Med. 1987;317(9):526-532.

9. Morgan GJ, Davies FE, Gregory WM, et al. First-line treatment with zoledronic acid as compared with clodronic acid in multiple myeloma (MRC Myeloma IX): a randomised controlled trial. Lancet. 2010;376(9757): 1989-1999.

10. Giuliani N, Colla S, Morandi F, et al. Myeloma cells block RUNX2/CBFA1 activity in human bone marrow osteoblast progenitors and inhibit osteoblast formation and differentiation. Blood. 2005; 106(7):2472-2483.

11. Tian E, Zhan F, Walker R, et al. The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. N Engl J Med. 2003;349(26):2483-2494.

12. D'Souza S, Del Prete D, Jin S, et al. Gfi1 expressed in bone marrow stromal cells is a novel osteoblast suppressor in patients with multiple myeloma bone disease. Blood. 12 011; 118(26):6871-6880.

13. Li X, Pennisi A, Yaccoby S. Role of decorin in the antimyeloma effects of osteoblasts.Blood. 2008; 112(1):159-168.

14. Giuliani N, Ferretti M, Bolzoni M, et al. Increased osteocyte death in multiple myeloma patients: role in myeloma-induced osteoclast formation. Leukemia. 2012.

Sarcopenia: Definition and Assessment

Robert McLean, DSc

ASBMR Meet-the-Professor Session Friday September 12, 2014

Sarcopenia: Definition and Assessment

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Significance of the Topic

The age-related loss of muscle mass and strength, termed sarcopenia, is common in older adults and leads to frailty, poor mobility, loss of independence and institutionalization. As such, sarcopenia is a substantial healthcare problem that will only continue to grow along with the rising number of older adults. Effective treatments and preventive measures are needed to reduce the public health and economic burdens attributable to sarcopenia.

Unfortunately, development of interventions has been severely hindered by the lack of a standard clinical or research definition of sarcopenia. During this session, the evolution and current state of sarcopenia assessment will be presented and discussed. The ongoing challenges to developing consensus sarcopenia criteria will be examined, and future directions for reaching this goal will be discussed.

Learning Objectives

As a result of participating in this session, attendees should be able to better understand:

1. The determinants and consequences of sarcopenia
2. Why a consensus sarcopenia definition is needed
3. The evolution of and latest developments in defining sarcopenia
4. Current challenges in assessment of muscle mass and strength in older adults

Discussion Points and References

1. What is sarcopenia and why do we care?

- Janssen, I., et al., The healthcare costs of sarcopenia in the United States. J Am Geriatr Soc, 2004. 52(1): p. 80-5.
- Walston, J.D., Sarcopenia in older adults. Curr Opin Rheumatol, 2012. 24(6): p. 623-7.

2. Why do we need a consensus sarcopenia definition?

- Roman, D., K. Mahoney, and A. Mohamadi, Sarcopenia: what's in a name? J Am Med Dir Assoc, 2013. 14(2): p. 80-2.

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b. *Upper vs. lower extremity?*

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e. *What are the relevant clinical and research sarcopenia outcomes?*

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Using Human iPS cells to Model Skeletal Diseases

Edward Hsiao, M.D., Ph.D.

Using Human iPS cells to Model Skeletal Diseases

2014 ASBMR Meet the Professor Session

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Introduction

Musculoskeletal conditions such as osteoporosis, fractures, and skeletal malformations are among the most frequently reported medical conditions in the USA and are the second-greatest cause of disability worldwide (1). Inherited skeletal disorders are among the most common genetic diseases (2) and affect 2.4 in 10,000 births with 23% of the affected presenting as stillbirths and 32% mortality in the first week of life (3). In adults, osteoporosis affect over 10 million people in the United States and results in over 2 million fractures each year (4). Being able to model these conditions in a ***human model system*** is one critical tool for developing therapies for these medically important diseases.

Major challenges hinder our understanding of human skeletal diseases

Achieving a better understanding of human skeletal development has several major challenges:

First, the genetic factors underlying skeletal diseases are complex. Many of the traits and diseases we associate with the skeleton (e.g., height; osteoporosis) are multi-genic in origin. In addition, some genes have distinct functions in humans that vary significantly from what occurs in model organisms such as rodents (5-7). Although model organisms provide valuable insights into biology, these genetic complexities indicate that having a continuous source of ***human tissues*** would be extremely valuable for understanding disease pathophysiology and translating our knowledge into new treatment strategies. Until recently, this has been a major hurdle since obtaining primary tissues from humans can be very difficult or impossible.

Second, a surprisingly large number of severe skeletal and non-skeletal medical conditions remain “undiagnosed” with only rudimentary molecular understanding of the disease pathogenesis. Patients with these rare or orphan conditions often face diagnostic and treatment delays, which can be improved when the disease process is discovered. Importantly, research into some of these rare presentations has identified key pathways leading to breakthrough discoveries and medications that benefit the wider population (e.g., the role of *SOST* in regulating bone mass). This demonstrates that ***rare disease models*** can highlight important pathways and help address the unmet medical needs of more complex polygenic diseases such as osteoporosis.

Third, during the past several decades, bone researchers have focused on autologous cells such as mesenchymal stem cells (MSCs) or adult stem cells (e.g., adipose-derived stem cells). These multi-potent cell types are finding applications in regenerative therapies. However, isolating large numbers of primary cells remains difficult: one report showed that 30 ml of human bone marrow yielded only 7-22x10^6 phenotypic MSCs after 4 weeks of culture, with some samples requiring extended culture (8). In addition, multiple donors are needed as sources for different cell types (i.e., MSCs, endothelial cells, muscle stem cells, etc.), introducing different genetic backgrounds as a new confounder. This also decreases the likelihood that a composite allograft could be created from a single donor, and increases the risk of allograft rejection if a multi-donor allograft was used. Finally, other cell types abundant in bone, such as neurons or

hematopoietic cells, cannot be easily generated from MSCs and thus their contributions are difficult to explore. Human iPS cells help address this challenge by allowing us to ***potentially generate any cell type of interest.***

Significance of pluripotent cells for skeletal research

Stem cells are defined as having two basic properties: the ability to self-renew and the potential to differentiate into one or more specialized cell types. Stem cells are critical for maintaining tissues that normally have high turnover such as skin and blood. However, it is increasingly recognized that many organs, even ones with low proliferative capacity, contain tissue-specific stem cells that contribute to their growth and maintenance. These tissue-specific cells typically have limited differentiation potential and can create only a subset of cell types (called multipotency). In contrast, cells in the mammalian early embryo can contribute to any tissue in the body (called pluripotency) (9; 10).

Pluripotent cells such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells are well suited for modeling human physiology, pathophysiology, and development since they can create any cell types that are needed, if the appropriate differentiation protocols are available. Although multi-potent stem cells like MSCs or adult stem cells are valuable for studying skeletal diseases, pluripotent cells would allow us to ***generate lineages that may be critical for bone formation***, ***but outside of the normal repertoire*** for lineage-restricted multi-potent cells (i.e., neural crest cells, neurons, immune cells). In addition, since many of the pathways that regulate skeletal development also have critical roles in other tissue types, human pluripotent cells can be used to ***study these functions in non-skeletal tissues***. Furthermore, starting from a pluripotent cell allows us to create a ***continuous supply of isogenic cell types***, thus minimizing the effects of variations in genetic background that may occur with primary cells.

Human embryonic stem cells (ES cells)

Human ES cells are derived from human embryos created from eggs fertilized *in vitro* (11; 12). Briefly, these cell lines are derived from blastocysts that have been plated on a tissue culture surface to allow the ***inner cell mass*** to expand. The surviving cells grow to create a renewable cell population. Cells that maintain a normal genetic background, and remain in a pluripotent state (i.e., do not differentiate into a terminal cell type), become an embryonic stem cell line. A number of human ES cell lines are currently available. NIH supports research using a select number of lines that have met specific quality control and ethical standards (***http:// escr.nih.gov***).

Human induced pluripotent stem cells (iPS cells)

Human ES cells have always been plagued by three potential limitations: 1) ethics surrounding the derivation of the ES cell lines (e.g., source of the donor oocytes and the need for a fertilized embryo); 2) the difficulty to directly model a specific patient's disease mutation; and 3) the technical challenges of isolating the inner cell mass cells to create the ES cell lines.

The ***discovery of mouse (13) and human iPS cells by Shinya Yamanaka's laboratory*** in 2007 (14) revolutionized the stem cell field by providing a relatively straight forward method to create pluripotent cells from a differentiated cell source. All of the current methods activate a pluripotency transcriptional network to convert a more differentiated cell into a pluripotent-like cell.

Many iPS cell induction methods are now widely used and demonstrate that there are many roads to pluripotency. Methods include retroviral transduction (14); DNA constructs (15); non-integrating episomes (16); non-integrating Sendai viruses (17); non-integrating modified mRNA transduction (18; 19), transposons (20), and small molecules (21). The field of reprogramming continues to innovate and many new methods are constantly being made available. Many of

these techniques have been used to reprogram multiple types of terminally differentiated cells. However, the two most common remain human skin fibroblasts and peripheral blood lymphocytes.

Directed differentiation of pluripotent stem cells

A tremendous library of protocols, too large to list here, is now available describing many ways to created differentiated cell lines from pluripotent stem cells. Over the past several years, new methods have been developed specifically for human iPS cells. These methods use different approaches, including robust small molecule directed differentiation protocols (i.e., cardiomyocytes, neurons, and endothelial cells), expression of master transcription factors (i.e., skeletal muscle), and culture in conditions that favor the formation of specific lineages (chondrocytes and mineralizing cells).

Directed differentiation methods continue to improve, particularly with the use of newer scaffolds and culture matrices. However, several factors need to be kept in mind: the specific protocols used in directed differentiation methods may be cell type specific; many commercial differentiation media are proprietary (i.e., osteogenic media often contains BMPs, and this is a problem if you need to manipulate the BMP pathway); and a detailed optimization process is usually necessary when applying the method to other cell lines. In addition, the use of specific media conditions can make co-cultures particularly challenging since the individual cell types may not survive together if the culture conditions are not compatible. Finally, human iPS cells appear to differentiate easily into immature cell types in a dish; however, more mature cell types may require advanced 3D or *in vivo* environments (22).

Despite these limitations, the ability to make specific cell types from iPS cells carrying a specific disease mutation is exciting for disease modeling since in many cases, the specific cell types that are affected by the mutation are not easily identified or obtained from primary samples.

Genetic manipulation of iPS cells

Although mouse ES cells have long been amenable to genetic engineering, human ES and iPS cells appear to be much more resistant to traditional approaches such as homologous recombination. New approaches using directed nucleases such as ***TALENs*** (23) and ***CRISPRs*** (24) to introduce nicks into genomic DNA show great promise as a way to induce recombination. These methods will help speed the process of genomic targeting for both mouse and human cells, but are associated with a risk of off-target nicks requiring careful analysis to assure that high quality isogenic cell lines are generated.

iPS cell pearls based on our experience

Several considerations should be kept in mind when considering an iPS cell project:

- ***The reprogramming process usually takes approximate 3-4 months after obtaining the patient cell sample,*** if things go well. Reprogramming is still highly dependent on the quality of the original source cells (blood or fibroblasts, for example). Earlier passage cells tend to reprogram better than more senescent cells.

- ***Method of reprogramming may be important:*** In our studies using iPS cells created from patients with fibrodysplasia ossificans progressiva (FOP), we found that retroviral and episomal methods could create FOP iPS cells (25). Prior reports using Sendai virus indicated that the FOP iPS cells were not able to maintain their pluripotent state (26). If one method of reprogramming doesn't work, trying a different method is important because a particular genetic mutation or background effect may affect the efficiency of one method vs. another.

- ***The characterization of iPS cells is an important assessment of quality.*** The basic

characterization should include pluripotency (ability to form representative cells from each of the 3 germ layers); silencing or absence of the iPS transcriptional factors; activation of stem cell genes; normal karyotype; ability to freeze and recover cells; promoter methylation consistent with pluripotency, and phenotypic stability with culturing. Although teratomas remains the gold standard for assessing the pluripotency of an iPS cell line, many other methods including *in vitro* embryoid body differentiation and genomic analysis methods are now available to assist with determining pluripotency of a cell line (27).

- ***Choice of culture conditions:*** Pluripotent cells are typically grown using three basic techniques: on a feeder cell layer, which may be mouse or human cells that provide nutrients and growth factors to the ES cells or iPS cells; using conditioned media; or in defined culture conditions using feeder-free methods. Each method has advantages and disadvantages and there is no one clearly better method (28). While feeder layers (i.e., SNLs, mouse embryonic fibroblasts, human dermal fibroblasts) are cumbersome to work with and introduce a large "black box" where the specific biological factors in the culture may not be known, the method is very well established and seems to work better for some cell lines that may be more unstable. In contrast, newer feeder-free conditions (i.e., mTeSR, E8, etc.) have the advantage of well-defined or recombinant components and potentially less frequent cell feedings. This helps minimize batch-to-batch variability in the culture media and increase experimental consistency. Unfortunately, iPS cell culture is still expensive (such as for media, growth surfaces, cytokines) and labor intensive (for changing cell media every 1-2 days, close monitoring of cell morphology, and maintaining clean culture conditions as cultures are done without antibiotics).
- ***Having a defined in vitro phenotype:*** It is important to understand what the *in vitro* assay will be, and how it relates to your *in vivo* phenotype. For example, what does a behavioral phenotype such as autism look like in a tissue culture dish?
- ***Need for close monitoring:*** Identifying iPS cells is still highly dependent on cellular morphology. Changes in morphology are often the first sign that culture conditions are no longer supporting pluripotency (i.e., that the bFGF2 has gone bad) or that differentiation is occurring. Although a number of molecular assays are now available, having someone with experience in looking at iPS cells is still critical for successful iPS cell culture.
- ***Significant functional diversity among cell lines:*** As the number of ES and iPS cell lines grows, it is clear that there are functional differences between cell lines derived from different patients (not really surprising) and also differences between cell lines derived from the same patients. Much of the molecular basis for this variation remains unknown but may be related to reprogramming efficiency or other factors such as epigenetics. These factors should be considered at the start of the study. In addition, methods such as correcting the mutation via gene editing with CRISPRs or TALENs, or introducing the mutations into a "normal" background, should be evaluated. While these strategies may have more up-front work, they may help reduce the biological variability of an assay.

Future directions

Human iPS cells are a promising way to generate human cell types from patients with genetic diseases, for disease modeling, drug screening, and for tissue engineering. iPS cells provide an important complement to adult stem cells and mesenchymal stem cells by allowing the creation of a broader array of cell types. More widespread application of iPS cells to musculoskeletal diseases will require the development of better directed differentiation protocols that exhibit high

yield, cellular uniformity, and ease of use, particularly for lineages directly relevant to musculoskeletal tissues. In addition, new marker and reporter lines for identifying skeletal gene expression; cell surface markers for purifying mesenchymal lineages; and libraries of diseased and genetically-corrected human iPS cells will be extremely valuable tools for advancing the application of pluripotent stem cells for musculoskeletal diseases.

Additional resources

International Society for Stem Cell Research (ISSCR): http://isscr.org
California Institute for Regenerative Medicine (CIRM): http://www.cirm.ca.gov/
National Institutes of Health (NIH): http://stemcells.nih.gov/Pages/Default.aspx
StemBook (one of many sources of protocols and reviews): http://www.stembook.org/

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Wnt Signaling in Bone

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WNT Signaling in Bone

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Bone homeostasis is tightly controlled by WNT signaling. The importance of canonical WNT signaling in particular is highlighted by

- the striking effects of rare human mutations in WNT pathway genes on bone
- an abundance of mouse genetic studies confirming its importance in bone
- genome-wide association studies identifying natural variants within WNT pathway genes to be associated to bone mineral density

Activation of the pathway leads to increased, and inhibition leads to decreased bone mass and strength. WNT signaling impacts cells of the osteoblastic lineage and osteoclasts and thus both bone formation and resorption. It is subject to complex tight regulation involving multiple ligands, cell surface receptors and facilitators, as well as a number of extracellular antagonists, some of which are relatively specific to bone. Consistent with what is observed in other tissues, extensive crosstalk exists between WNT signaling and other pathways in bone - amongst them PTH and BMP signaling as major bone anabolic pathways.

Given its pivotal role in regulating adult bone homeostasis, canonical WNT signaling is currently explored for generation of therapeutic agents for treatment of common and rare and bone fragility disorders such as postmenopausal osteoporosis and certain types of osteogenesis imperfecta. The most promising approach to date encompasses neutralizing antibodies to the osteocyte secreted WNT antagonist sclerostin which have been demonstrated to increase bone mineral density in postmenopausal osteoporotic women.

As a result of participating in this session, attendees should be able to

- understand the implication of WNT and there specifically canonical WNT signaling in bone homeostasis
- appreciate its impact on osteoblasts, osteocytes and osteoclasts and on cancellous and cortical bone
- recognize the therapeutic potential of targeting canonical WNT signaling for the treatment of common and rare bone diseases
- realize also the limitations and open questions in respect to therapeutic targeting of the pathway for bone diseases and to the use of serum levels of WNT antagonists for prediction of local bone turnover

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Bone Cells and Energy Metabolism

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Meet-the-professor session: Bone Cells and Energy Metabolism
Fanxin Long

Significance of the Topic:

While recent studies have implicated bone in the regulation of whole-body metabolism, this session will focus on cellular metabolism of osteoblasts.

Energy metabolism is fundamental to the cell's existence. Cellular metabolism is not only generates ATP but also provides essential intermediate metabolites to be used for anabolic reactions. Whereas the general metabolic pathways are well described, each specific cell type may utilize the different pathways differently, exhibiting unique metabolic features.

The energy and carbon source for osteoblasts are not well understood. Studies in the 1960s revealed that bone slices utilize glucose briskly in cultures, but produce mostly lactate as the end product, even in the presence of abundant oxygen. This phenomenon is known as aerobic glycolysis, best known to occur in cancer cells. Interest in this area of research waned during the past several decades, but is now poised for a resurgence. Remaining questions are numerous. What is the functional significance of aerobic glycolysis? What is the molecular basis for such significance? Since glucose is used in an energy-insufficient manner (aerobic glycolysis produces 2 ATP verus ~30 ATP by Krebs cycle), what other energy source do osteoblasts rely on?

A clear understanding of cellular metabolism in osteoblasts will not only advance basic biology, but may also provide novel mechanistic insights to bone frailty associated with disease conditions and aging.

Learning Objectives:

Through this session, attendees are expected to achieve the following goals:

1) appreciate the importance of cellular metabolism
2) be familiar with the current knowledge about glucose metabolism in osteoblasts
3) learn about examples of remaining questions in the field

Figure 1

Energetics of glucose metabolism

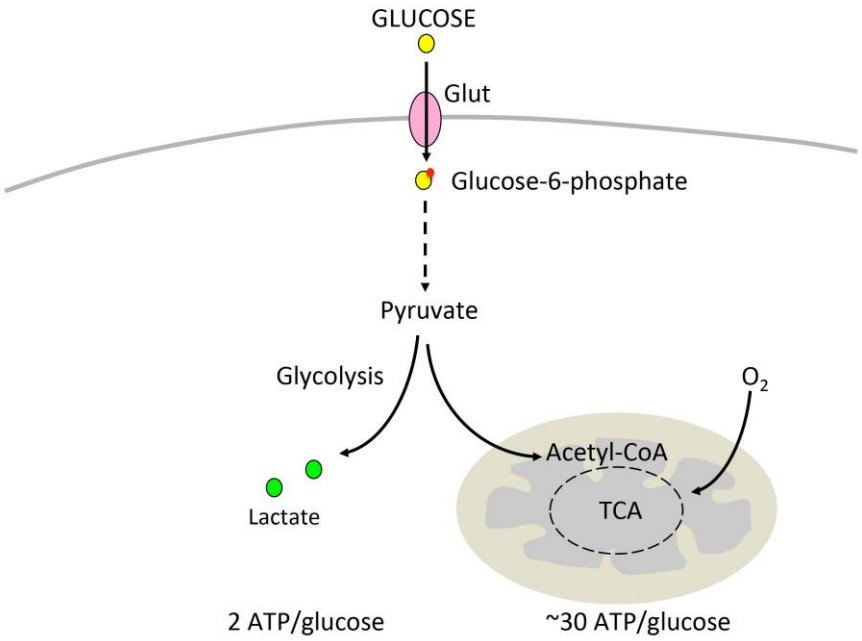
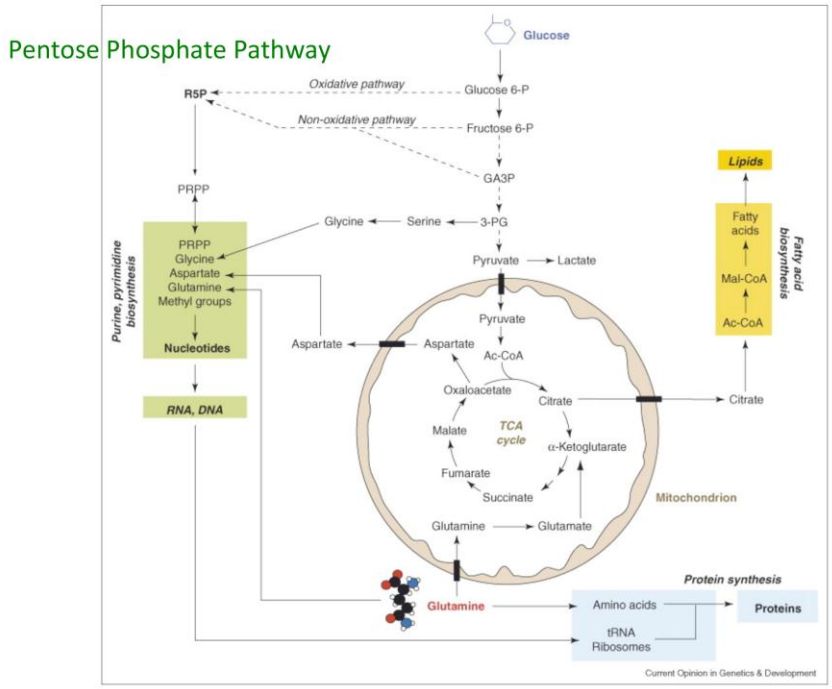


Figure 2

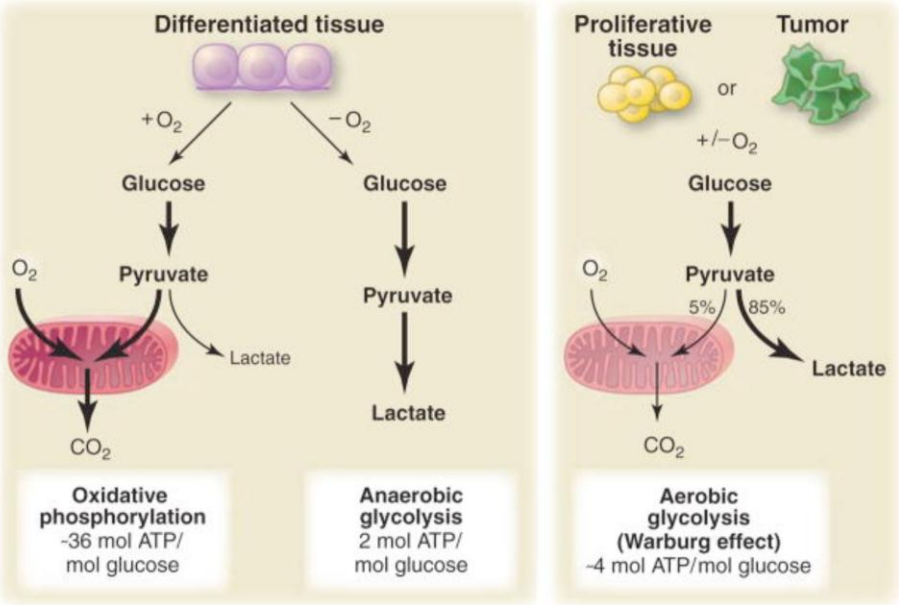
Glucose metabolism provides biosynthesis precursors



DeBerardinis et al., 2008, Current Opinion in Genetics &Development 18, 54-61

Figure 3

The Warburg Effect (proliferative metabolism)



Vander Heiden, Cantley and Thompson, 2009, Science 324: 1029-33

Figure 4

Borle, Nichols and Nichols, 1960, Metabolic studies of bone in vitro, I. normal bone, JBC

TABLE II

Lactic and citric acid production and glucose uptake in normal bone

Values are expressed as μmoles per hour per mg of cell N and represent the means of 2-hour incubations in bicarbonate buffer at pH 7.4. Glucose, when present, was added to the medium at a final concentration of 2 mg per ml. Differences between aerobic and anaerobic conditions were statistically significant only in the case of lactate production in the presence of glucose ($p = 0.001$).

	Lactate		Citrate	Glucose uptake
	Glucose present	Glucose absent	Glucose present	
Aerobic				
Mean	2.56	0.37	0.035	1.52
s.d.	0.42	0.12	0.014	0.48
No. of incubations	7	5	5	5
Anaerobic				
Mean	5.46	0.48		1.47
s.d.	0.69	0.04		0.38
No. of incubations	6	2		6

TABLE III

Aerobic production of lactate by normal bone

Values are expressed as μmoles per hour per mg of cell N and represent the means of 2-hour incubations. Glucose, when present, was added to the medium at a final concentration of 2 mg per ml. A significant difference ($p = <0.01$) was observed between the two systems only when glucose was present.

	Glucose present	Glucose absent
With HCO_3^- buffer		
Mean	2.56	0.37
s.d.	0.42	0.12
No. of incubations	7	5
With P_i buffer		
Mean	1.93	0.35
s.d.	0.12	0.05
No. of incubations	5	10

DISCUSSION

Although the observations reported here are insufficient to provide a detailed understanding of the metabolism of adult bone cells, certain general conclusions concerning the over-all metabolic pattern of this tissue can be drawn from them. First, bone cells have a very active metabolism consuming glucose from the medium at a rapid rate. Indeed, their rate of glucose uptake was greater, even under anaerobic conditions, than rates reported by others for liver (18), or any other tissue so far studied with the exception of adipose tissue (17).

In contrast to kidney, muscle, and liver which have relatively high rates of oxygen consumption, bone, like cartilage (19), appears to have chiefly an anaerobic or glycolytic metabolic pattern. Thus, the major portion (84%) of the substrate consumed by adult bone in the presence of oxygen passes only through the glycolytic pathways to form lactate as an end product leaving but 16% to go through the tricarboxylic acid cycle or for synthetic processes.

The rate of citrate release into the medium in our preparation (0.035 μmole per hour per mg of cell N) was almost identical to the rate which can be calculated from the data reported by Kenny *et al.* (20) by means of a completely different experimental system (0.033 μmole per mg of bone N per hour). This comparatively extremely small rate of citrate release suggests that mobilization of bone mineral by production of this acid plays at most a minor role in the maintenance of extracellular fluid Ca and P

Figure 5

Borle, Nichols and Nichols, 1960, Metabolic studies of bone in vitro, II. The metabolic patterns of accretion and resorption, JBC 235, 1211-1214

TABLE II

Lactate production of bone

Values are expressed as μmoles per hour per mg of cell nitrogen calculated from the means of 2-hour incubations in HCO_3^- buffer at pH 7.4. Glucose, when present, was at a final concentration of 2 mg per ml of medium.

	Glucose present		Glucose absent	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Control				
Mean	2.56	5.46	0.37	0.48
s.d.	0.42	0.69	0.12	0.04
No. of incubations	7	6	5	2
PTE				
Mean	3.44	4.42	0.33	0.36
s.d.	0.62	0.74	0.10	0.32
No. of incubations	8	8	5	6
Estradiol				
Mean	2.07	2.21		
s.d.	0.55	0.26		
No. of incubations	7	6		

Figure 6

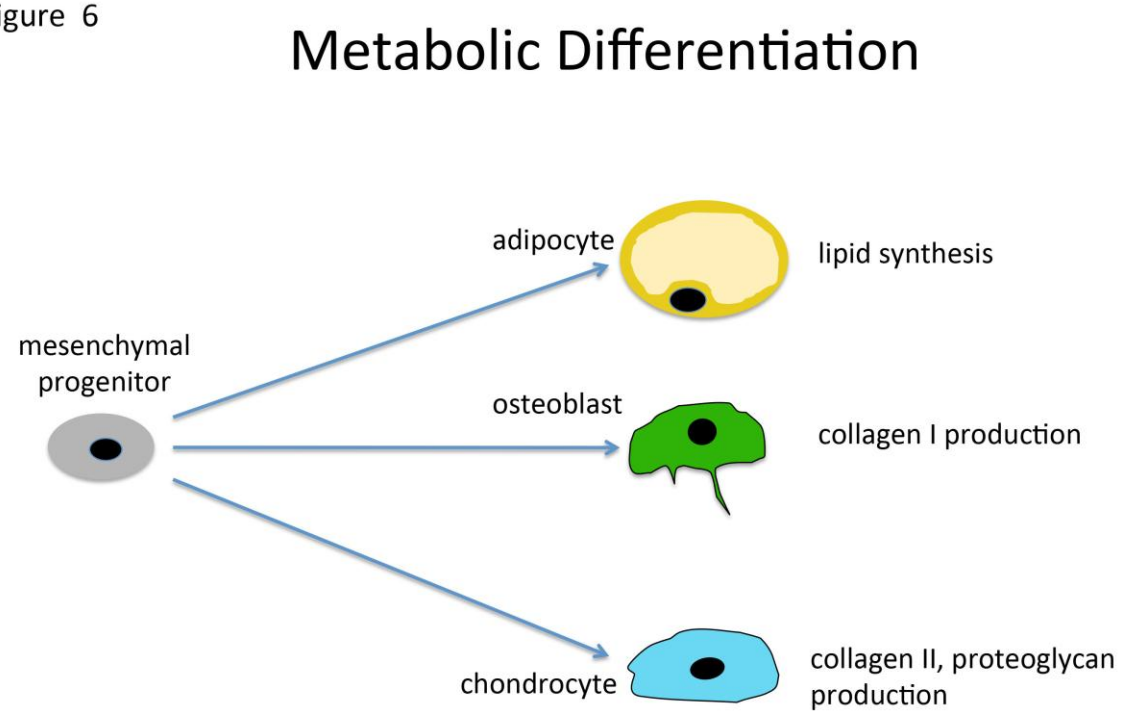
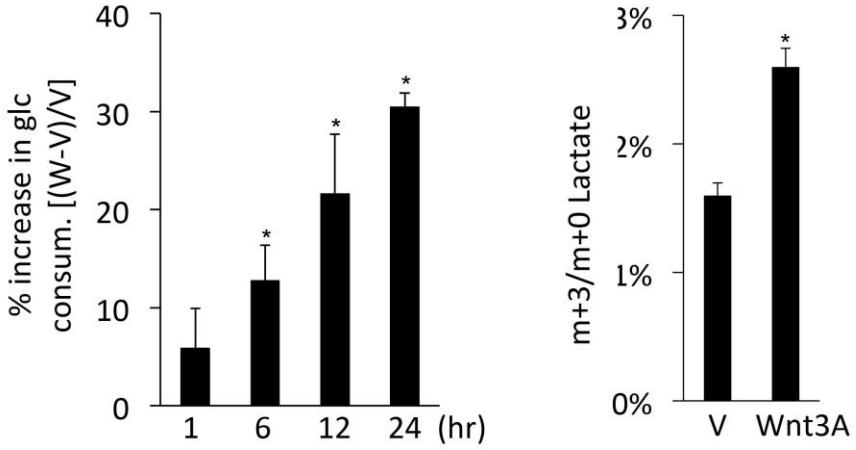


Figure 7

WNT induces aerobic glycolysis during osteoblast differentiation



Esen et al., 2013, Cell Metab 17, 745-55

Figure 8

Examples of remaining questions

- How does aerobic glycolysis affect osteoblast differentiation and function?
- Metabolic regulation by other signals, transcription factors?
- Amino acids, fatty acids?
- Does osteoblast metabolic profile change in diseases conditions, with aging?
- How does osteoblast metabolic profile differ from related cell types (adipocytes, chondrocytes)?

HIV and Bone

Michael Yin, M.D.

Meet the professor session "**HIV and Bone**"

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A. Significance

With advances in antiretroviral therapy, HIV-infected individuals in resource-rich countries are living longer and dying more from non-AIDS complications (cardiovascular disease, cancer, liver and kidney failure) than opportunistic infections. Guidelines now recommend treatment of all HIV-infected individuals with CD4<500 cells/ml. Moreover, in certain populations, such as age>50, hepatitis co-infection, nephropathy, or pregnancy, treatment is recommended at time of diagnosis regardless of CD4. Antiretroviral therapy choices evolve rapidly with development of new drugs and new one-pill combinations, but in general it involves the pairing of two nucleoside reverse transcriptase inhibitors (NRTIs) with either a non-nucleoside reverse transcriptase inhibitor (NNRTI), a ritonavir-boosted protease inhibitor (PI/r) or an integrase inhibitor (INSTI) (DHHS and IAS guidelines). In the United States, half of the approximately 1 million HIV-infected individuals in U.S will be over age 50 with extensive antiretroviral exposure. Endocrinologists have to be aware of increased risks of osteoporosis and fracture in this population and help guide HIV care providers in decisions regarding screening for osteoporosis and treatment for fracture prevention.

In many cross sectional studies, HIV-infected individuals have lower BMD than uninfected individuals matched by age and sex [1]. However, group differences are attenuated by adjustment for weight/BMI, which is generally lower in HIV-infected [2]. There are limited data on change in BMD in HIV-infected individuals right after infection or before initiation of antiretroviral therapy (ART), but from many different clinical trials, it is clear that within the first 1-2 years after ART initiation there is a net 2-4% loss in aBMD by DXA at either the lumbar spine or hip (Table 1). Similar dynamics occur with different combinations of antiretrovirals, with the peak loss occurring within the first 6 (spine) to 12 (hip) months with stabilization in years 2-3. The best evidence come from clinical trials comparing 2 or more ART regimens, and is supported by changes in bone turnover marker levels. From multiple studies, it appears that certain antiretrovirals are associated with greater bone loss than others when used in combination for treatment of ART-naïve individuals: tenofovir more than abacavir or raltegravir or TAF (a new formulation of tenofovir) and ritonavir-boosted protease inhibitors more than efavirenz or raltegravir (Table 1). Data also come from switch studies, in which patients who have virologic control are switched off regimens for convenience or toxicity. Again, tenofovir associated with more bone loss than zidovudine, abacavir, and raltegravir (Table 2).

Most longitudinal cohort studies of patients on established ART regimens show that BMD is stable [3], confirming the observation from clinical trials that bone loss is largely limited to the 1-2 years after initiation or switch of antiretrovirals. Data are limited in older HIV-infected individuals, but in postmenopausal women on established ART, rates of bone loss still exceed that of uninfected controls[4]. Despite these reassuring BMD data, fracture rates are higher among HIV-infected individuals in comparison to uninfected or population based controls (RR=1.56 all fractures; RR=1.36 fragility

fractures) [5], and in some of the larger studies, an association with antiretrovirals has also been detected[6, 7].

The etiology of low bone density and fractures in HIV-infected individuals is undoubtedly multifactorial and may include (a) host factors such as higher prevalence of smoking and frailty; low body weight; (b) hepatitis C coinfection[8, 9]; (c) chronic immune activation and upregulation of pro-resorptive cytokines such as TNFa, IL6, RANKL[10]; (d) direct effects of HIV-1 viral proteins on bone cells[11-14]; (e) direct effects of antiretrovirals on bone cells or vitamin D metabolism[14-18].

Table 1. Rates of bone loss in larger clinical trials using contemporary antiretroviral regimens

Study	Sample size/ Duration	ART regimens	Change in LS BMD	Change in TH or FN BMD
Stellbrink, ASSERT 2010[19]	N=385 48 weeks	**TDF**/FTC + EFV ABC/3TC + EFV	**-3.6%*** -1.9%	**-2.4%*** -1.6%
McComsey, ACTG 5223s 2011[20]	N=269 96 weeks	**TDF**/FTC ABC/3TC **ATV/r** EFV	**-3.3%*** -1.3% **-3.1%*** -1.7%	**-4.0%*** -2.6% -3.4% -3.1%
Reynes, PROGRESS 2013 [21]	N=206 96 weeks	**TDF/FTC+LPV/r** RAL+LPV/r	**-2.5%*** +0.7%	
Sax, 292-1012 2014[22]	N=170 48 weeks	E/C/F/**TDF** E/C/F/TAF	**-3.4%*** -1.0%	**-.2.4%*** -0.6%
Brown, ACTG 5260s 2014[23]	N=328 96 weeks	TDF/FTC+ATV/r TDF/FTC+DRV/r TDF/FTC+RAL	-4.0% -3.6% **-2.4%****	-3.9% -3.4% **-1.8%****

Abbreviations: TDF tenofovir disoproxil fumarate; TAF tenofovir alafenamide; FTC emtricitabine; 3TC lamivudine; ABC abacavir; EFV efavirenz; ATV/r atazanavir; with ritonavir boosting; LPVr lopinavir; with ritonavir boosting DRVr darunavir with ritonavir boosting; E/C/F elvitegravir/cobicistat/emtrictabine;
* p<0.05 TDF containing group against ABC or RAL; ATVr against EFV
** p<0.05 RAL group against ATV/r and DRV/r groups combined

Table 2. Rates of bone loss after switching ART regimens

Study	Sample/ Duration	ART regimens	Change in LS spine	Change in FN or TH BMD
Martin, STEAL 2009[24]	N=357 96 wks	AZT/3TC to **TDF**/FTC AZT/3TC to ABC/FTC	**8.5/100py** T<-1.0* 4.4/100py T<-1.0	
Cotter PREPARE 2013 [25]	N=84 48 wks	AZT/3TC to **TDF**/FTC Stay on AZT/FTC	**-2.0%*** -0.2%	

Bloch TROP 2014 [26]	N=37 48 wks	TDF+PI/r to RAL+PI/r	+3.0%	+2.5%
Haskelberg SECOND LINE 2013 [27]	N=210 96 wks	LPVr+**2-3 NRTIs** LPVr+RAL	**-4.9%*** -3.5%	**-4.1%*** -2.2%
Curran, SPIRAL-LIP, 2012 [28]	N=74 48 wks	NRTIs+LPVr to NRTIs+RAL Stay on NRTIs+LPVr		**+0.01 g/cm^2*** no change
* p<0.05 comparing one regimen to another				

B. Learning Objectives/Clinical Pearls: As a result of participating in this session, attendees should be able to:

1. Appreciate the additional risk for osteoporosis and fracture conferred by HIV infection and/or antiretroviral therapy and recognize the need for earlier screening and preventive measures
2. Recognize potential adverse effects of antiretrovirals on bone metabolism, especially the role of tenofovir disoproxil fumarate (TDF)
3. Manage osteoporosis in HIV-infected individuals, including working knowledge of antiretroviral switch strategies
4. Diagnose and treat TDF-associated osteomalacia

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C. Cases

Case 1. Management of osteoporosis

50 year-old African American HIV-infected postmenopausal woman referred for management of osteoporosis. She was diagnosed with HIV10 years ago after presenting with bacterial pneumonia and started on tenofovir/emtricitabine/atazanavir/ritonavir and has had an excellent virological and immunologic response. Her current CD4=550 cells/ul. Her last menstrual period was at age 48, she has no history of falls or fractures, no bony pain, and does not have a parenteral history of fracture, and does not have renal insufficiency, diabetes, rheumatoid arthritis. Her BMI=26 kg/m^2. She is not taking systemic glucocorticoids, smokes ½ ppd, and takes 800 IU Vitamin D3 and 1000 mg calcium carbonate supplementation daily.

Would you recommend risk stratification with FRAX or a screening DXA?

By FRAX ,based on just risk factors, her absolute 10-year risk of hip or major osteoporotic fracture was 0.1% and 1.3%, respectively. She was referred for a screening DXA which reveals the following T scores: LS -2.9; TH -2.8; FN -2.7; 1/3R -2.1. No vertebral fractures were detected on VFA. Her work-up for secondary osteoporosis reveals normal PTH=35 pg/dl (8-51 pg/dl); TSH=1.2 IU/ml (0.3-3 IU/ml); 25OHD=25 ng/ml (30-80 ng/ml); serum phosphate=3.0 mg/ml (2.5-4.3 mg/dl).

How would you manage?
Would you recommend switching her antiretroviral regimen or starting treatment for osteoporosis immediately? Bisphosphonate, teriparatide, denosumab or other?

Comments: There have been several randomized clinical trials that have demonstrated the safety, tolerability and short term efficacy of weekly alendronate and parenteral zoledronate in HIV-infected patients [29, 30]. Several cases of treatment with teriparatide have been reported in literature, but there are no clinical trial data. Reservations still exist for use of denosumab given concern for increased risk of skin/soft tissue infections in the major osteoporosis phase 3 registration study. However, given the 2-3% increase in BMD that may occur as a result of switching off of tenofovir, and potentially more with switching off of ATV/r as well, it may be reasonable to switch patient to a regimen such as ABC/3TC/RAL and monitor BMD in 1-2 years to see if bisphosphonate therapy could be delayed.

Case 2. Bone pain

55 yo postmenopausal woman with HIV infection was diagnosed 12 years ago after presenting with Pneumocystic jiroveci (PCP) pneumonia. At that time, her CD4 count was 180 cels/ul and she was started on stavudine, lamivudine, and lopinavir/ritonavir (D4T/3TC/LPVr) after treatment of her PCP with trimethoprim/sulfamethoxazole and prednisone. She had excellent virologic and immunologic response. She developed peripheral neuropathy 5 years ago and was switched to fixed-dose tenofovir/emtricitabine/efavirenz (atripla), which she has tolerated. Now she presents with 3 month history of bone pain starting from lower extremities and migrating to upper torso, most severe in spine and ribs. On physical exam, she has tenderness to palpation over ribs and long bones of arms and legs, and also proximal muscle weakness. Reflexes and sensory exams are normal. Laboratory tests reveal: Calcium 8.8 mg/dl (reference range 8.7-10.2 mg/dl); PTH 45 pg/dl (8-51 pg/dl); 25OHD 28 (30-80 ng/ml); $1,25(OH)_2D$ 45 pg/ml (15-75 pg/ml), TSH 1.0 IU/ml (0.3-3 IU/ml); phosphate 1.4 mg/dl (2.5-4.3 mg/dl); Alkaline phosphatase 239 U/l (40-135 U/l); BSAP 133.5 µg/L (5.6-29 µg/L); CTX 788 pg/ml (40-465 pg/ml).

Additional confirmatory studies included spot urine phosphate (52.2 mg/dL) and creatinine (158.0 mg/dL). Tubular Reabsorption of Phosphate (%TRP) was calculated [100 x [1-(urine phosphate/urine creatinine) x (serum creatinine/serum phosphate)]=72%, which is much lower than expected given her serum phosphate level. She also has proteinuria (1276 mg/24 h) but no hypercalciuria (129 mg/24 h), glucosuria, or aminoaciduria.

What is the diagnosis? What is the treatment?

Comments: Tenofovir use is associated with proximal tubular dysfunction (PRTD), which results in increased fractional urinary excretion of phosphate in 30% to 40% of subjects, including elevations in serum alkaline phosphatase[31]. However, development of hypophosphatemia is uncommon (eg, in < 5% in clinical trials) and osteomalacia occurs even less frequently. Since one of the compensatory effects of the body to hypophosphatemia is to increase 1-alpha hydroxylation of 25(OH)D, which in turn increases absorption of phosphate from the intestines, some investigators have theorized that vitamin D supplementation may mitigate some of demineralization effects of tenofovir-associated PRTD [17], but this has yet to be proven in a prospective study. Treatment includes stopping tenofovir and repletion of phosphate and calcium, and potentially vitamin D supplementation as well.

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Connexins and Cell-to-Cell Signaling In Bone

Teresita Bellido, Ph.D. and

Pierre Marie, Ph.D.

Meet the Professor session – 2014 ASBMR, Houston

Connexins and Cell-to-Cell Signaling In Bone

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Significance of the topic

Connexins (Cx) are structurally conserved proteins that form membrane channels. Cx43 plays a central role in cell-to-cell communication in bone, through gap junction channels that mediate intercellular communication and hemichannels that communicate cells with their extracellular milieu. Through its intracellular C-terminus domain, Cx43 also serves as a hub for structural and signaling molecules thus regulating intracellular signaling, independently of channel activity. During this Meet-the-Professor session, we will discuss the evidence demonstrating that via these diverse mechanisms Cx43 is a key component of the intracellular machinery responsible for signal transduction in bone in response to pharmacologic, hormonal and mechanical stimuli.

Learning objectives

As a result of participating in this session, attendees should become familiar with the mechanisms by which Cx43 regulates bone cell functions.

Outline and/or points of interest

1- Gap junction channels, hemichannels, and channel independent functions of Cx43
2- Cx43 and osteoblast and osteoclast differentiation and function
3- Cell-autonomous actions of Cx43 in osteocytes
4- Role of Cx43 in intracellular signaling by bisphosphonates, PTH, and mechanical force.

References and recommended reading (reviews)

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Figure 1. Cx43 hemichannels and gap junction channels in bone cells

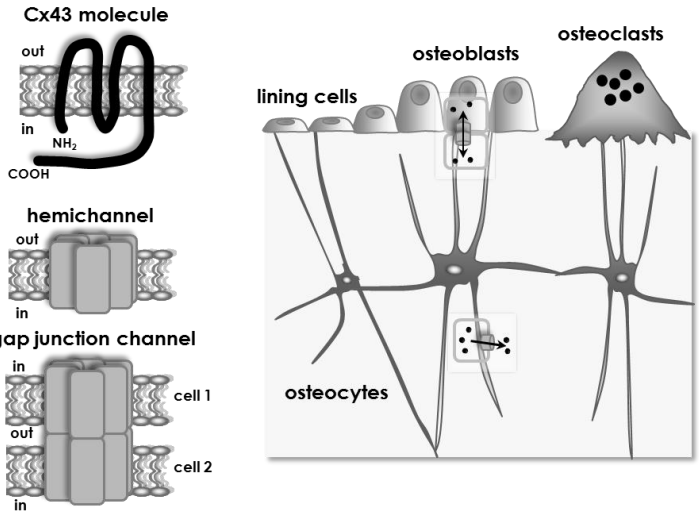


Figure 2. Cx43 as a hub of anti-apoptotic signaling pathways in bone cells

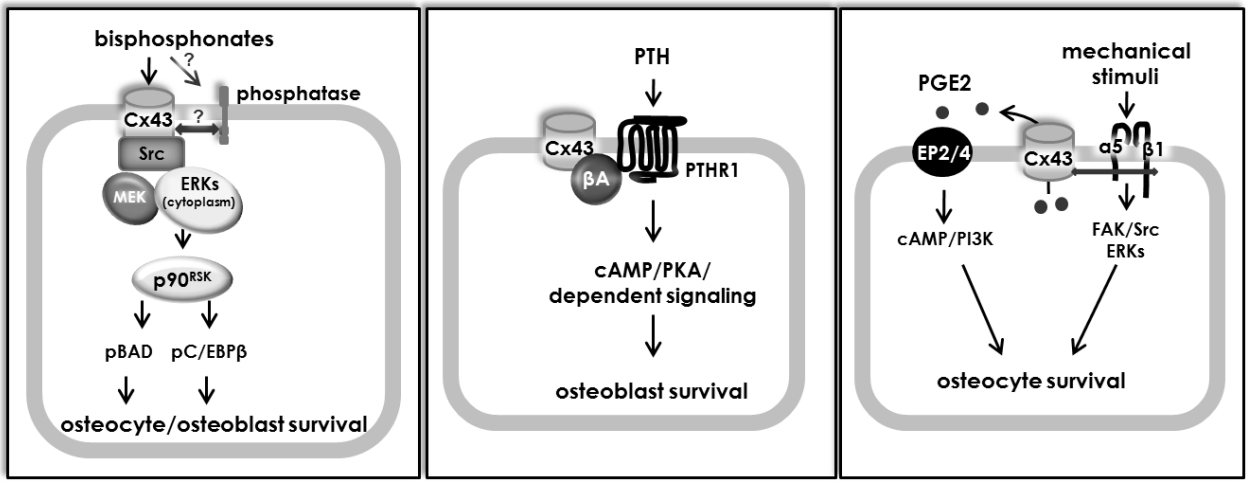
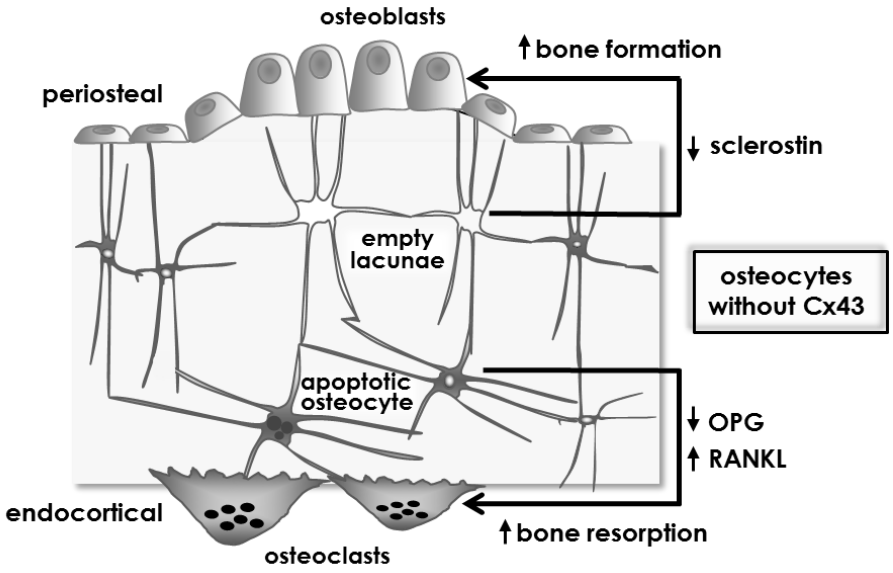


Figure 3. Osteocytic Cx43 and the control of osteoblast and osteoclast function



Meet the Professor session – ASBMR 2014, Houston, Texas, USA

Cadherins and Cell-to-Cell Signaling In Bone

Pierre J. Marie, Ph.D.

Director of Research at CNRS
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Significance of the Topic

Cadherins are calcium-dependent cell adhesion molecules that play major roles during morphogenesis and tissue formation. Osteoblasts express a repertoire of cadherins, some of which are expressed differently at various stages of differentiation. In vitro studies predicted that cadherin-mediated cell-cell adhesion controls osteoblast differentiation. However, the mechanisms involved were unknown.

Recent studies have highlighted the importance of the interactions between cadherins and Wnt signaling in the control of osteoblastogenesis and bone mass in mice. During this meet-the-professor session, we will discuss the notion that cross-talks between N-cadherin and Wnt signaling in osteoblasts control cell fate, bone formation and bone mass.

Learning Objectives

As a result of participating in this session, attendees should be able to better understand the interactions between cadherins and Wnt signaling and their importance in the control of bone formation.

Outline and/or Points of Interest

1. Control of bone formation and bone mass by cadherin-mediated cell-cell adhesion
2. Mechanisms by which N-cadherin interacts with Wnt signaling
3. Mechanisms by which cadherin controls osteoprogenitor cell fate in bone niches

References and recommended reading (reviews)

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Figure 1. Cadherin-mediated cell-cell adhesion controls bone formation (ref. 6)

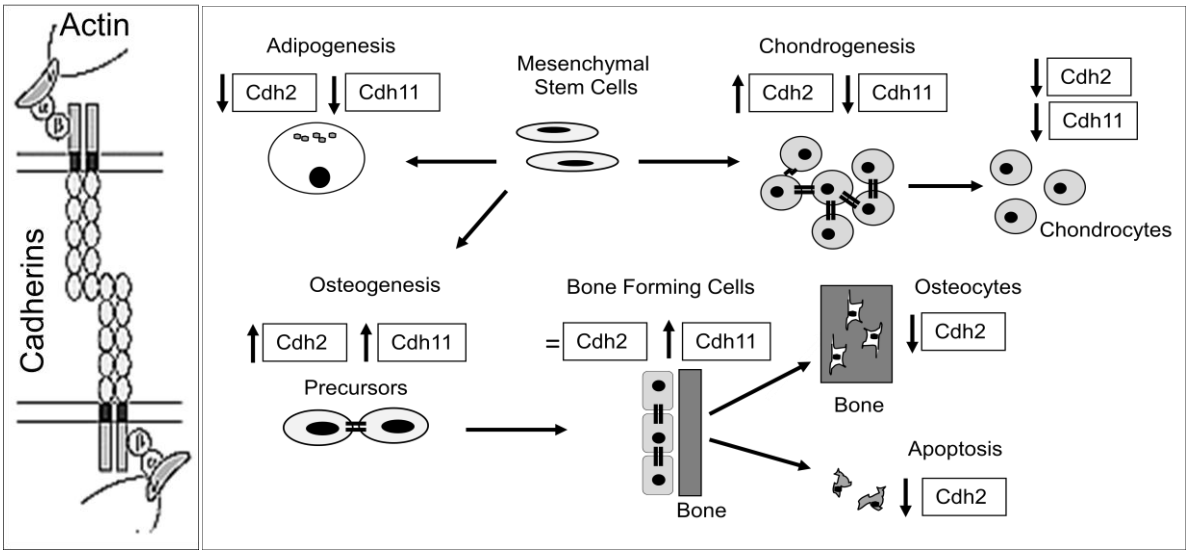


Figure 2: Interactions between N-cadherin and Wnt signaling (ref. 6).

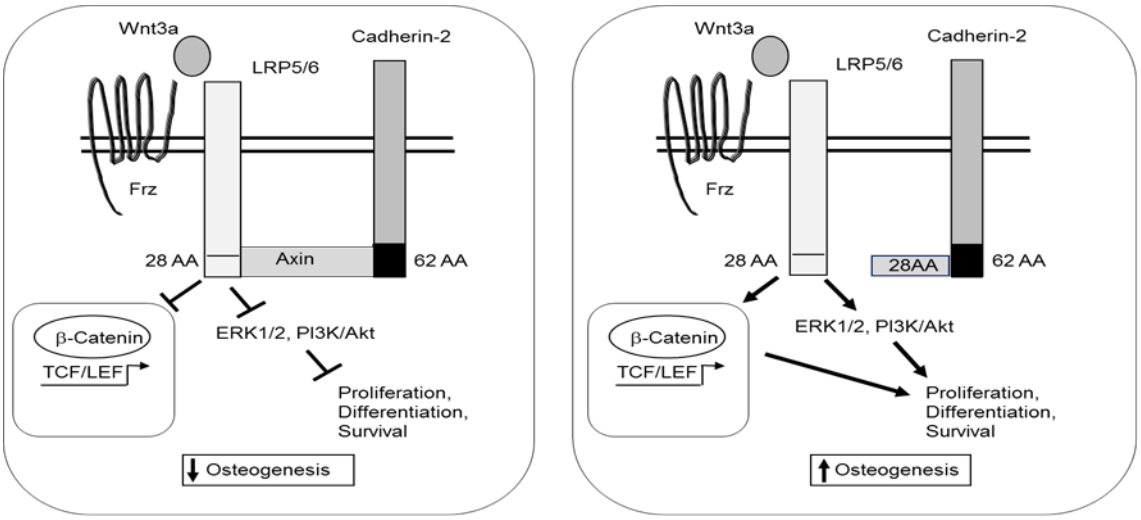
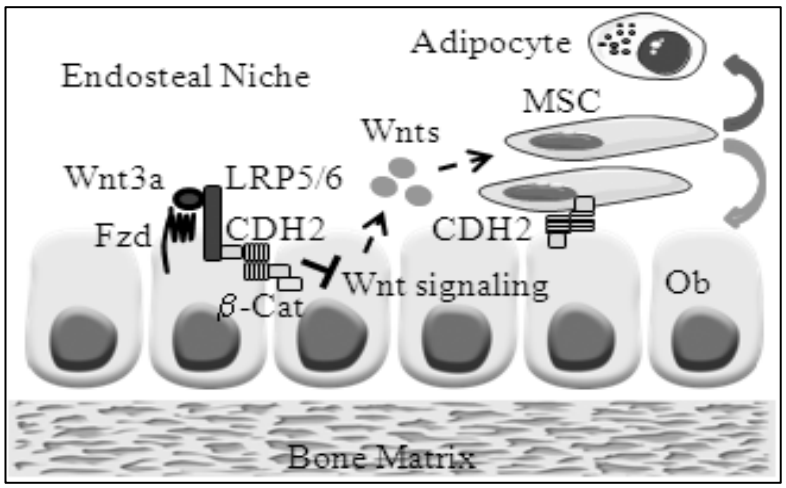


Figure 3 : N-cadherin controls mesenchymal cell adherence and fate in endosteal niches (refs. 6 and 9)



Diet, Microbiome, and Bone Health

Connie Weaver, Ph.D.

Diet, Microbiome, and Bone Health

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Learning Objectives

1. To share the first observation of a diet-induced change in the gut microbiome associated with a bone health benefit in healthy individuals.
2. To discuss future research needs to understand the relationship of the gut microbiome to bone health.

Interactions between diet, the gut microbiome, and individual characteristics that influence health are beginning to be explored. Most studies have evaluated changes in the gut microbiome in relation to disease. We have shown feeding dietary fiber (resistant to digestion) is associated with changes in gut microbiota that are capable of fermenting the fiber in the lower gut to short chain fatty acids in healthy adolescents. The changes in gut microbiota were significantly correlated with increases in calcium absorption and the observed delayed timing of the increase in calcium absorption is consistent with lower gut effects. Thus, it appears that diet can lead to shifts in the gut microbiome that have functional bone benefits in healthy people.

We conducted a series of studies in animal models and humans. From a feeding study of 0, 2, 4, 6, or 8% of the prebiotic fiber, galactooligosaccharides (GOS), in growing male Sprague-Dawley rats, we developed a regression model (Fig. 1) (1).

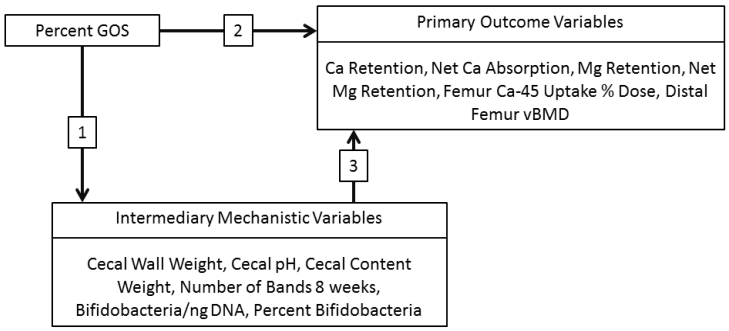

Figure 1. Model for regression analysis: 1, influence of GOS on mechanisms that influence skeletal health; 2, dose-response effect of GOS on primary end points; 3, effect of predictors on primary end points. GOS supplementation influenced all intermediary and primary end points. Cecal characteristics and some microbial profile characteristics influenced primary endpoints.

Dietary GOS significantly decreased cecal pH and increased cecal wall and content weight in a dose-dependent manner ($P<0.0001$). Quantitative PCR of fecal DNA showed an increase proportion of bifidobacteria with GOS ($p=0.0001$). Calcium and magnesium absorption and retention and femur and tibia breaking strengths, distal femur total and trabecular vBMD and area and proximal tibia vBMD increased ($p<0.02$) with GOS supplementation. We then studied the effect of GOS at 0, 2.5, or 5 g/day in a smoothie drink for 3-wk periods given in randomized order to 31 healthy girls (2). Fractional calcium absorption using stable isotopes was increased about ~10% with both levels of GOS; a dose response effect was not observed. Fecal bifidobacteria increased as a result of GOS feeding.

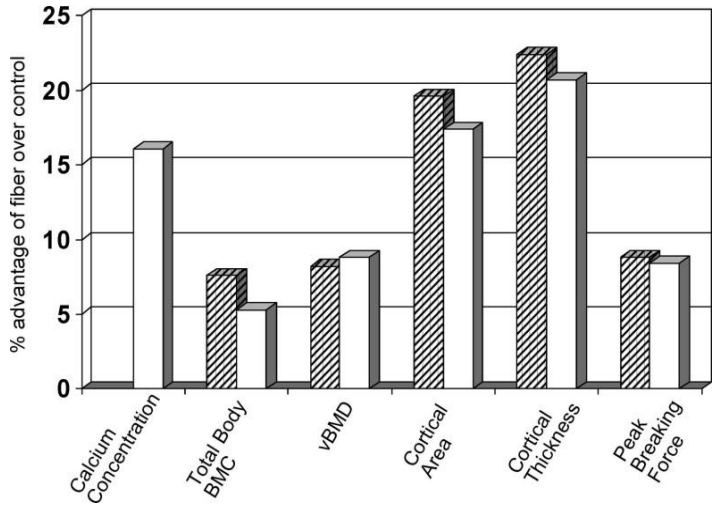

Fig. 2 Benefits to femur of soluble fibers over control fed rats; $p<0.05$ by Dunnett's test (slashed bars are SCF and open bars are SFD).

In an evaluation of 8 pre-biotic fibers in a male weanling rat model we saw increases in SCFA, mineral absorption and bone density and strength (3). Soluble corn fiber (SCF) and soluble fiber dextrin had the greatest benefit to bone properties including whole body BMC and distal femur vBMD, cortical thickness and area, and peak breaking strength (Fig. 2).

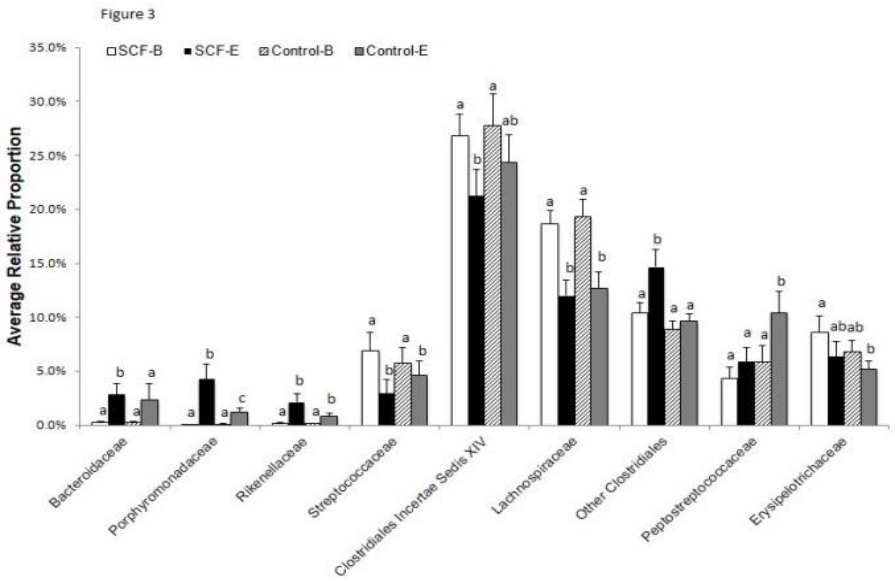

Fig. 3. Comparison of average relative proportions of bacterial families in pubertal girls (N=23) at the beginning (B) and end (E) of clinical sessions where diets included soluble corn fiber (SCF) vs. control (CON). At time E, significant differences between SCF and CON treatments were observed for Bacteroidaceae, Porphyromonadaceae, Streptococcaceae, Other Clostridiales, Peptostreptococcaceae. Only families representing >1.0% of the total community in at least one treatment and had significant differences in relative proportions are depicted. Error bars represent standard errors of means. Letters depict significant differences within each family ($p < 0.05$)

The relationship of prebiotic fibers and bone health have been reviewed (5,6). The future will bring an understanding of microbial signaling pathways that are associated with changes in mineral absorption and bone through metagenomics. Effects likely depend on characteristics of the host. For example, sex differences in the gut microbiome influenced sex steroid hormone driven regulation of immunity (7).

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Osteopetrosis

Uwe Kornak, M.D., Ph.D.

Osteopetrosis Meet-the-Professor Session
Uwe Kornak

Significance of the Topic

1. Background

Bone tissue has to be constantly remodeled by the joint action of bone-forming osteoblasts and bone-resorbing osteoclasts in order to ensure proper growth, mechanical stability, calcium/phosphate-homeostasis, hematopoiesis, immune function and glucose metabolism. Impaired bone resorption disturbs all these processes in a variable fashion. The consequent accumulation of bone tissue results in an increased radiological bone density, the typical hallmark of osteopetrosis (marble bone disease). The osteopetroses are a group of monogenic disorders with autosomal recessive, autosomal dominant and X-chromosomal inheritance patterns, respectively. Depending on the affected genes, osteopetrosis can arise from an osteoclast differentiation defect, giving rise to osteoclast-poor forms, or from an impairment of resorptive function, which is typical for the osteoclast-rich forms [1]. Autosomal recessive osteopetrosis (ARO) often leads to severe complications and a dramatically reduced life expectancy, mostly due to hematological and central nervous system problems. ARO has a frequency of around 1:300.000 live births and so far the only curative therapy is transplantation of hematopoietic stem cells (HSCT), from which the osteoclast lineage is derived. However, this treatment strategy has some limitations since it shows considerable lethality, mainly due to toxicity of the aggressive condition regimen used to favour the engraftment of HSC donor cells, graft-versus-host reaction or graft rejection, especially if the donor is not well matched [2, 3]. The European Group of Blood and Marrow transplantation (EBMT) registry reported that 5-year survival rates after transplantation of HSCs from HLA-matched sibling donors are significantly higher compared to HSCs from unrelated or mismatched donors (80-88% vs 66%) [2]. The products of the genes most commonly mutated in ARO, *TCIRG1* (55% of cases), *CLCN7* (12%), and *OSTM1* (6%), are all involved in proton secretion by the osteoclast, which is crucial for its resorptive activity [4]. Due to its high morbidity and mortality, the current HSCT strategy is not appropriate for the milder *CLCN7*-related autosomal dominant form type 2 (ADO type II), which does not display shortened life expectancy, but reduced quality of life due to recurrent fractures and additional complications [5]. The incidence of ADO type II has been estimated to be 1:20.000.

2. Learning Objectives

As a result of participating in this session, attendees should be able to
1. know about novel molecular diagnostics approaches using gene panels
2. know about possible gene therapy strategies for osteopetrosis
3. understand the pros and cons of these different strategies

3. Novel strategies for diagnostics of osteopetrosis

Currently, the following genes are known to cause autosomal recessive forms of osteopetrosis: *TCIRG1, CLCN7, OSTM1, SNX10, TNFSF11, TNFRSF11A, CA2, PLEKHM1*. Autosomal dominant osteopetrosis type 2 is due to *CLCN7* mutations and X-linked osteopetrosis is cased by *IKBKG* mutations. Furthermore, there are overlapping high bone mass disorders: Raine syndrome, endosteal hyperostosis type Worth, pycnodysostosis, etc.. The recessive osteopetrosis genes alone cover more than 70 exons and conventional testing can therefore be time- and money-consuming. This bottleneck can be easily resolved by gene panel diagnostics.

4. Novel strategies for treatment of osteopetrosis

As outlined above ARO can be treated by transplantation of bone marrow or enriched hematopoietic stem cells. Best results are obtained if transplantation occurs as early as possible.

The group of Anna Villa demonstrated that *in utero* transplantation of wildtype bone marrow can efficiently rescue the osteopetrosis phenotype in a mouse model of *TCIRG1*-related ARO, the osteosclerotic (*oc*) mouse mutant [6, 7]. The group of Dr. Richter went one step further and transplanted *oc/oc* lineage depleted (lin^-) bone marrow cells that were transduced by a retrovirus containing the wildtype cDNA of the defective *Tcirg1* (*Atp6v0a3*) gene [8]. Likewise, using lentiviruses rescue of osteoclast function was shown in human *TCIRG1*-deficient CD34+ cells in vitro [9]. The ideal therapy would be the compensation or even repair of the individual genetic defect in the relevant cell type - in the case of osteopetrosis the hematopoietic stem cells (Fig. 1).

The TAL effector nucleases (TALEN), and the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system allow for site-specific integration through double-strand break enhanced recombination in a highly efficient manner [10]. CRISPR is based on an endogenous sequence-specific nuclease system in which transcribed RNA specific for a foreign DNA guides the Cas9 protein for the cleavage of the target sequence. This system is extremely easy to handle and is highly efficient in inducing small deletions. If an engineered donor DNA containing any sequence flanked by short regions of homology is added, this foreign sequence can get inserted into the target locus by homologous recombination. However, the efficiency of this double strand break-facilitated homologous recombination is lower. Therefore, enrichment of the targeted cells might be a necessary step for *in vivo* application. The ideal target locus, a so-called safe harbour, should support expression of the inserted construct in the majority of cell types, but not influence any neighbouring genes. A frequently used safe harbour is the human AAVS1 or the murine ROSA26 locus [11]. The potential problem of using genome engineering are the off-target effects.
A real restoration of gene function can be achieved by a specific correction of individual mutations by genome editing in the affected cell type. While it has recently been shown that a mutated allele can be corrected by CRISPR in embryonic stem cells and in liver cells in vivo it remains to be demonstrated that this is also possible in HSCs with the necessary efficiency [12, 13]. However, low efficiency can be compensated by selection of the correctly targeted clone if the respective type of stem cell can be cultured. For cell types like HSCs that cannot be propagated in culture it is possible to first generate induced Pluripotent Stem Cells (iPSCs), which are then differentiated into the desired cell type after correction by genome editing and selection. Alternatively, somatic cells could be directly differentiated toward HSC by the simultaneous expression of 6 specific factors, although it is not yet clear at which stage a non-viral gene correction could be performed [14].

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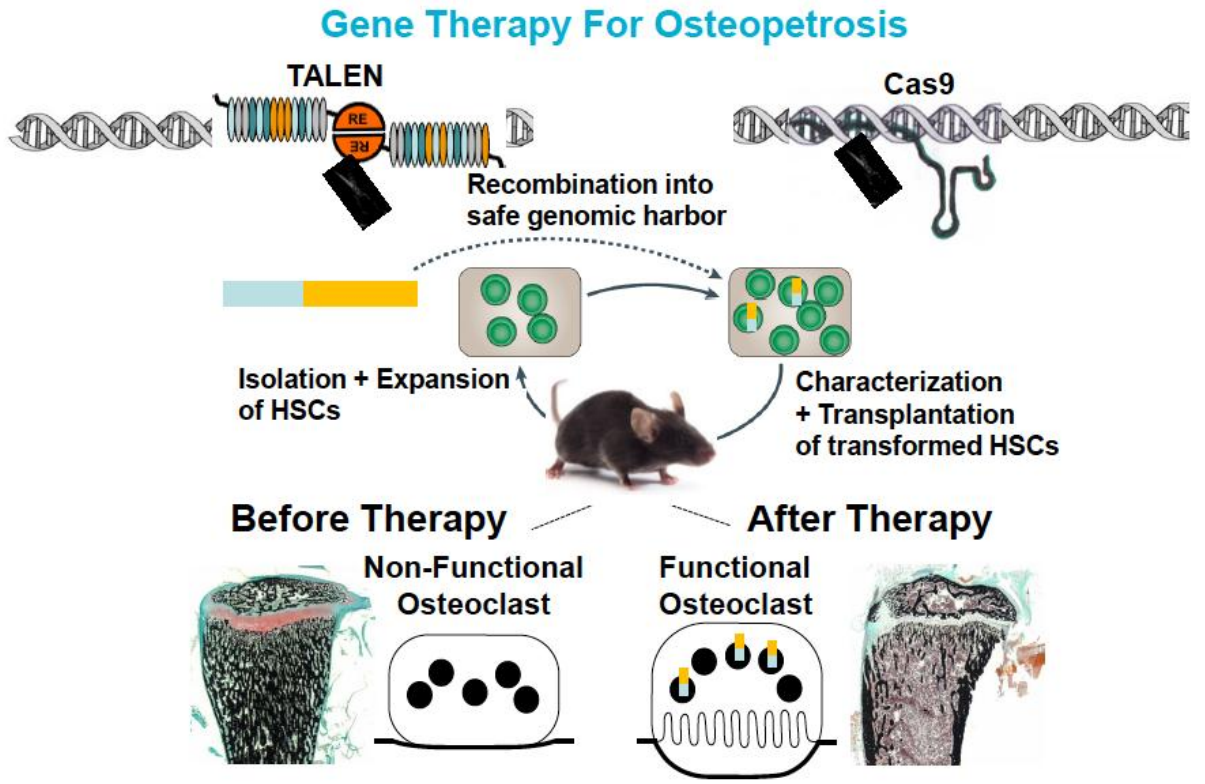
Fig. 1



Strong Risk Factors for Hip Fracture for Clinical Practice

Steven Cummings, M.D.

Strong Risk Factors for Hip Fracture for Clinical Practice

Steven R. Cummings[1]

BACKGROUND AND INTRODUCTION

Prescriptions of drug treatments for osteoporosis have declined by over 50% in the United States since 2008. It is important to recognize patients who warrant treatment to reduce the risk of fracture, particularly hip fracture because hip fracture is the major cause of preventable death and disability. Furthermore, several treatments reduce the risk of hip fracture by about 40%. Thus, it is important that clinicians increase their identification and treatment of patients who have a high risk of hip fracture. The approach outlined here may supplement the current emphasis on screening with BMD and risk tools, such as FRAX.

STRONG RISK FACTORS FOR FRACTURE

Several strong risk factors are very well known, including age (accelerates especially after 60) bone density (particularly in the proximal femur), gender, past fracture, regardless of trauma.(1) The FRAX algorithm (http://www.shef.ac.uk/FRAX/) estimates 10-year probabilities of hip and 'major clinical fractures' (hip, humerus, wrist, and clinical vertebral fractures) based on data pooled from international cohorts that generally consisted of volunteers rather than patients in clinical practice. Its components, BMI, parenteral history of hip fracture, smoking, use of corticosteroids, and 3 or more alcoholic drinks daily, rheumatoid arthritis, and femoral neck BMD, independently increase the risk of hip fractures.(2)

Medical conditions that increase fracture risk

In this session, I highlight several clinical conditions that are common and/or carry a high risk of hip fracture. Patients with medical conditions strongly associated with risk of hip fracture should generally have an evaluation and strong consideration of treatment that has been established to reduce the risk of hip fracture. This list does not include some risk factors for fracture that have been controversial or found to have more modest effects.

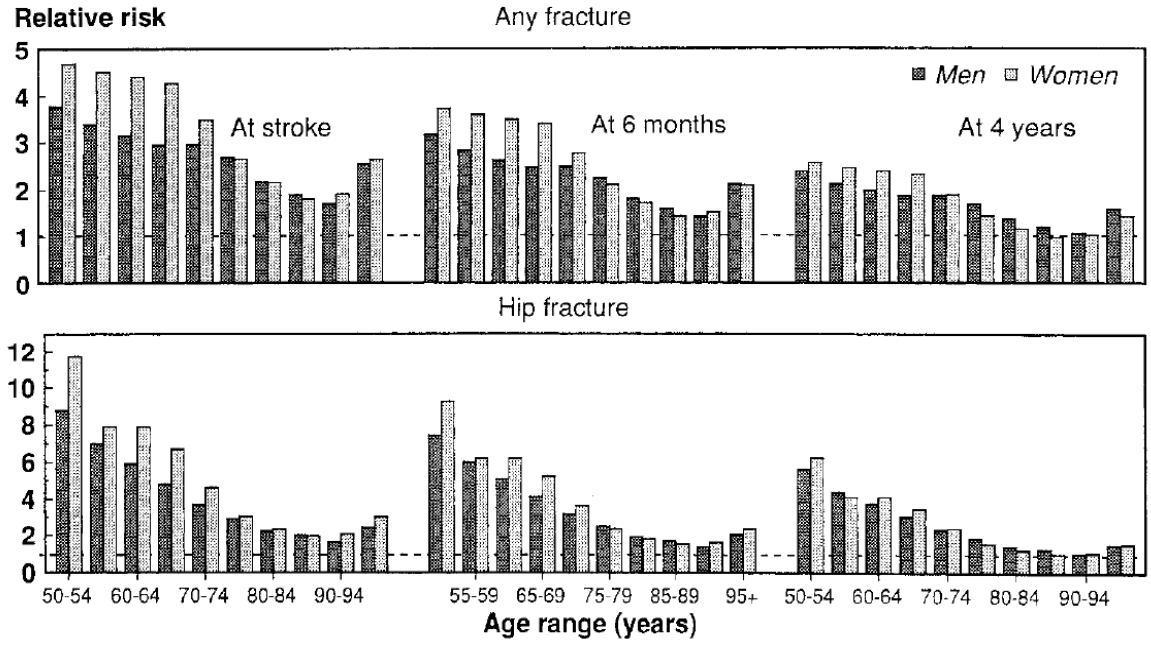
Renal insufficiency has been associated with an increased risk of hip fracture risk: about a 2.5-fold increase in women for eGFR < 60 ml/min defined by *cystatin-c* but the association may be less strong for eGFR based on creatinine.(3) There is a 4-fold increased risk with stage 5 renal failure and dialysis.(4) FDA-approved anti-resorptives appear to retain their efficacy on BMD in patients with stage 4 renal insufficiency who have normal calcium and parathyroid hormone levels, but the management of fracture risk with end stage renal disease is controversial.(5)

HIV infection: Two very large studies of national medical databases found that patients diagnosed as having HIV infection had 6- and 9-fold increases in risk of

[1]The review of individual risk factors for fracture was written with contributions from Richard Eastell.

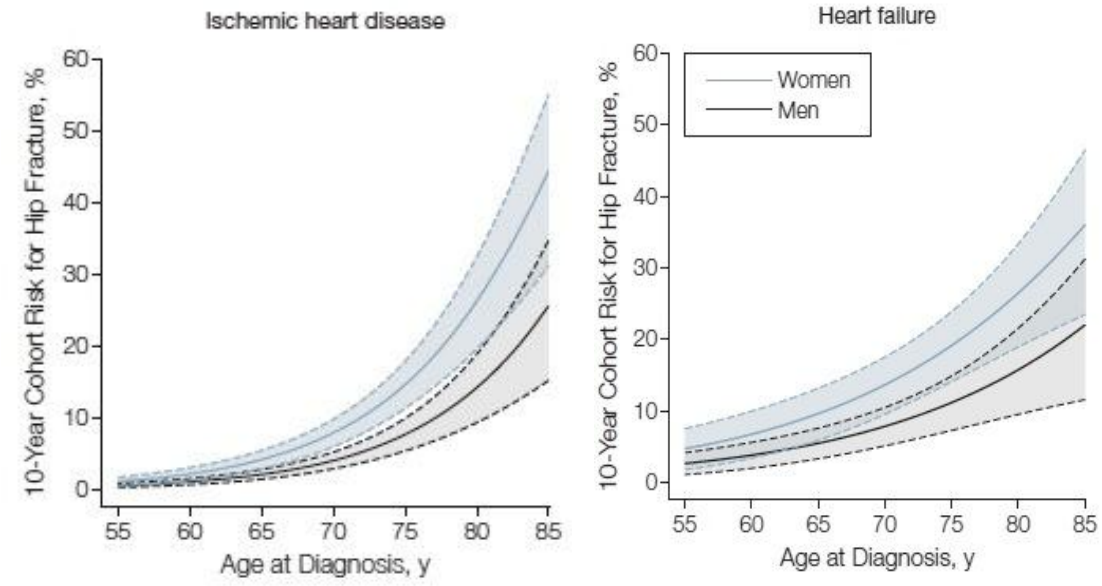
hip fracture.(6,7) The increased risk might be due to several factors including lower BMI and lower BMD. Antiretroviral therapy also decreases BMD.(8) Guidelines recommend BMD testing for those age 50 years.(8)

Stroke occurs in about 600,000 people in the US and 1.1 million people in Europe per year, largely in people > age 65. A stroke, increases the risk of all fracture, but particularly hip fracture and the risk is greatest soon after the stroke (figure).(9,10) Thus, any patient ≥ age 65 years should be considered for drug treatment but very few (<5%) receive treatment.



From Kanis (9)

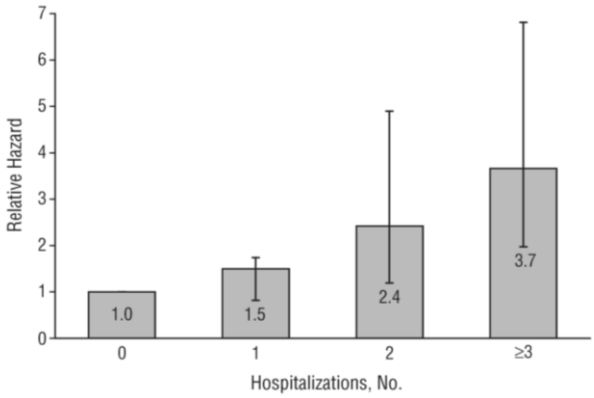
Patients with cardiovascular disease, have a 2-4 fold increased risk of hip fracture.(10,11) The risk is particularly high in patients with heart failure and ischemic heart disease. The 10-year risk of hip fracture is high in patients with these conditions who are at least 60 years old (figures from reference 11). Interestingly, twins of patients with CVD who do not have a diagnosis of CVD, have increased risk of hip fracture that is similar to the member of the pair who has the diagnosis.(11). Patients with CVD warrant evaluation and consideration of treatment to reduce the risk of hip fracture.



Parkinson's disease indicates an increased risk of nonvertebral fracture with a 2 to 4-fold increased risk of hip fracture.(13,14) Some of the increased risk is due to lower BMD (by about -0.7 T-score). Thus, all patients with Parkinson's warrant evaluation of BMD, risk, and consideration of drug treatment.

Type 2 diabetes has been much discussed as a risk factor for fracture. The diagnosis of Type 2 DM has has been associated with a 20-70% increased risk of hip fracture in most studies (15,16) but a recent large study from Scotland found no association.(17) On the other hand, Type 1 Diabetes is a very strong risk factor for hip fracture carrying a 4- to 7-fold, increased risk of hip fractures.(15) Patients with Type 1 DM warrant evaluation and consideration of treatment to reduce fracture risk.

Hospitalizations: The risk of hip fracture in men and women increases with the number of non-elective hospital admissions: 2.4-fold with 2 and 3.7-fold with ≥ 3 admissions. Evaluation of fracture risk and consideration of treatment should be part of discharge after 2nd or more hospital stays.(18)



'Vital signs' for hip fracture risk

Several simple clinical measurements have been found to be associated with an increased risk of hip fracture, including height and height loss, rapid pulse, and inability to stand up from a chair without pushing up with arms. Slow usual walking speed has also been associated with an increased risk and it has been promoted as a simple 'vital sign' that should be routinely assessed in elderly patients.

We have analyzed a combination of 'vital signs' that can be quickly assessed before the clinician sees a patient. In combination, these tell the clinician a patient's risk of hip fracture. A simple score sheet (attached) can be marked during the initial part of a clinical visit can be used to estimate the 10 year risk of

hip fracture. Observations not made simply add no points and the resulting score may underestimate the patient's risk of hip fracture.

This index was derived and confirmed in the prospective Study of Osteoporotic Fractures (9,704 women ≥ age 65) and MrOs Study (5,995 men ≥ age 65 years) with 10 years of follow-up after the measurements. It applies to Caucasian patients age 65 or older.

However, the participants in these studies are generally healthy volunteers. *The risk of hip fracture will be substantially higher in patients who have one of the chronic diseases included in this review.*

HIP FRACTURE 'VITAL SIGNS'

Age 70-74 years	1
Age 75+ years	3
Female	1
BMI < 25	1
Lost ≥ 5 cm since age 25	1
Pulse ≥ 80 / min	1
Walks 5 m in > 5 sec	1
Unable to rise from chair	1
TOTAL	

Circle the risk level

Total points	10 year hip fracture risk %
0	0.3
1	1.6
2	3.0
3	4.4
4	7.1
5	11.4
≥6	19.3

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Bone Metastasis and the Bone Microenvironment

Roberta Faccio, Ph.D.

Meet-the-Professor: Bone Metastasis and the Bone Microenvironment

Roberta Faccio, Associate Professor, Departments of Orthopedic Surgery and Cell Biology and Physiology, Washington University School of Medicine, St Louis, Missouri, USA.

Sunday, September 14 from 11:30am to 12:30pm

Significance

Bone metastases are an enormous cause of morbidity in cancer patients, and an estimated 350,000 patients die with bone metastases each year in the United States. As many as 70% of breast cancer patients and 30% of lung cancer patients eventually have spread of their primary tumors to bone. The problematic effects of bone metastasis, known as skeletal related events (SREs), include pathologic fractures, bone pain, hypercalcemia, spinal cord compression, and a need for palliative radiotherapy treatment to bone [1]. In the U.S. alone, more than 20,000 patients are treated for epidural spinal cord compression from vertebral metastases each year. As cancer patients are living longer after their initial diagnosis, there is a high likelihood that metastatic bone disease will increase in prevalence. Treating the fractures, pain, and other sequelae that result from these metastases will place additional strain on the health care system. Thus, finding new methods for preventing and slowing bone metastases will greatly benefit cancer patients, providing better quality of life and potentially increasing longevity.

The current paradigm for bone metastasis is that there is a mutual interaction between osteoclasts and cancer cells, known as the tumor/bone vicious cycle. This model is based largely on findings in animal models showing amelioration of bone metastases by targeting the osteoclasts. Thus, osteoclast inhibitors, such as Zoledronic Acid (ZA) and anti-RANKL-Ab denosumab, are the standard of care in breast cancer bone metastases. However, clinical studies demonstrate only a partial reduction in skeletal related events (SREs) in breast cancer patients with bone metastases treated with bisphosphonates (BPs) and currently there is limited evidence supporting anti-resorptive therapies in reducing the overall incidence of bone metastasis or extending survival. These data indicate that other cells, in addition to the osteoclasts, control tumor growth in bone. This discussion will address the role of the osteoclast in regulating tumor growth in bone in light of the recent clinical trials evaluating the anti-tumor effects of ZA in breast cancer bone metastases. We will also discuss the role that immune cells may play in modulating responsiveness to ZA and how they affect, directly or indirectly, tumor growth in bone.

Learning Objectives:

1. Importance of osteoclast targeting agents to modulate tumor growth in bone and tumor-associated bone loss.

We will be discussing findings in animal models of bone metastases treated with OC inhibitors and/or with genetic ablation of osteoclastogenic pathways (Fig.1). We will compare these animal findings to recent clinical trials using ZA as adjuvant therapy in breast cancer patients (Fig.2). We will also comment on the limitations of the available animal models of bone metastases based on the results from the clinical studies.

2. Emerging concepts of immune regulation of tumor growth in bone. We will be discussing the importance of CD8+ T cell anti-tumor responses in fighting tumors in bone (process called tumor elimination) (Fig.3). We will also examine how the tumor can modulate the immune system and change it from tumor “hostile” to tumor “friendly” (a process called tumor escape). We will conclude our discussion by focusing on the tumor edited immune suppressor cells (MDSC, M2 macrophages, subsets of CD4+ T cells) and their ability to create a favorable microenvironment within bone where the tumor can grow unabated (Fig.4). Finally, we will discuss how these findings can be translated into new therapeutic approaches for patients with incurable bone metastases.

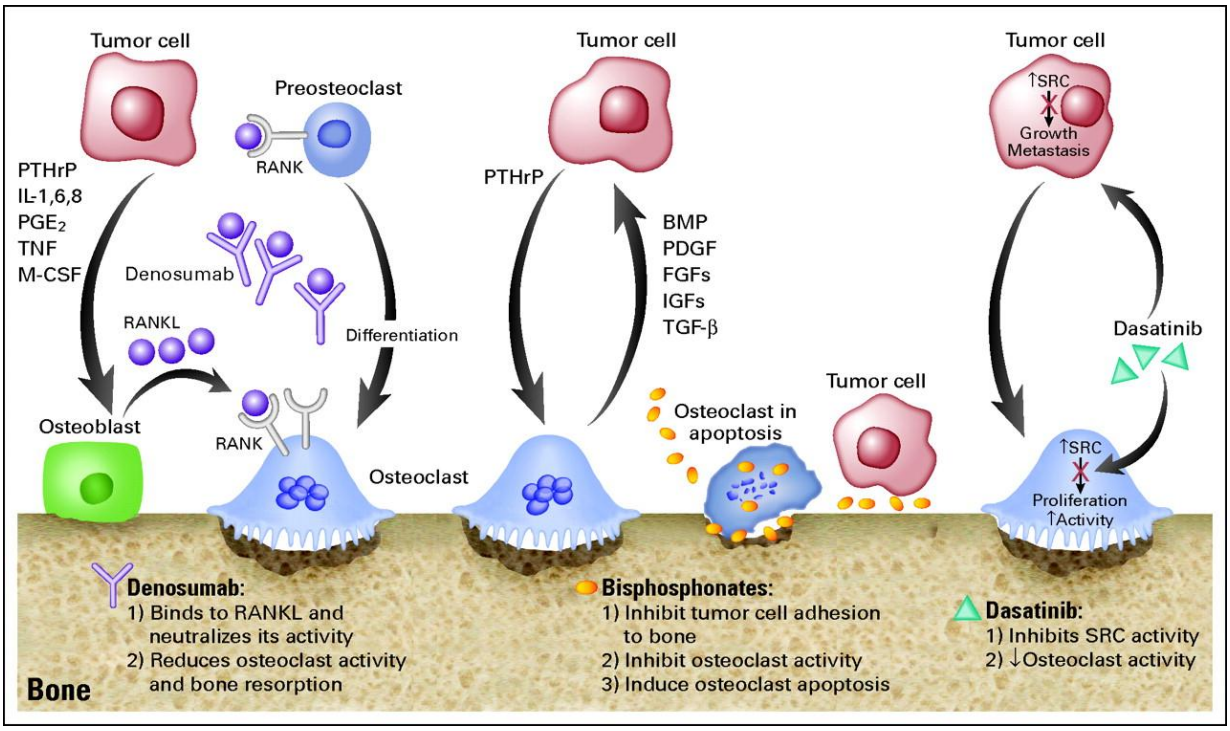

Figure 1. Tumor invasion into bone is associated with osteoclast and osteoblast recruitment (reviewed in [2]). Tumor cells secrete osteolytic factors (such as, parathyroid hormone-related protein (PTHrP), interleukin-1 (IL-1), IL-6, IL-8, tumor necrosis factor (TNF), Macrophage Colony Stimulating Factor (M-CSF)) that stimulate osteoclastic bone resorption either directly or indirectly by increasing the ratio of receptor activator of NF-κB ligand (RANKL) to osteoprotegerin (OPG). Osteoclastic bone resorption causes the release and activation of growth factors (transforming growth factor-β (TGFβ), insulin-like growth factors (IGFs), bone morphogenic proteins (BMPs) and others) that are stored in mineralized bone matrix to further enhance tumor recruitment and proliferation. Further, tumor cells can also target bone marrow stromal cells to stimulate production of factors that further promote tumor growth including, but not limited to, interleukin-6 (IL-6), and osteoclast recruitment, such as RANKL. This creates a 'vicious cycle' in which tumor-derived factors, deregulate bone remodeling, while stimulating the production of pro-tumorigenic factors. This self-perpetuating cycle results in increased tumor burden and bone destruction. Current therapeutic approaches to prevent or delay SREs target the bone resorbing osteoclasts and include RANKL neutralizing Antibody (Denosumab), Bisphosphonates, and Src kinase inhibitors (Dasatinib), just to name a few.

Study	Drug	DFS	Comments
Diel (1998)	Clodronate	No	There was an initial benefit (55 months of follow-up, *P* <.001), but it disappeared at 103 months of follow-up.
Powles (2002)	Clodronate	No	
Saarto (2004)	Clodronate	No	
NSABP-B34	Clodronate	No	
GAIN	Ibandronate (Boniva)	No	
AZURE (2011)	Zoledronic Acid	No	DFS benefit was observed in the post-menopausal women for more than 5 years (HR = 0.75; CI, 0.59-0.96, *P* =.02).
ZO-FAST (2011)	Zoledronic Acid	Yes	No significant DFS benefit was observed in women receiving delayed ZA treatment.
ABCSG-12 (2011)	Zoledronic Acid	Yes	Multivariate analyses showed no significant DFS benefit in patients < 40 years old.

Figure 2. Because of the central role of osteoclast-mediated bone disruption in creating a hospitable niche for tumor colonization and growth in the bone microenvironment, Bisphosphonates are largely utilized for treatment of bone metastases (reviewed in [3]).

The first generation of clinical studies testing the anti-tumor role of BPs in early breast cancer evaluated oral Clodronate in various randomized trials. Meta analysis studies demonstrated that Clodronate did not provide any significant benefit in bone metastasis-free survival, or Disease Free Survival (DFS). Similarly, the GAIN trial, which included 3,023 randomized patients receiving oral Ibandronate or placebo, failed to show improvement in DFS.

The Azure trial addressed the role of adjuvant Zoledronic Acid (ZA for 5 years) in chemotherapy treated stage II/III breast cancer. Although the Azure study failed to show that adding ZA to chemotherapy improves disease-free survival in the overall patient population, DFS was improved in postmenopausal patients (5 years or more) with the addition of ZA.

The ZO-FAST trial included Stage I-III, ER positive postmenopausal patients who were treated with letrozole and were randomized to receive either immediate or delayed ZA. At 5 years follow up, a DFS benefit of immediate ZA treatment has been reported with a trend for an Overall Survival (OS) gain. No benefits were observed in the ZA delayed group.

The ABCSG12 study included 1,803 premenopausal women with stage I/II breast cancer, who were randomized to receive 3 years of ZA versus observation; added to endocrine therapy. 36% reduction in the relative risk of disease progression was observed in ZA group. Importantly, and unlike the earlier Clodronate studies, the therapeutic gain obtained by ZA was maintained at 84 months median follow-up, with a significant benefit in DFS and OS, although no significant DFS benefits were observed in patients < 40 years old. Of notice, the patients in the ABCSG12 and ZO-FAST study were treated with endocrine therapies known to induce a profound estrogen poor environment and significant bone loss.

In an integrated analysis of 3 randomized clinical trials, Denosumab (anti-RANKL AB) was shown to be superior to ZA for prevention or delay of SREs in advanced cancer patients with bone metastases. Denosumab prolonged bone metastasis free survival and delayed the time to first bone metastasis in men with non-metastatic castration resistant prostate cancer. Currently, the anti-tumor effects of denosumab are being tested in the adjuvant breast cancer setting (reviewed in [4])

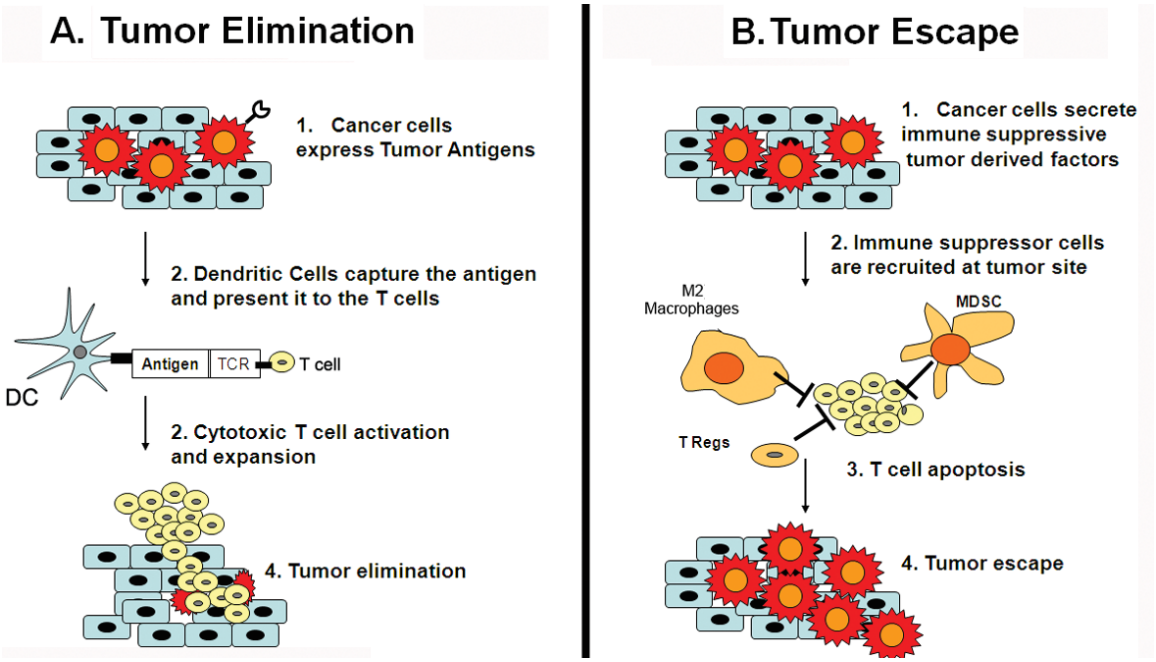

Figure 3. Cancer immune surveillance occurs when the immune system identifies tumor specific antigens on transformed cells that have escaped cell-intrinsic tumor-suppressor mechanisms and eliminates them before they can establish malignancy (reviewed in [5]. In this tumor elimination phase, dendritic cells (DC) capture and present tumor antigens to T lymphocytes leading to anti-tumor-specific T cell activation (A).

Unfortunately, anti-tumor immune responses are not always efficient in eliminating incipient tumors thus allowing the transformed cells to escape immune control. Many mechanisms are involved in the escape phase including intrinsic cancer cell alterations and tumor induced immune suppression. In the tumor escape phase, factors secreted by the tumor itself can lead to accumulation and activation of M2 macrophages, Myeloid Derived Suppressor Cells (MDSC), and regulatory T cells (T_{reg} cells), which suppress anti-tumor-specific T cell activation (B). The result of the escape phase is the tumor outgrowth and dissemination to distant sites.

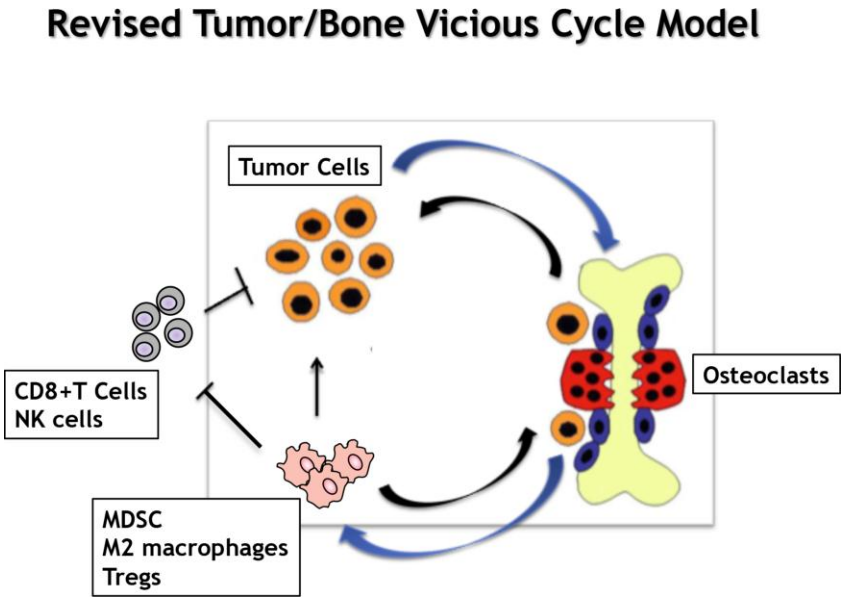

Figure 4. Osteoclasts have long been considered to be the central players in bone metastasis, and current therapeutic approaches target their generation and function. However, despite some significant clinical success in certain subgroups of patients, we are far from either prevention or cure for bone metastasis. Immune cells are emerging as additional contributors to the bone-tumor vicious cycle, acting both in concert with and independent of osteoclasts. Specifically, $CD8^+$ T cells inhibit bone metastasis independent of the status of the osteoclasts [6] [7]. However, the anti-tumor function of T cells can be inhibited by immune suppressor cells, such as MDSC and M2 macrophages [8]. Thus, expansion of these myeloid populations is associated with tumor progression, including to bone, and poor response to therapy. Future anti-tumor therapeutic strategies for patients with bone metastases should then consider enhancing anti-tumor immune responses while suppressing factors and/or cellular players involved in cancer immune resistance, together with anti-resorptive agents to prevent pathological bone loss.

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Bone Microdamage

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Bone Microdamage

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Significance

Recent interest in the pathogenesis of atypical femoral fractures has highlighted the importance of tissue level ductility in the etiology of clinical fractures [1]. Ductility is a mechanical property different from strength and is associated with the degree to which a can deform without breaking. Bone tissue with low ductility is quite brittle, and cracks can readily form and propagate in the tissue, often along a relatively linear path such as the transverse fracture plane seen in atypical femoral fractures.

Impaired tissue level ductility may play a role in typical age-related fractures. Vertebral fractures and other insufficiency fractures common in the elderly are often not the result of a single fall or other discrete overload and are instead associated with damage accumulated during multiple loading events. Bone tissue with reduced ductility is more likely to accumulate microscopic cracks and other tissue damage, known collectively as "microdamage". Microscopic tissue damage formed in bone in vivo has been observed in humans and is more prevalent in older patients [2]. While microdamage generated in vivo has been observed in many independent studies, the importance of microdamage to clinical fracture remains poorly understood. Microdamage may weaken bone tissue to the point where failure occurs during activities of daily living or mild trauma such as a fall from standing height. Additionally, microdamage may contribute to bone loss by stimulating bone resorption and remodeling.

The discussion has two parts: a tutorial meant to introduce participants to the concept of tissue ductility and how it differs from strength and stiffness. The tutorial session ends with a review of recent studies examining the mechanical consequences of pre-existing microdamage on bone mechanical performance as well as the response of the body to microdamage, when present.

Learning Objectives: At the completion of the session attendees will be able to:

- Specify the differences between strength, toughness, fracture toughness and fatigue life.
- Associate a material property with different failure modes in bone.
- Learn the challenges associated with measuring microscopic tissue damage in bone tissue.
- Be familiar with the state of the art regarding mechanical consequences of microdamage in bone

Outline:

- Tutorial: What is Mechanical Failure of Bone and Why are There Measures Other Than "Strength"?
- What is Microdamage in Bone?
- What Does Microdamage Do To Bone Mechanical Performance?
- How Does the Body Respond to Microdamage?

Basic Questions:

Question: Is "Strength" the Bottom Line?
Answer:
In the bone research community, mechanical failure of bone is commonly attributed to insufficient "bone strength," but in engineering and materials science, the parameter "strength" is just one of many mechanical properties used to describe mechanical failure (see table below and Figure 1,2). Failure from cyclic loading is characterized by the "fatigue life" of a material, while the ability of the material to resist fracture in the presences of a flaw or crack is referred to as "fracture toughness." Fracture toughness should not to be confused with the term "toughness," a different parameter that expresses the energy absorbed while a specimen is deformed.

Mechanical Property Describing "Failure"	Definition
Yield Strength	The stress at which plastic deformation begins
Ultimate Strength	The maximum stress carried by the material
Toughness	Energy absorbed by material to specified deflection
Fracture Toughness	Resistance to crack extension
Fatigue Life	Number of cycles of loading that may be applied prior to failure (requires applied load magnitude)

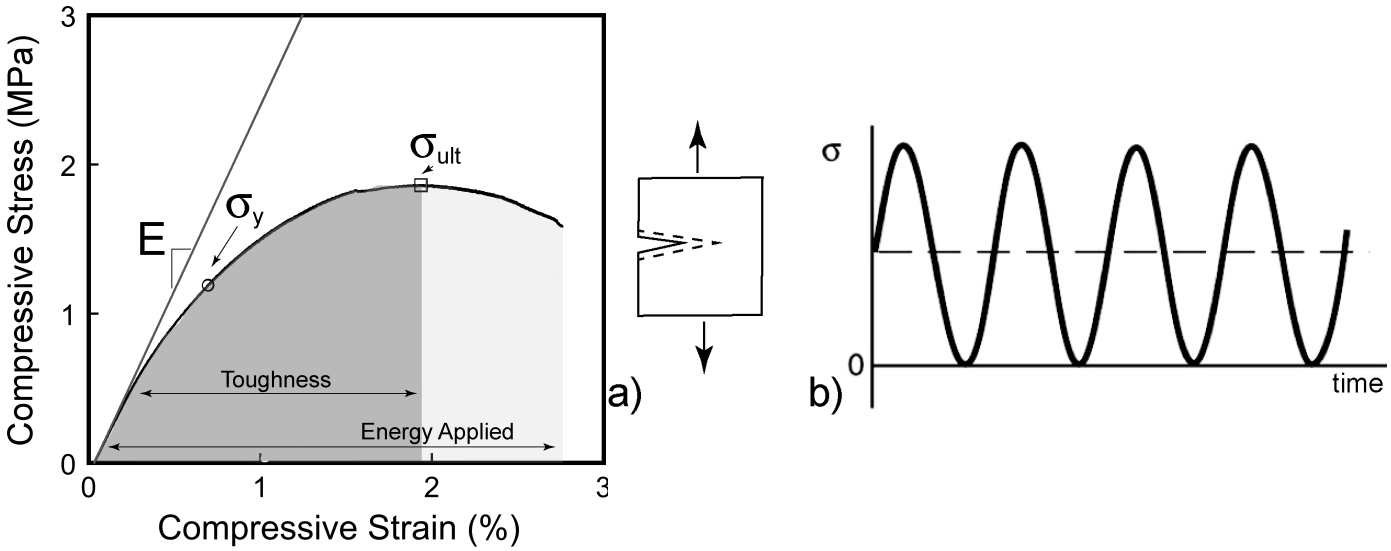

Figure 1. (Left) An example stress-strain curve generated during a compression test of cancellous bone is shown. Definitions of mechanical properties including Young's modulus (E), yield strength (σ_y), ultimate strength (σ_{ult}), toughess (darker shaded region) and energy applied during loading (lighter shaded region). (Right) Illustrations of measurement of a) fracture toughness and b) fatigue life are shown.

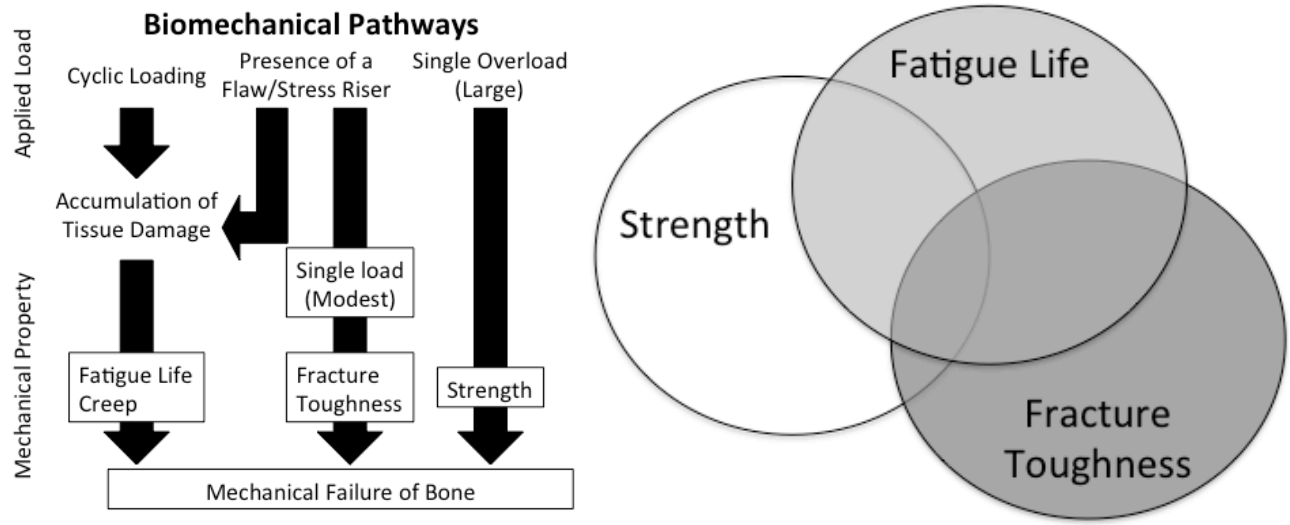

Figure 2. (Left) Mechanical failure of bone is not always due to a single overload such as a fall. Failure may also occur from excessive cyclic loading and/or the presence of a natural stress riser (Haversian canal, resorption cavity or other morphological characteristic). Each biomechanical pathway is related to a different mechanical property (Fatigue life, Fracture Toughness, Strength). (Right) Each mechanical property that describes failure of bone is evaluated with a distinct measurement, yet there is overlap among them. For example, resistance to crack growth is most directly assessed by fracture toughness, but can also influence measures of strength and fatigue life.

Question: What Is Microdamage?
Answer:
Microdamage is a term that refers to cracks and other tissue damage that are smaller than 1mm in size. Microdamage is most commonly observed in histology slides and has been reported to appear in the form of microcracks, cross-hatching microcracks, diffuse damage (a region in which microdamage stain is taken up and consists of a mix of sub-microscopic cracks) and trabecular microfracture [2]. More recently non-light microscopy approaches have been used to characterize diffuse damage at a the nanoscale [3].

Question: How Do We Measure Damage in Bone?
Answer:
There are two methods of assessing damage in bone: Microscopic Examination and Mechanical Examination. Microscopic examination requires directly visualizing the presence of microcracks and other forms of tissue damage, often performed using cut sections and stains used to identify microdamage (and differentiate it from damage caused during cutting). Mechanical examination of damage involves loading the material and determining the degree to which mechanical properties are impaired.

Microscopic Examination of microdamage is traditionally done by hand counts on two-dimensional sections and is therefore subjective in nature. Recently three-dimensional methods of visualizing stained microdamage have been presented. In our experience, measures of microdamage using three-dimensional techniques are systematically larger than those determined using two-dimensional methods and somewhat less subjective since microdamage is assessed with image thresholding rather than direct observation by a histologist.

Question: Does Microdamage Make Bone Weaker?
Answer:
Yes. There have been many studies demonstrating that cortical and cancellous bone submitted to cyclic loading experienced reductions in Young's modulus that were correlated with the amount of microdamage generated by the loading. More recently, microdamage stained with fluorochromes has been used to determine how the amount of microdamage in bone tissue alters bone tissue strength. Modest amounts of microdamage (damage volume fraction DV/BV = 1.5%) were associated with 50-60% reductions in cancellous bone strength [4].

Question: How Does Bone Respond to Microdamage?
Answer:
The generation of microcracks in cortical bone has been shown to be a strong stimulus for the initiation of new bone resorption and remodeling. The effect has been shown to be so strong that it can initiate Haversian remodeling in rodents that don't typically display Haversian remodeling [5] and has been shown to be regulated by osteocytes [6]. Diffuse damage in cortical bone has not been associated with such a strong response and may be repaired through passive mechanisms [7]. Little is known regarding the response to microdamage in cancellous bone. Presumably, microdamage in cancellous bone will trigger bone resorption and remodeling in cancellous bone (using the same mechanisms as in cortical bone) but there is also evidence that microdamage (trabecular microfractures) in cancellous bone can trigger new bone formation in the form of a microcallus (a small callus like structure on a trabecula).

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Brown Fat and Bone

Beata Lecka-Czernik, Ph.D.

Meet-the-Professor Session: Brown Fat and Bone

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Significance of the Topic

In recent years we are witnessing a remarkable explosion of research illuminating a relationship between bone and energy metabolism. Although central and sympathetic nervous systems as well as gastrointestinal and pancreatic axes play an essential role in systemic regulation of energy metabolism, the role of fat tissue in this regulation is the most prominent due to its fundamental function in storing and dissipating energy. In the last two decades significant progress has been made in understanding fat tissue origin, its diverse functions, and pathophysiological consequences of its impairment. These advances lead to the finding that fat tissue metabolism is linked to bone homeostasis.

Objectives

As a result of participants in this session, attendees should be able to:

- Discuss clinical and translational research findings on the association between fat metabolic status, and specifically brown/beige fat metabolic status, and bone mass.
- Have a perspective on the bone marrow fat metabolism and its potential role in regulation of local milieu supporting bone homeostasis.

Outline

Different types of fat tissue

Adipocytes accumulate energy in the form of lipids and burn it in the process of fatty acid β-oxidation. In addition, fat cells produce adipokines, among them leptin and

adiponectin, which in endocrine manner regulate calorie intake and insulin sensitivity. The multiplex of fat functions is sequestered throughout different fat depots. A role of mitochondria-sparse white adipose tissue (WAT), which is represented by visceral and subcutaneous fat, is to store energy in the form of lipids and endocrinal regulation of insulin sensitivity and glucose metabolism in liver and muscle. In contrast, a role of mitochondria-enriched brown adipose tissue (BAT), which is distributed in adult humans as discrete deposits located in the neck, supraclavicular, paravertebral, and suprarenal regions, is to dissipate energy to support adaptive thermogenesis.

It has been recognized that BAT may come from two different origins. The classical preformed BAT originates from Myf5-positive dermomyotomal progenitors, which also give rise to skin and muscle, and functions in non-shivering thermogenesis. In contrast, the Myf5-negative progenitors can differentiate to white adipocytes with function in energy storage or to BAT-like or “beige” adipocytes, which have characteristics of both brown and white fat cells. The BAT-like phenotype can be induced in WAT-type adipocytes by several mechanisms comprising either cold exposure, endocrine action of FGF21, irisin, or transcriptional regulators including FoxC2, PRDM16, and PPARγ that is activated with specific agonists which cause SirT1-mediated deacetylation of PPARγ protein. Beige fat possesses strong anti-obesity and anti-diabetic activity.

Bone marrow adipose tissue (BMAT) constitutes of a distinct population of adipocytes with mixed WAT and BAT phenotype or a heterogenous population of both WAT- and BAT-type of fat cells. A gene expression profile of epididymal and bone marrow adipocytes shows significant difference in the expression of genes controlling biological processes and molecular functions including adipocyte differentiation, and lipid and carbohydrate metabolism. Interestingly, genes associated with brown adipocyte

phenotype were over-represented in the bone marrow as compared to epididymal adipocytes. BMAT profile for white and brown adipocyte gene markers showed elevated expression of several BAT markers including PRDM16, FoxC2, PGC1α and Dio2. However, this profiling also showed low levels of UCP1 and β3AR, known BAT markers, and low levels of WAT markers including adiponectin and leptin (Figure 1){Krings, 2012 #1289}.

Brown / Beige fat activity associates with higher bone mass

With identification of functional BAT and existence of beige adipocytes in adult humans, the evidence is growing for a positive correlation of these fat types with bone mass. Increased BAT activity correlates with increased bone mineral density in young women, but not in men {Bredella, 2012 #1290}, children and adolescents {Ponrartana, 2012 #1300}. In addition, the bone mass in women recovering from anorexia nervosa is higher in those who possess cold-induced BAT foci as compared with those who lost BAT function {Bredella, 2012 #1290}. Moreover, BAT activity in these patients was in an inverse relationship to circulating levels of Pref-1, a marker of impaired osteoblast differentiation, indicating that BAT activity has a positive association with bone formation {Bredella, 2012 #1290}. Recently, it has been shown that BAT volume is a positive predictor of femoral bone structure including total and cortical cross section area and correlates positively with thigh muscle and subcutaneous fat {Bredella, 2014 #1400}.

With respect to rodents, there are several models indicating a positive function of BAT on bone. Recently, an association of functional BAT with bone mass has been demonstrated in a model of Misty mice. In this model, a mutation in DOCK7 causes impairment of BAT activity. Misty mice have low bone mass and accelerated bone loss with aging {Motyl, 2013 #1399}. Interestingly, bone loss can be partially prevented by β-

blocker propanolol suggesting involvement of sympathetic nervous system and adrenergic signaling in control of bone mass in this model.

Conversely, heterotopic bone formation induced by BMP2 injection to muscle provides evidence that brown adipocytes may have a positive effect on bone formation {Olmsted-Davis, 2007 #1132}. As demonstrated, an accumulation of adipocytes expressing UCP1 at the early stages of heterotopic bone formation is prerequisite for this process perhaps by providing an environment supporting angiogenesis, innervation, and chondrogenesis.

Because evidence of a presence of brown/beige adipocytes in bone {Krings, 2012 #1289} and evidence of WAT-derived beige adipocytes contribution to the systemic energy metabolism {Cohen, 2014 #1393}, it is plausible to expect that beige fat may positively contribute to the regulation of bone mass. Indeed, mice with targeted expression of forkhead box C2 (FoxC2) in adipocytes, which converts white-type adipocytes to beige-type, have high bone mass {Rahman, 2013 #1384}. Closer examination revealed that $FoxC2_{AD}^{+/Tg}$ mice have increased bone formation associated with high bone turnover, lower expression of Sost, and higher expression of RANKL in osteocytes {Rahman, 2013 #1384}. It has been shown that FoxC2-expressing beige adipocytes secrete bone anabolic factors including IGF-1, IGFBP2, Wnt10b and BMP4. Interestingly, besides bone remodeling, these factors also control energy metabolism in adipocytes, providing additional evidence for close association between bone and energy metabolism.

Conclusion

The close association between bone and fat leads to the conclusion that fat metabolic status has the ability to regulate bone homeostasis by modulating bone remodeling either directly at the level of MSCs differentiation, or indirectly by providing a milieu in bone marrow environment controlling bone remodeling. If beneficial effect of marrow fat

on bone is confirmed, one can expect a possibility to develop bone therapies which will target fat metabolic status instead of bone cells.

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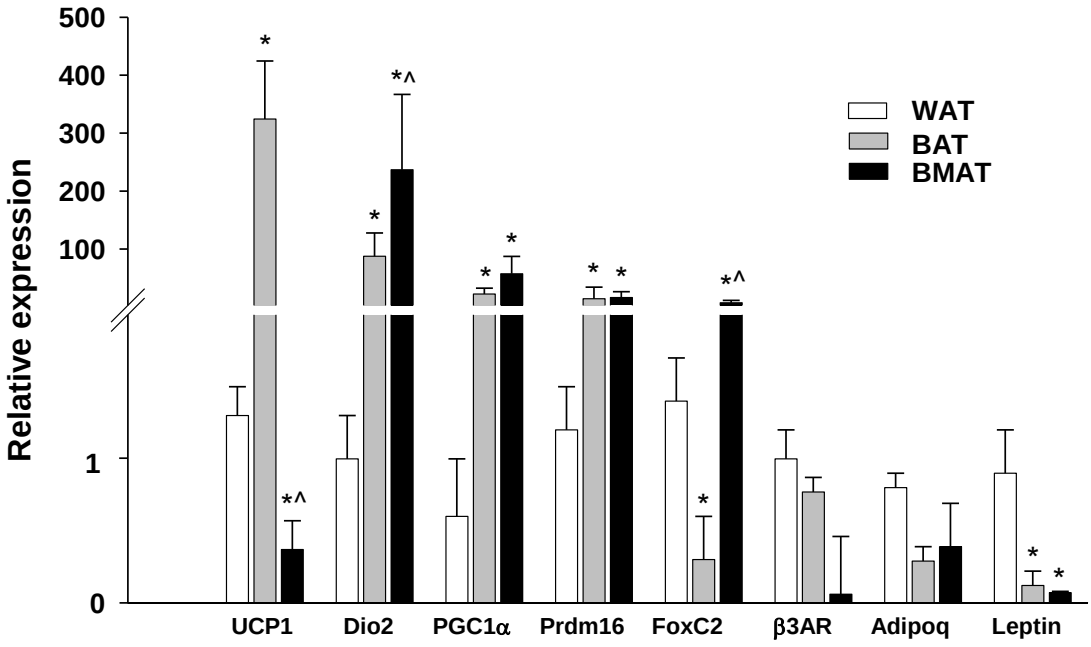
Figure Legend

Figure 1. Relative expression of adipocyte-specific gene markers in BAT and BMAT as compared to WAT {Krings, 2012 #1289}. RNA was isolated from epididymal WAT, interscapular BAT and bone marrow isolated from femora of 6 mo old C57BL/6 male

mice (n=4). Gene expression was analyzed using real time PCR and normalized to the level of 18S RNA in each sample. The values from bone marrow analysis were further normalized to the levels of FABP4/aP2 expression in WAT and BAT. * p<0.05 *vs.* WAT; ^ p<0.05 BMAT *vs*. BAT



Clinical Management of Phosphorus Disorders

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Clinical Management of Phosphorus Disorders

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Significance of the Topic

Since phosphorus is one of the most abundant constituents of all tissues, disturbances in phosphate homeostasis provoke a wide variety of complications. Indeed, a deficiency or excess of this mineral can have profound effects on a wide variety of tissues. These effects include osteomalacia, rickets, red cell dysfunction, rhabdomyolysis, metabolic acidosis and cardiomyopathy. By contrast, hyperphosphatemia may lead to soft tissue calcification, hypocalcemia, tetany, and secondary hyperparathyroidism. In many cases, it has proven difficult to establish whether the consequences of hypo- or hyperphosphatemia are singularly related to this abnormality or are modified by changes in complementary hormones or metabolic factors. Attempts to discriminate between these possibilities often has been sought by evaluating of the therapeutic response to phosphate supplementation or depletion. Such studies indicate that few of the phosphate homeostatic disorders respond adequately to therapeutically induced alterations in phosphate alone, but do regress upon coincident modification the complementary abnormalities.

Hypophosphatemia

Hypophosphatemia is a common clinical occurrence and is observed in up to 5% of hospitalized patients. In fact, alcoholic patients and those with severe sepsis have up to a 30-50% prevalence of this disorder. Other common clinical settings in which severe hypophosphatemia occurs include critical illness, treatment of diabetic ketoacidosis, and nutritional repletion in at risk individuals.

The three major mechanisms by which hypophosphatemia can occur are: 1) increased urinary excretion; 2) decreased intestinal absorption; and 3) redistribution of phosphorus from extracellular fluid into cells. The causes of hypophosphatemia are found in Table 1. Amongst the diseases causing hypophosphatemia, those genetic and acquired disorders, in which abnormal renal phosphate reabsorption results in increased urinary excretion (e.g. X-linked hypophosphatemic ricktets [XLH]; Autosomal dominant hypophosphatemic rickets [ADHR]; Autosomal recessive hypophosphatemic rickets [ARHR] and Hereditary hypophosphatemic rickets with hypercalciuria [HHRH]) , present the most challenging diagnostic and therapeutic dilemmas. However, recent literature has improved the understanding of the pathogenesis of such diseases and provided new insights to targets for effective treatment.

Hyperphosphatemia

Like hypophosphatemia, hyperphosphatemia is a relatively common clinical occurrence observed in a significant proportion of the 11-15% with chronic kidney disease. Indeed, hyperphosphatemia is a paradigmatic finding in late-stage chronic kidney disease and a frequent occurrence in stage 3-5 moderate kidney failure. Other common clinical disorders, in which hyperphosphatemia is present, include hypoparathyroidism, tumoral calcinosis (TC), and rhabdomyolysis.

Clinically, hyperphosphatemia occurs most commonly as a result of: 1) impaired renal excretion; and 2) transcelluar shift of phosphorus from cells to the extracellular fluid compartment. The causes of hyperphosphatemia are presented in Table 2. Amongst the diseases causing hyperphosphatemia, Tumoral Calcinosis is most interesting as the pathophysiology of this disorder is closely tied to the pathophysiology of the genetic and acquired diseases in which abnormal renal phosphate excretion results in hypophosphatemia.

Table 1. Causes of Hypophosphatemia

- Increased Renal Excretion
  - Primary Renal Phosphate Loss
    - Hereditary Hypophosphatemic Rickets with Hypercalciuria
  - Secondary Renal Phosphate Loss
    - X-linked hypophosphatemic rickets
    - Autosomal dominant hypophosphatemic rickets
    - Dent's disease
    - Tumor-induced osteomalacia
    - Fanconi syndrome
    - Fibrous Dysplasia
    - Linear nevus sebaceous syndrome
    - Hyperparathyroidism
      - Primary
      - Secondary
      - Tertiary
- Decreased Intestinal Absorption
  - Vitamin D Deficiency
  - Vitamin D Metabolic Defects
    - Vitamin D-dependent rickets type 1
    - Vitamin D-dependent rickets type 2
  - Nutritional Deficiency
  - Alcoholism
  - Anorexia
- Acute Volume Expansion
- Medications
  - Calcitonin; Diuretics; Glucocorticoids; Bicarbonate
- Transcellular Shift of Phosphorus
  - Sepsis
  - Salicylate Intoxication
  - Insulin Therapy
  - Leukemia Blast Crisis
  - Hungry Bone Syndrome
  - Nutritional Repletion – Refeeding Syndrome

Table 2. Causes of Hyperphosphatemia

- Impaired Renal Excretion
  - Primary Renal Phosphate Retention
    - Tumoral Calcinosis
  - Secondary Renal Phosphate Retention
    - Hypoparathyroid Disorders
      - Idiopathic hypoparathyroidism
      - Surgical hypoparathyroidism
      - Pseudohypoparathyroidism
    - Acromegaly
    - Diphosphonate therapy
    - Hyperthyroidism
- Increased Intake/Enhanced Absorption
  - Oral Administration - NeutraPhos
  - Rectal Administration – Phosphosoda Enemas
  - Intravenous Administration
  - Vitamin D intoxication
- Transcellular Shift of Phosporus
  - Cytotoxic Therapy – Tumor Lysis
  - Malignant hyperthermia
  - Rhabdomyolysis
  - Hemolytic Anemia
  - Metabolic or Respiratory Acidosis

Focus of the Meet-the-Professor Session

Given the evident gaps in knowledge regarding the pathophysiology and treatment of XLH, AHDR, ARHR, and TC, highlights of the contemporary advances made in managing these diseases will be the focus of the meeting. In addition, important insights will be shared regarding the targets of future treatment strategies.

Learning Objectives

As a result of participating in this session, attendees should be able to:

1. Improve diagnostic skills for differentiating between genetic forms of hypophosphatemic rickets (XLH, ADHR, ARHR, HHRH) and discriminating acquired forms of hypophosphatemic rickets (TIO) from the genetic variants.
2. Increase understanding of the recently obtained information regarding the pathogenesis of the hypophosphatemic disorders, and the impact of these new data on the limits of contemporary treatment strategies and the potential targets for new therapies.
3. Appreciate that Tumoral Calcinosis has a pathophysiology that, like hypophosphatemic disorders, has an abnormality related to FGF-23 activity, but the multiple gene defects underlying the disease result in FGF-23 abnormalities and variable upstream effects to the FGF-23, and thereby provide enhanced understanding of the mechanisms underlying normal FGF-23 function.

Outline/Points of Interest/Clinical Pearls

I. Diagnostic Skills

A. X-Linked Hypophosphatemic rickets

1. Onset of a “rare” disease 1:20,000 live births
2. Skeletal abnormalities and growth retardation (lower extremeties)
   a. Spectrum: isolated hypophosphatemia to severe lower extremity bowing; onset 6-12 months of age
   b. Enlarged wrists/knees due to rickets
   c. Late dentition, tooth abscesses (poor mineralization of interglobular dentine), cranial synostosis; enthesopathy
   d. Rickets/osteomalacia with no relationship to gender; radiographically detectable rickets-varable
   e. Hypophosphatemia due to FGF-23 dependent renal phosphate wasting
3. Inheritance
   a. X-linked dominant; often missed diagnosis in mother; relatively frequent spontaneous mutation; only 1/3 of affected patients aware of disease
   b. Genetic testing available worldwide (GeneDX, Gaitherburg, MD; Athena Diagnostics Inc., Worcester, MA; Center for Human Genetics, Ingelheim, Germany; University Hospital Antwerp, Edegem, Belgium; and Royal Devon and Exeter Hospital, Exeter, United Kingdom)

B. Autosomal Dominant Hypophosphatemic Rickets

1. Full expression: hypophosphatemia due to renal phosphate wasting, an inappropriately low or normal serum calcitriol level, and rickets/osteomalacia
2. Incomplete penetrance and variable age of onset; disease presence marked by increased FGF-23
3. 50% with early onset presenting with hypophosphatemia, lower extremity deformities and dental abnormalities in childhood
4. In some patients abnormalities persist into adulthood
5. The remaining 50% have late onset of clinically evident disease (hypophosphatemia, bone pain, weakness, and pseudofractures), presenting primarily in females during puberty and after pregnancy (consistent with iron deficiency enhanced increase in FGF-23)
6. Inheritance
   a. Autosomal dominant; often missed in affected parent because of incomplete penetrance
   b. Genetic testing available: 4 different mutations documented, each affecting the arginines within **R176**XX**R179**/S180, a subtilisn-like proprotein consensus sight.
   c. Disease in childhood requires differentiation from XLH; disease onset in adulthood requires differentiation from TIO

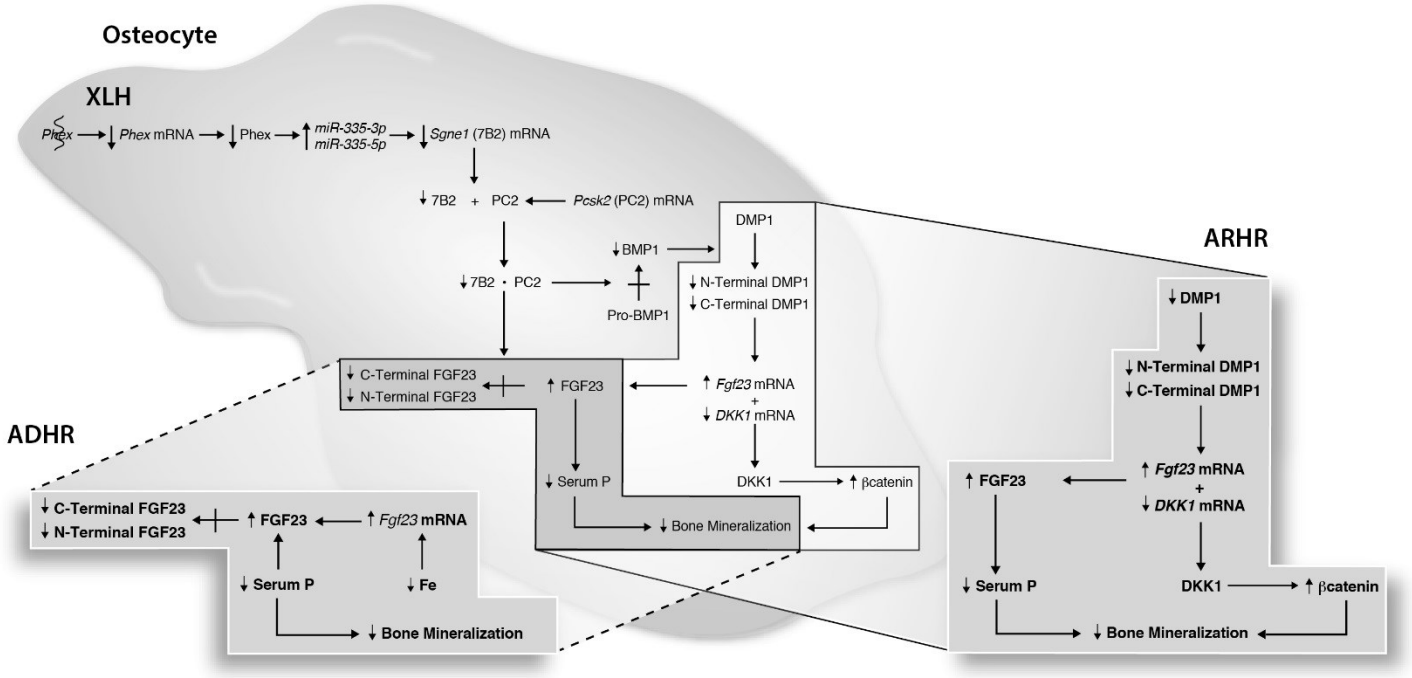
C. Autosomal Recessive Hypophosphatemic Rickets

1. Extremely rare and described only in 10 kindreds worldwide
2. Classic physical/laboratory findings of XLH/ADH; however, unlike these diseases the symptoms appear to depend largely upon the severity and chronicity of the associate phosphate depletion and hypophosphatemia
   a. Widely varying serum FGF-23
   b. Unusual findings occasionally encountered include osteosclerosis at the base of the skull, rib and long bone hyperostosis, complete ankyloses and degenerative arthritis
3. Inheritance

a. Autosomal recessive
b. Diagnosis requires DNA sequencing of leukocytes present in a small blood sample; the coding sequences of DMP1 are amplified through PCR reactions, and all products are fully sequenced

II. Pathogenesis of the Hypophosphatemic Rickets, Impact on Therapy, and New Therapeutic Targets

A. The Inter-related Biomolecular Abnormalities Underlying the Abnormal Phosphate Homeostasis and Bone Mineralization in XLH, ARHR, and ADHR

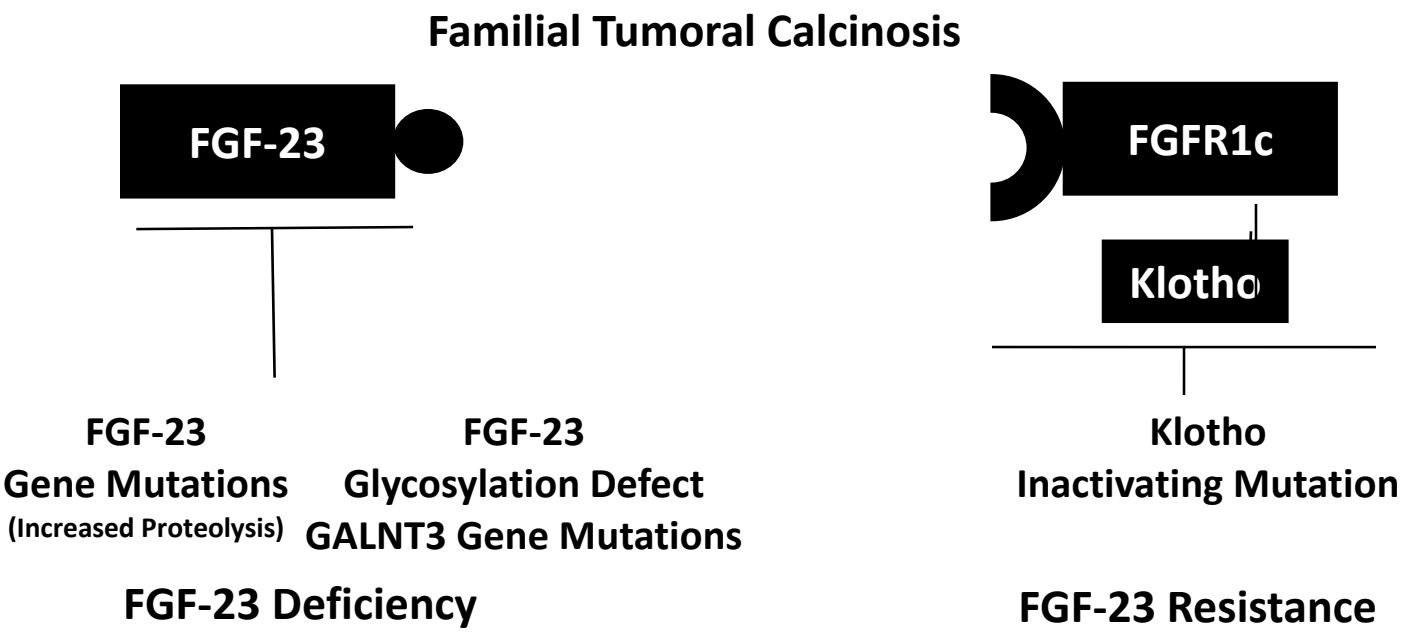


B. Impact That the Pathogenesis of the Diseases Shown Above Has on Contemporary Therapy

1. **ADHR**: (Gene Mutation – FGF-23) Abnormal mineralization appears due to FGF-23 mediated hypophosphatemia
   a. Arrest of the disease with normalization of bone mineralization occurs concurrent with normalization of FGF-23 and phosphate homeostasis, suggesting that treatment resulting in a normal serum phosphorus concentration would effectively manage the disease.
      (1). High dose therapy with calcitriol and phosphorus may therefore be successful
      (2). Treatment with the FGF-23 antibody at appropriate doses may also heal the bone disease
2. **XLH**: (Gene Mutation – PHEX) Abnormal mineralization appears due to FGF-23 mediated hypophosphatemia and increased βcatenin, a known inhibitor of bone calcification
   a. Contemporary treatment with calcitriol and phosphorus, while healing the rachitic disease, fails to result in normal mineralization of the osteomalacic bone; results reported to date indicate that use of the FGF-23 antibody likewise fails to completely heal the osteomalacic bone abnormality; such ineffective therapeutic response may be due to persistence of the increased βcatenin.
   b. By contrast treatment of *Hyp*-mice with D6R, which normalizes the miR-335-3p and miR-335-5p, resulting in changes in FGF-23 and, in accord, normophosphatemia, and likewise normalizes the βcatenin, rescues the phenotype, including normal mineralization of bone
   c. The role of βcatenin in bone mineralization has been supported in several studies
      (1). Transgenic overexpression of βcatenin in mice produces osteomalacia
      (2). Transgenic overexpression of *DKK1* (a potent Wnt inhibitor) suppresses βcatenin in osteomalacic states and rescues the phenotype
      (3). Injecting an anti-βcatenin compound in osteomalacic models improves bone mineralization
3. **ARHR**: (Gene Mutation – DMP1) Like in XLH, abnormal mineralization appears due to FGF-23 mediated hypophosphatemia and increased βcatenin

a. The few attempts to treat affected subjects with calcitriol and phosphorus have not healed the osteomalacia
b. In contrast, complete rescue of the phenotype in DMP1 knockout mice has occurred with transgenic expression of the carboxy-terminal fragment of DMP1, which resulted in restoration of normal phosphate homeostasis and normalization of bone βcatenin.

C. Pathophysiology of Familial Tumoral Calcinosis



1. Familial tumoral calcinosis (TC) is a rare autosomal recessive disorder distinguished by the development of ectopic and vascular calcified masses that occur in settings of hyperphosphatemia (hFTC) and normophosphatemia (nFTC).
2. Mutation in various genes (see above figure) cause this disorder
   a. Since the FGF-23 subtilisin-like proprotein convertase recognition sequence (^{176}RHTR$^{179}\downarrow$) is allegedly protected by O-glycosylation through ppGalNAc-T3 (GALNT3) activity, inactivating GALNT3 mutations are assumed to render FGF23 susceptible to proteolysis, thereby reducing circulating intact hormone levels and leading to hyperphosphatemic familial tumoral calcinosis. However, recent studies indicate that O-glycosylation by Galnt3 is only necessary for proper secretion of intact Fgf23 and, once secreted, does not affect Fgf23 function. Thus, the GALNT3 mutations decrease FGF-23 levels by preventing normal secretion.

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Nutrition and Bone Health in Adolescents

John Pettifor, MBBCh, Ph.D.

Nutrition and bone health in the adolescent

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Significance of the topic:

Optimizing bone health during adolescence is of importance for two major reasons:

1. To prevent or reduce the risk of low-trauma fractures, particularly during the adolescent growth spurt
2. To optimize peak bone mass, which is an important factor in influencing the risk of osteoporosis in later life

Nutrition is thought to play an important role in optimizing peak bone mass, although in the normal course of events the magnitude of the effect is probably relatively small compared to the roles played by genetics and hormonal changes. The nutrients which are frequently considered to be important include total energy intake, protein, calcium and vitamin D, although severe deficiencies of other nutrients may be associated with alterations in bone mass.

Learning objectives:

As a result of participating in this session, attendees should be able to

- Appreciate the role that low energy availability plays in the female athlete triad of menstrual dysfunction, estrogen deficiency and low bone mass, and in the pathogenesis of low bone mass in adolescents with eating disorders
- Understand the possible role that protein intake plays in optimizing bone health during adolescence
- Consider the possible deleterious effect of calcium supplementation in adolescents who are on habitually low dietary calcium intakes

Outline:

The adolescent athlete: Although physical activity, especially sports involving high impact loading, such as gymnastics, hurdles, judo, karate and volley ball, are typically associated with higher bone mineral density and enhanced bone geometry, female athletes are prone to what is known as the female athlete triad.

The female athlete triad comprises three features: 1) Low energy availability (EA) with or without disordered eating, 2) menstrual dysfunction, and 3) low bone mineral density. The condition occurs in physically active girls but particularly in those who are in sports associated with intense training and the need to maintain low body fat, such as ballet dancing and gymnastics. Low energy availability is associated with low body fat, disturbances in hypothalamic-pituitary –gonadal axis resulting in menstrual disturbances, hypoestrogenemia, and delayed pubertal development. Overt signs of low EA are a BMI of <17.5 kg/m^2 or EBW of <85%, however low EA may be present in an athlete whose weight is stable and who may be in apparent energy balance. An index of EA may be calculated by determining:

$$\frac{\text{energy intake (kcal) - exercise energy expenditure}}{\text{Fat free mass (kg)}}$$

which if <30kg/kg FFM is associated with bone deficit.

The 2014 Female Athlete Triad Coalition Consensus document provides information on how to diagnose the various components of the triad, and who should get DXA scans for bone mass assessments:

≥1 'High Risk' Triad risk factors: OR	**≥2 'Moderate Risk' Triad risk factors:**
➢ History of DSM-V diagnosed ED	➢ Current or history of DE for 6m or greater
➢ BMI ≤17.5, <85% EBW or wt loss ≥10% in 1m	➢ BMI between 17.5 and 18.5, <90% EBW, or recent wt loss of 5-10% in 1m
➢ Menarche ≥16 y	➢ Menarche between 15 and 16y
➢ Current or history of <6 menses over 12m	➢ Current or history of 6-8 menses over 12m
➢ 2 prior stress #s, 1 high risk stress #, or low energy non-traumatic #	➢ One prior stress #/reaction
➢ Prior DXA Z-score of <-2.0	➢ Prior DXA Z-score of -1.0 and -2.0

Osteoporosis in children and adolescents has been recently defined by an expert panel of paediatricians as the following:

- One or more vertebral compression fractures in the absence of high energy trauma, irrespective of the DXA Z-scores.
- The presence of both a clinically significant fracture history and a BMD Z-score of ≤2.0. A clinically significant fracture history is one or more of the following:
    - Two or more long bone fractures by age 10 y
    - Three of more long bone fractures at any age up to 19 y.

When assessing the results of the DXA, always use Z-scores. The sites recommended are the lumbar spine and whole body (less head). Adjustments should be made for growth delay (height adjustment) and if possible for pubertal development (bone age).

There is good evidence that an increase in energy intake, which may or may not be accompanied by a reduction in training intensity, if associated with weight gain (5-10%), leads to recovery of menstrual regularity and an improvement in bone mass. The difficulty in achieving this is related to having to personalise the approach for each athlete, involving the trainer, sports dietician and sports psychologist. Achieving this may be difficult in there is evidence of an ED.

Oestrogen replacement in the form of oestrogen-progesterone combination pills has not been shown to be an effective method of increasing BMD in most patients with AN or eating disorders, even though regular menstrual cycles may be achieved. The same appears to hold true for female athletes with menstrual dysfunction.

Whether athletes with low bone mass should be treated on pharmacological agents is unclear. Paediatricians are generally conservative in the management of low BMD in adolescents. Despite the higher risk for fractures athletes involved in high-impact sports are exposed to, and the possible need for higher BMD to withstand these forces, there is no clear evidence to support the use of pharmacological agents, besides ensuring vitamin D sufficiency and a generally nutritious diet. Rather the use of bone specific anti-resorptive agents and possible anabolic drugs should only be considered on an individual patient basis after weighing up all the pros and cons.

Protein intake and bone mass: There have been concerns expressed that high protein diets might lead to osteoporosis due to an increase in the acid load and thus the need for increased buffering by bone, and due to an increase in calciuria. It is also suggested that the negative effect of high protein intakes is influenced by the calcium content of the diet, with low calcium intakes being detrimental. However, in children and adolescents, as in the elderly, there is evidence that dietary protein stimulates IGF-1 production and consequently bone formation. Even with protein intakes considered to be within the recommended range of 0.8-1.5 g/kg body weight/day for children and adolescents, there is evidence that protein intake can positively influence bone growth and thus possibly influence the peak bone mass. It is also suggested that protein intake influences the effect of calcium supplementation, such that calcium supplementation is more apparent in pre-pubertal children on low protein intakes. Further, the reported positive effects of milk supplements in children may be due to the increased protein intake over and above the effect of the calcium content of the supplement. Protein intakes account for some 3-4% of the variance of bone variables in prepubertal children.

Further interventional studies are required to assess the effect of varying dietary protein intakes on bone mass in pre- and pubertal adolescents.

Calcium supplementation in children on habitually low calcium intakes: The IOM has defined the RDA for calcium in adolescents as 1300 mg/d with an EAR of 1100 mg/d. Yet it is only in a small number of countries, especially North America and those in northern Europe, that adolescent intakes approach these recommendations. In the majority of the developing world calcium intakes among children and adolescents have been estimated to vary between 300 and 500 mg/d, with some intakes being as low as 150-200 mg/d. The majority of calcium supplementation studies in children and adolescents have been conducted in developed countries, and the majority of these studies have shown no long term benefits on bone health. It is not known if similar results are applicable to children on habitually low calcium intakes.

There is accumulating evidence that very low dietary calcium intakes (approximately 200 mg/d) may be associated with nutritional rickets. However, several questions should be asked around the need for increasing calcium intakes in adolescents with habitually low calcium intakes. Firstly is there any evidence of long term adverse sequelae on bone health of calcium intakes in the range of 300-500 mg/d, and secondly is there any evidence of a benefit on bone mass from increasing calcium intakes? In South Africa, fracture rates in black children and adolescents on low dietary calcium intakes are approximately half those found in their white peers. Further, size adjusted BMC is similar in black and white children except at the femoral neck, where it is higher in black children.

A 12 year follow-up study conducted in the Gambia on prepubertal boys aged 8-12, who were supplemented with calcium carbonate (1000mg Ca/d for 5 days per week) for a period of 1 year, provides evidence that there are no long term beneficial effects of calcium supplementation on size adjusted BMC, however the study did find that the supplemented children entered their pubertal related peak height velocity 8 months earlier than the controls, but ended up 2 cm shorter as young adults. The authors conclude that there is a need for caution when applying international recommendations to different populations.

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Cortical Bone Modeling and Remodeling

Ego Seeman, M.D., FRACP

Meet the Professor Session ASBMR 2014

Cortical Bone Modeling and Remodeling – the neglected source of most bone loss and bone fragility

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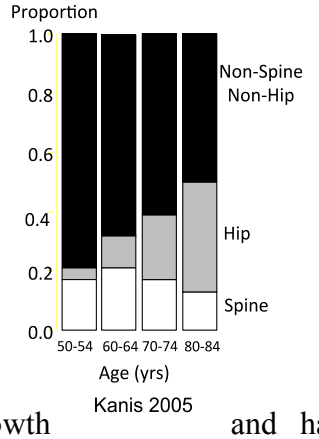
Introduction

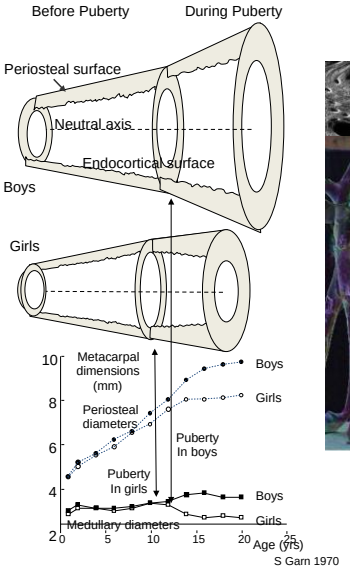
Historically, vertebral fractures and trabecular bone loss are the hallmarks of osteoporosis. Recent studies of the epidemiology and pathogenesis of fractures show that 80% of all fractures are non-vertebral 20% are vertebral. Few studies demonstrate non-vertebral anti-fracture efficacy. Of the few that do, the risk reduction is 20-30% at best; non-vertebral fracture prevention is a very important unmet need.

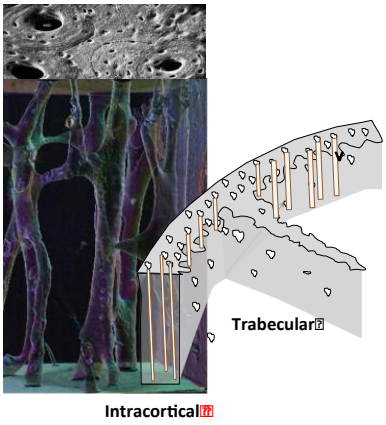


The skeleton serves paradoxical needs; it must be strong to tolerate loading and stiff – able to resist bending - to serve as a lever. Yet it must also be light to facilitate mobility, and flexible, able to deform at the microscopic level to absorb energy during loading.

Strength can be achieved by bulk (high mass), but bulk takes time to growth and has a high energy cost. Nature achieves these paradoxical needs by its macro- and microarchitectural design, its configuration in space by the clever use of void volume – void space.

Modeling (bone deposition without prior resorption) upon the outer periosteal surface during growth widens the bone while concurrent modeling based resorption (not followed by formation) upon the inner (endocortical) surface both removes bone and shifts the cortex outward which increases its resistance to bending (a 4th power of the radial distance from the neutral axis) using less mass *relative* to their diameter; their volumetric apparent density is lower. The cortical area and compressive strength are maintained as the thinner cortex has a larger perimeter.





Mass is also minimized by intracortical remodeling forming the cortical osteonal structures which have a central Haversian canal and Volkmann canals (right panel above figure) that form most of the void volume of the cortical bone. Voids are also formed by the osteocytic lacunar canalicular system and voids within and between collagen fibers and fibrils.

Cortical porosity – the main source of bone loss and bone fragility

Most of the skeleton, ~ 80% is cortical. Only 20% is trabecular. Cortical bone is remodeled, 'turned over', more slowly than trabecular bone, but the slow loss of 4 times more cortical bone than the rapid loss of the smaller volume of trabecular bone results in 70% of all bone loss being cortical.

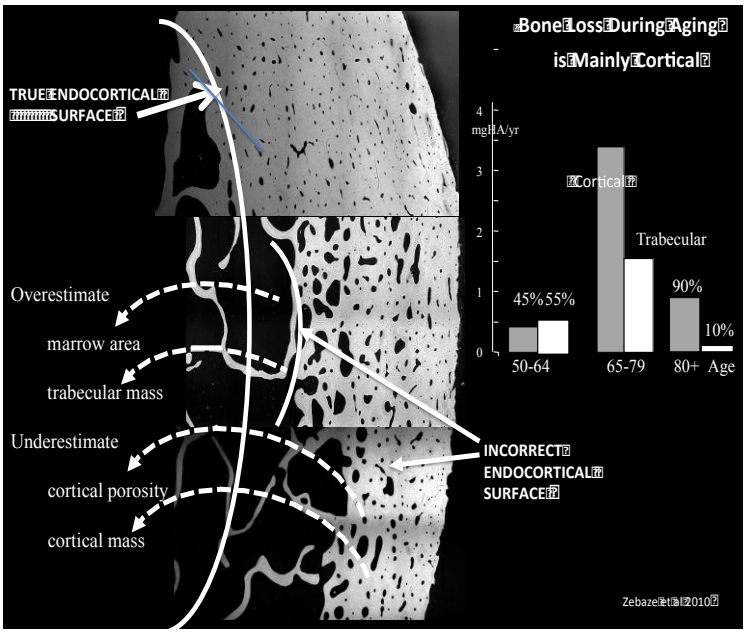
Of this loss, cortical bone loss, most is lost by *intracortical* remodeling initiated at points upon the Haversian and Volkmann canals. As remodeling balance is negative after midlife, more peri-Haversian canal volume is removed than deposited during each remodeling transaction. The canals enlarge focally, they approximate and coalesce, especially on the inner section of the cortex which thins from 'within'.

The inner cortex cavitates producing fragmentation of cortical bone (trabecularization}. Several errors result if the transitional zone is erroneously regarded as being 'trabecular bone' in the medullary canal.

a. Cortical fragments look like trabeculae so they are measured as 'trabecular' bone overestimating trabecular number and thickness in older persons or in disease.

b. The decrease in trabecular density across menopause or disease is underestimated so persons at risk are not identified correctly.

c. The cortical porosity created by intracortical remodeling is erroneously seen as medullary void leading to an underestimate of the rise in porosity with age because only the porosity of the remaining compact appearing cortex is calculated.



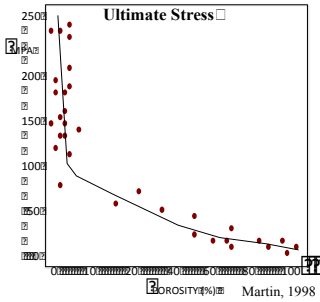
d. The decrease in cortical bone across menopause and with age is over-estimated because the cortical fragments are 'seen' as trabecular bone.

e. The combination of underestimating trabecular loss and over estimating cortical loss leads to the erroneous idea that menopause causes cortical not trabecular bone loss.

2.

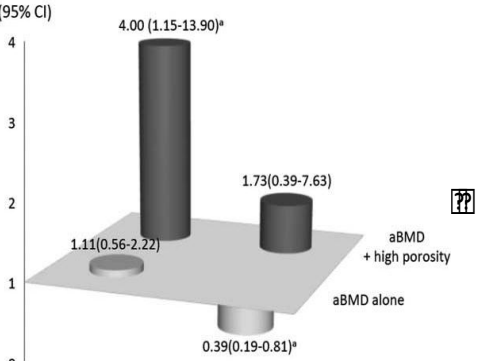
Measuring porosity

Porosity of the cortex is primarily formed by cross sections of the canals. These canals are about 50-100 microns in diameter. If scanners have a resolution of 80 microns it is not possible to measure the porosity without the pixel containing some mineralized bone (called a partial volume effect). The attenuation of the photons is increased by the mineralized matrix and if this attenuation is above the threshold designated to be 'porosity', that pixel is excluded with its contents. That's why porosity is underestimated by current methods of image analysis.



Clinical relevance

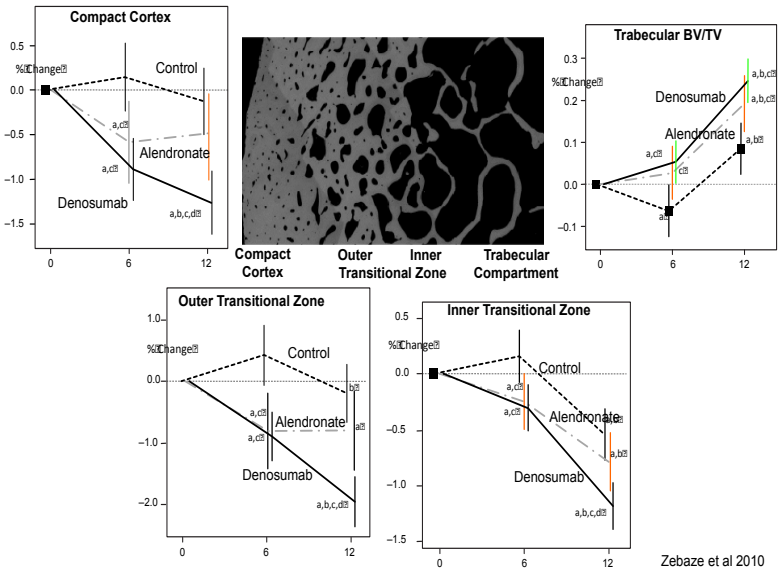
Bone Strength A small increase in porosity reduces bone strength exponentially. Failure to quantify porosity during growth, aging and disease underestimates the compromise in strength so produced.



Osteopenia Patients with osteopenia account for 50% of fractures in the community. This is not only because more persons have osteopenia than osteoporosis. The measurement of porosity identifies persons with osteopenia with fractures and so helps target individuals with osteopenia that would otherwise not be offered therapy

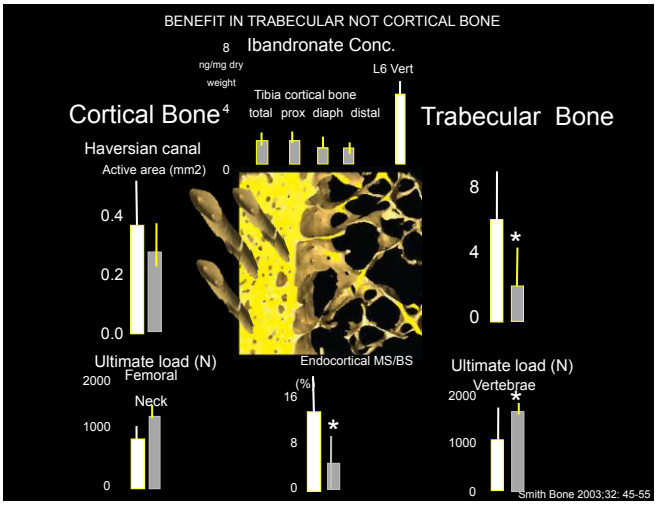
Antiresorptive therapy Reduces porosity and within 6 months. The reduction is greater with denosumab than alendronate, perhaps because denosumab can access deeper intracortical remodeling sites than alendronate, a bisphosphonate which is highly bound to superficial matrix.

Denosumab and Alendronate effects on cortical porosity and trabecular BV/TV



Zebaze et al 2010

Ibandronate achieves higher concentrations in trabecular than cortical bone and suppresses remodeling more trabecular bone and upon the endocortical surface because it can absorb at these locations but less so in thicker cortical bone.



Lessons

1. **Consider structure, not only 'bone mineral density'.**
2. **Consider cortical bone, not only trabecular bone.**
3. **Consider non-vertebral fractures, not only vertebral fractures.**
4. **Consider bone surfaces, its outer or periosteal surface, and the three (intracortical, endocortical, and trabecular) components of its inner or endosteal surface. This is were the cellular activity changes its size and shape and strength.**
5. **Consider the relevance of cortical porosity, a measureable fingerprint of most bone loss during aging and a key structural defect causing bone fragility.**

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Further reading

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Seeman E, Delmas PD. Bone quality. N Engl J Med. 2006;354:2250–61.

In Vivo Microindentation

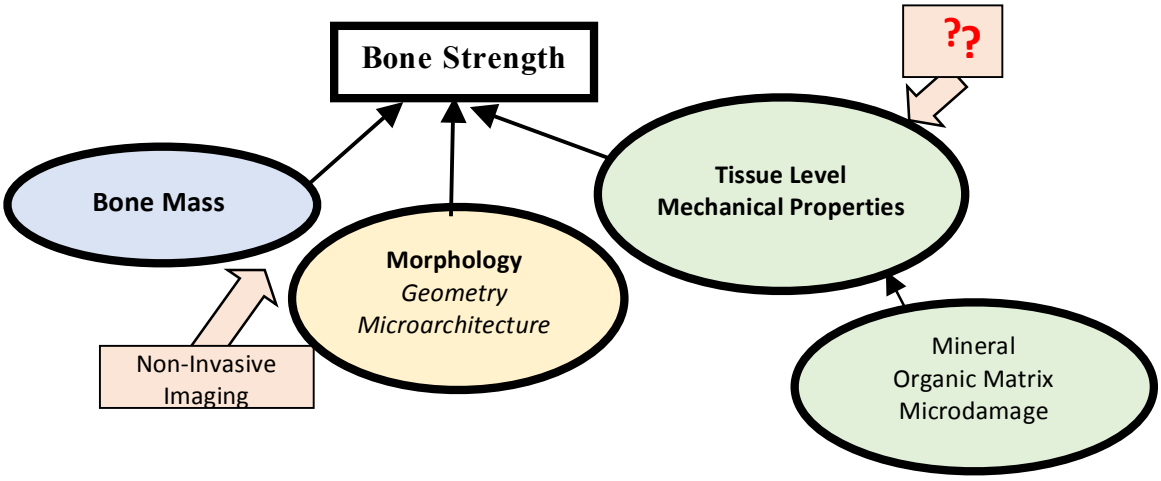
Mary Bouxsein, Ph.D.

"In vivo microindentation"
11:30 am – 12:30 pm, Monday, 15 September 2014
Mary L. Bouxsein, PhD
Harvard Medical School, Boston, MA

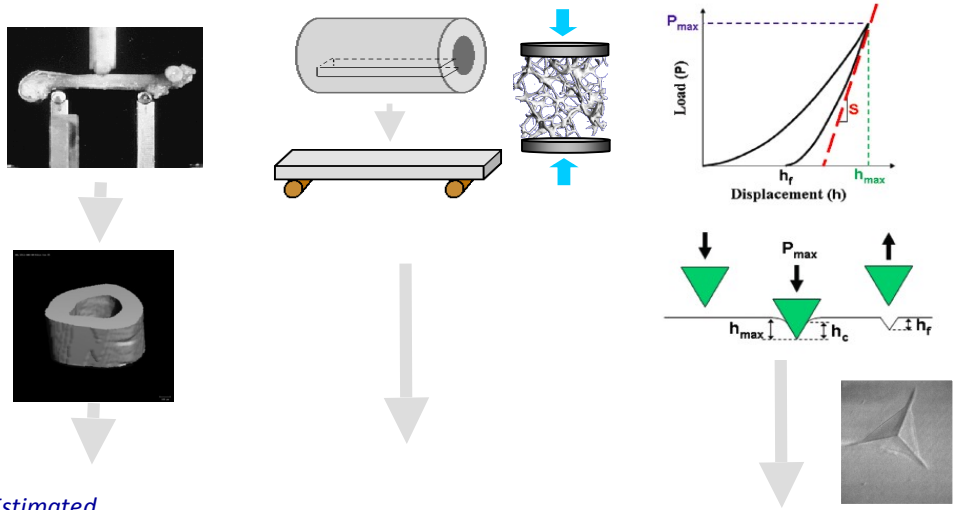
National Institutes of Health
R21 AR063253
NIAMS National Institute of Arthritis and Musculoskeletal and Skin Diseases

A. INTRODUCTION

From a mechanical perspective, fractures represent a structural failure of the bone whereby the forces applied to the bone exceed its load-bearing capacity. The forces applied to the bone will depend on the specific activity, and will vary with the rate and direction of the applied loads. The load-bearing capacity of a bone (or "whole bone strength") depends on the amount of bone (i.e., size), the spatial distribution of the bone mass (i.e., shape), and the intrinsic properties of the materials that comprise the bone. We have many non-invasive methods to assess bone mass, geometry and microarchitecture. Until development of reference point indentation, we had few options for assessing intrinsic bone material properties in vivo.



Traditional methods to measure material properties



Estimated
- elastic modulus
- ult. strength
- toughness

- elastic modulus
- ult. strength
- toughness

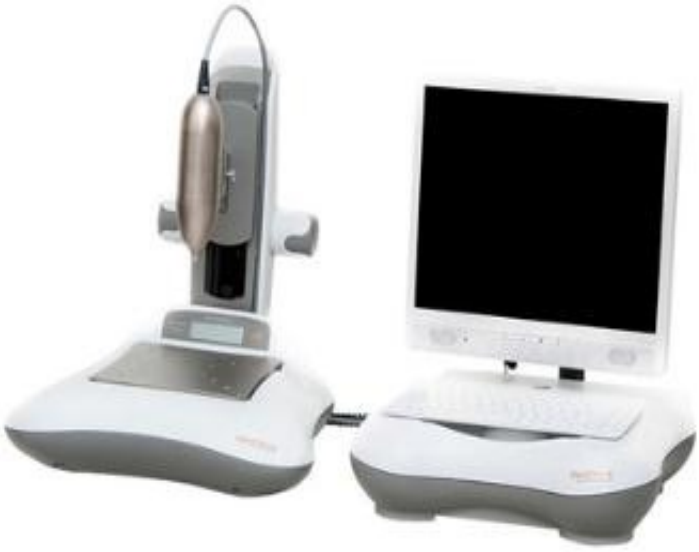
- elastic modulus
- hardness

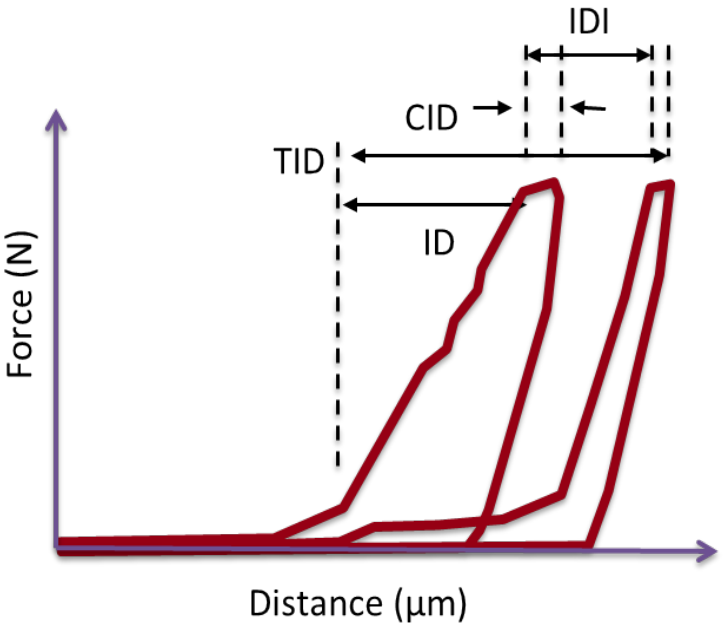
B. REFERENCE POINT INDENTATION (RPI): BIODENT VS OSTEOPROBE

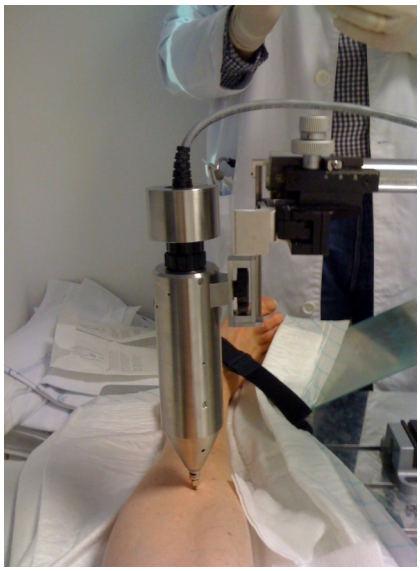
RPI measures distance that the test probe indents into the bone relative to a reference that is located on the bone surface.

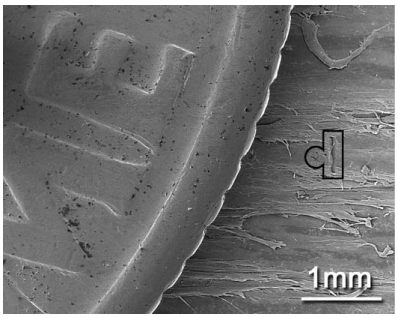
• Does not require special surface preparation

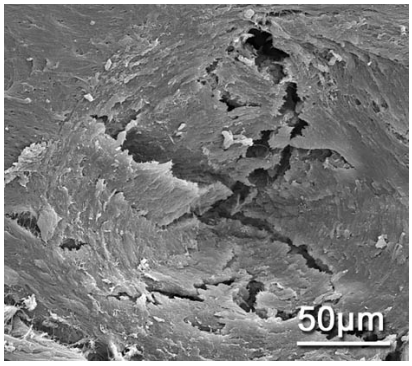
BIODENT [1-3]







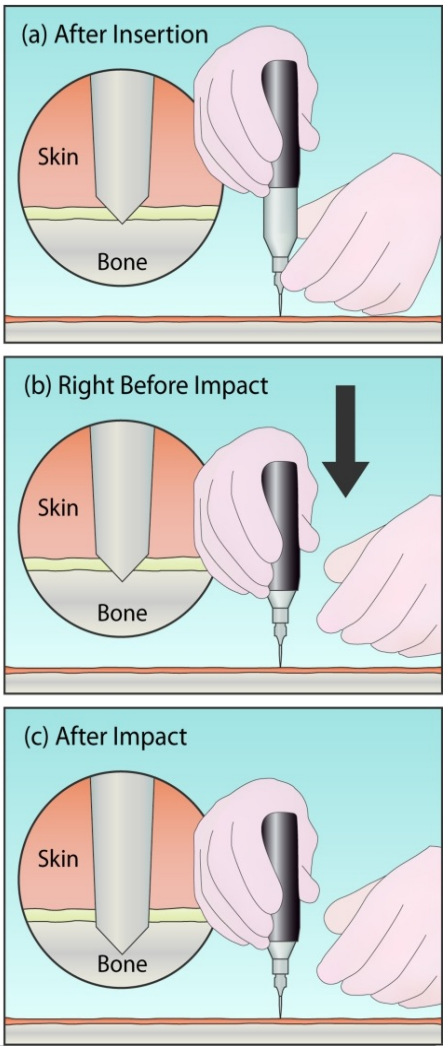


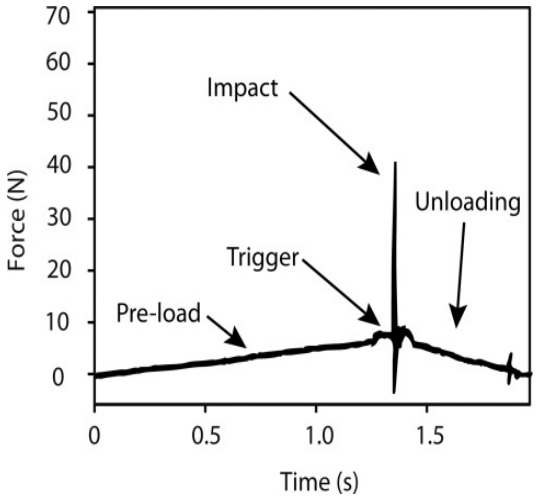


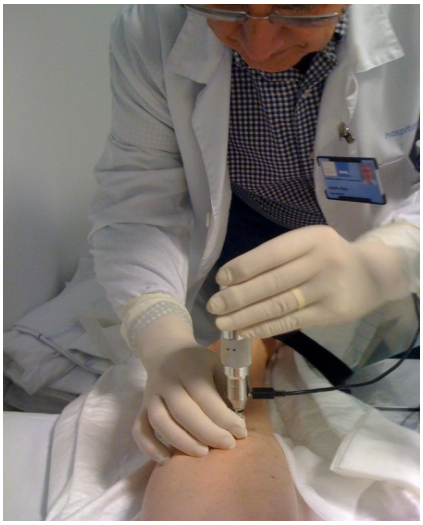
OSTEOPROBE [4,5]

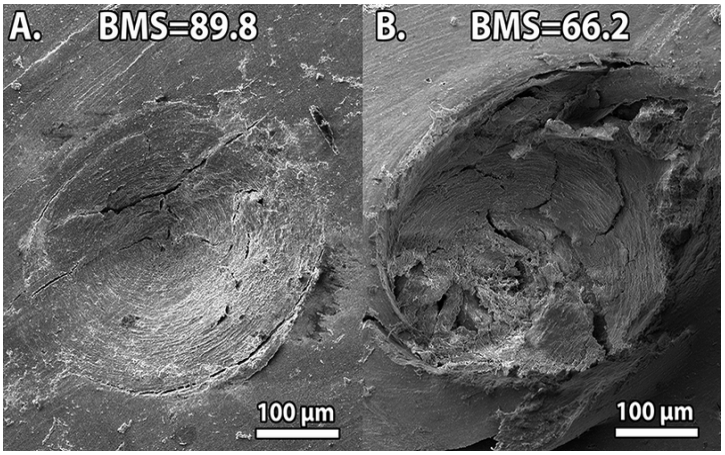
http://www.activelifescientific.com/how-osteoprobe-works/

- Hand-held
- Single impact (40N) indentation per measurement
- Outcome = Bone Material Strength Index (BMSi)
  Indentation distance in bone normalized to that of PMMA block









C. PUBLISHED STUDIES in HUMANS

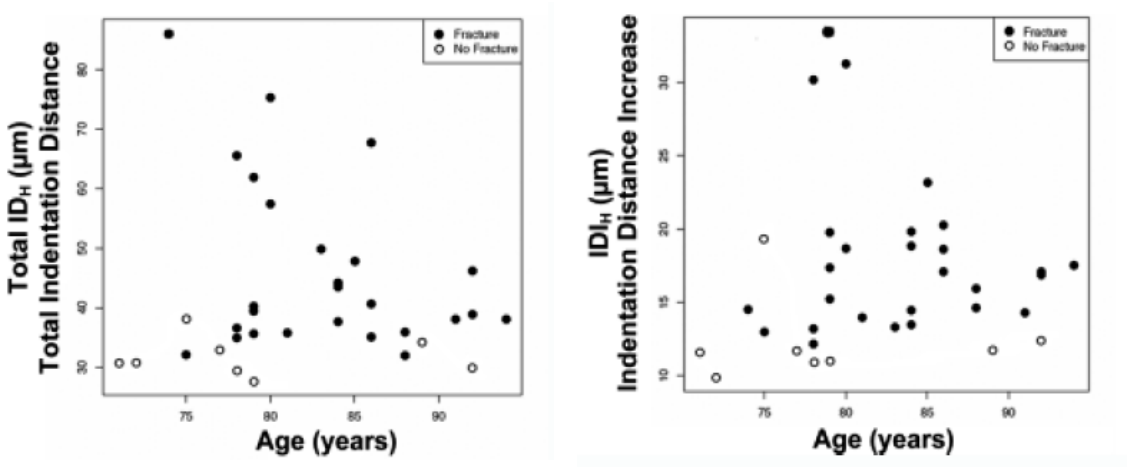
Diez-Perez et al, Microindentation for in vivo measurement of bone tissue mechanical properties in humans, JBMR, 2010. – Biodent [6]

Subjects: 27 postmenopausal women with OP-related fractures, 8 non-fracture controls

Design: Cross-sectional study with Biodent: 20 indentations, 2 Hz, 11 N

Take home points: Total indentation distance (46.0 +/- 14 versus 31.7 +/- 3.3 µm, p = .008) and indentation distance increase (18.1 +/- 5.6 versus 12.3 +/- 2.9 µm, p = .008) were greater in fracture patients than in controls. *Femoral BMD was measured in only a subset of patients, so difficult to know whether indentation differences were independent of BMD.*

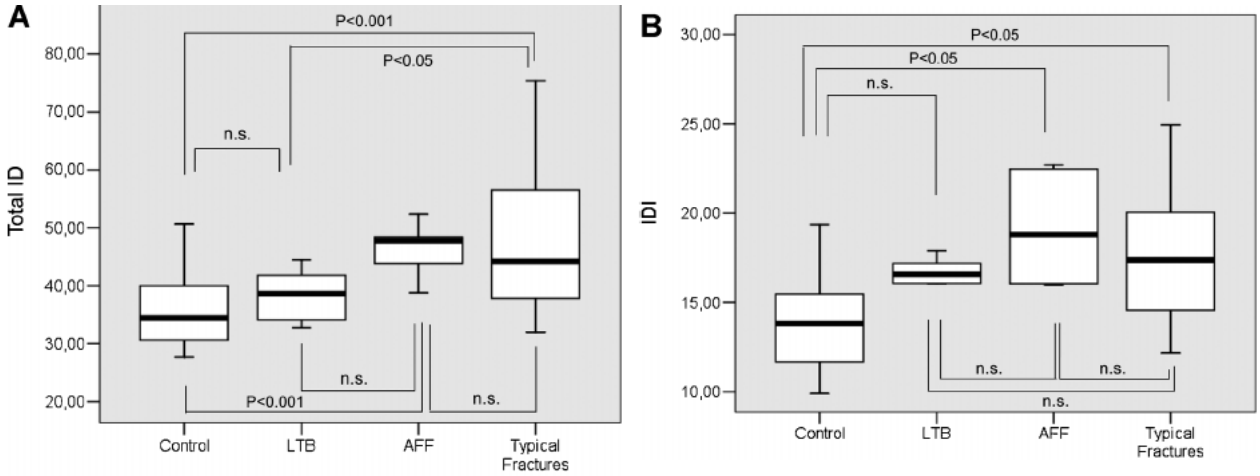
- *FIRST STUDY to report in vivo microindentation.*



Guerri-Fernandez, et al. Microindentation for in vivo measurement of bone tissue material properties in atypical femoral fracture patients and controls. JBMR 2013 – Biodent [7]

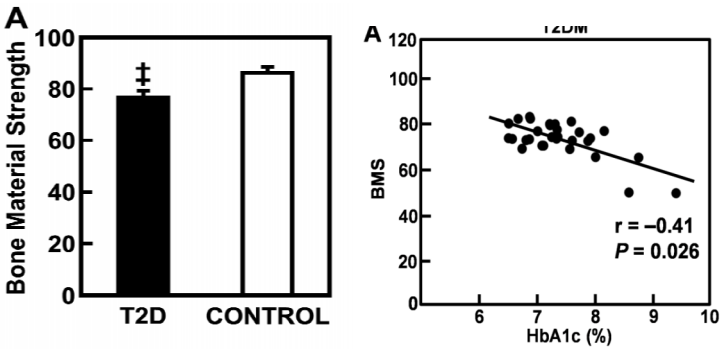
Subjects: postmenopausal women, including 6 AFF, 38 typical OP fractures, 6 long-term bisphosphonate, and 20 controls without fracture.

Design: Cross-sectional study



Take home points: Patients with AFF have deterioration in cortical bone properties similar to that for the OP fracture group. The LTB group shows levels that are in between controls and both type of fractures. No indication that BP treatment puts majority of patients at risk for AFF.

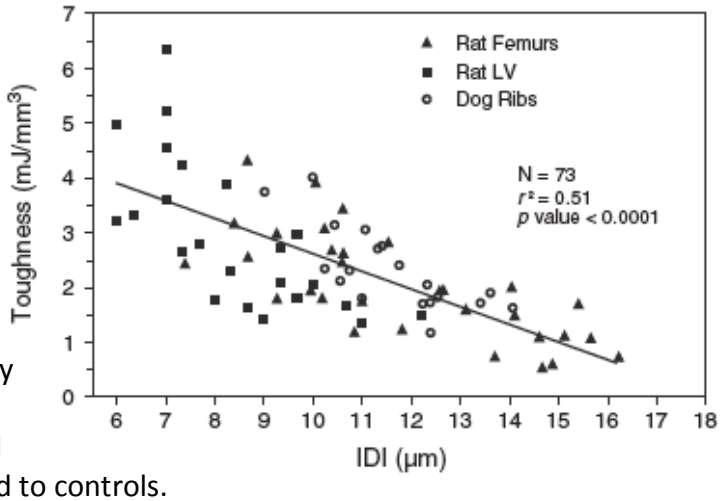
Farr et al, In vivo assessment of bone quality in postmenopausal women with type 2 diabetes, JBMR 2014 – Osteoprobe [8]



Subjects: 30 postmenopausal women with T2DM, 30 age-similar controls
Design: Cross-sectional study
Take home points: Women with longstanding (>10 yrs) T2DM had 11% lower bone material strength (BMS), even after adjusting for BMI differences. BMD and bone microarchitecture was largely similar between groups after adjustment for higher BMI in women with T2DM.
FIRST STUDY to report in vivo microindentation in humans with Osteoprobe.

D. OTHER KEY STUDIES

Gallant et al, Reference-point indentation correlates with bone toughness assessed using whole-bone traditional mechanical testing. Bone 2013 – Biodent [9]



Study deisgn: different animal models (rats) – T2DM, BP treatment
Take home points: Using different animal models, authors report that apparent bone toughness obtained from 3-point bending and axial compression is inversely correlated with the indentation distance increase (IDI) obtained from RPI (r2 = 0.50 – 0.57). Conditions or treatments previously shown to cause differences in toughness, including diabetes and BP treatment, had significantly different IDI values compared to controls.

Aref et al, In vivo reference point indentation reveals positive effects of raloxifene on mechanical properties following 6 months of treatment in skeletally mature beagle dogs. Bone 2013 – Biodent [10]

Study design: in vivo assessment (single timepoint) following 6 mo treatment with RAL in 12 dogs; assessed anterior tibial midshaft
Take home points: IDI (-16%) and energy absorption (-21%) were significantly lower in RAL-treated dogs than VEH. **First study to report in vivo Biodent measures in large animal model*

E. ABSTRACTS AT THIS MEETING

#1064 Bone Material Strength as measured by microindentation in vivo is decreased independently of BMD in patients with fractures. Frank Malgo, Neveen Hamdy, Socrates Papapoulos, Natasha Appelman-dijkstra

#FR0290 Microindentation in vivo captures elements of bone fragility independently of BMD. Natasha Appelman-dijkstra, Frank Malgo, Socrates Papapoulos, Neveen Hamdy

#SU0020 Association Between Reference Point Indentation Measures and Cortical Bone Composition, Bending Properties, and Fracture Toughness. Lamya Karim, Nathalie Portero-Muzy, Daniel Brooks, Evelyne Gineyts, Pascale Chavassieux, Roland Chapurlat, Mary Bouxsein

#SU0023 Differences in Assessment of Micro-Indentation Resistance between BioDent and OsteoProbe. Mathilde Granke, Sasidhar Uppuganti, Mary Katherine Manhard, Mark Does, Donald Lee, Daniel Perrien, Jeffry Nyman

#MO0028 Patients with stress fractures exhibit impaired bone material properties by microindentation. Daysi Duarte Sosa, Erik Fink Eriksen

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3. Randall C, Mathews P, Yurtsev E, Sahar N, Kohn D, Hansma P 2009 The bone diagnostic instrument III: testing mouse femora. Rev Sci Instrum **80**(6)**:**065108.
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6. Diez-Perez A, Guerri R, Nogues X, Caceres E, Pena MJ, Mellibovsky L, Randall C, Bridges D, Weaver JC, Proctor A, Brimer D, Koester KJ, Ritchie RO, Hansma PK 2010 Microindentation for in vivo measurement of bone tissue mechanical properties in humans. J Bone Miner Res **25**(8)**:**1877-85.
7. Guerri-Fernandez RC, Nogues X, Quesada Gomez JM, Torres Del Pliego E, Puig L, Garcia-Giralt N, Yoskovitz G, Mellibovsky L, Hansma PK, Diez-Perez A 2013 Microindentation for in vivo measurement of bone tissue material properties in atypical femoral fracture patients and controls. J Bone Miner Res **28**(1)**:**162-8.
8. Farr JN, Drake MT, Amin S, Melton LJ, 3rd, McCready LK, Khosla S 2014 In vivo assessment of bone quality in postmenopausal women with type 2 diabetes. J Bone Miner Res **29**(4)**:**787-95.
9. Gallant MA, Brown DM, Organ JM, Allen MR, Burr DB 2013 Reference-point indentation correlates with bone toughness assessed using whole-bone traditional mechanical testing. Bone **53**(1)**:**301-5.
10. Aref M, Gallant MA, Organ JM, Wallace JM, Newman CL, Burr DB, Brown DM, Allen MR 2013 In vivo reference point indentation reveals positive effects of raloxifene on mechanical properties following 6 months of treatment in skeletally mature beagle dogs. Bone **56**(2)**:**449-5

Management of Atypical Femoral Fractures

Angela Cheung, M.D., Ph.D.

Management of Atypical Femoral Fractures

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Significance of the Topic:

In the past ten years, various publications (case series, case reports, cohort studies) have described atypical femoral fractures (AFFs) as being a potential adverse effect of long-term bisphosphonate use and more recently, denosumab use. This led to the creation of an ASBMR Task Force in 2010, in which a group of international experts addressed key questions related to AFFs (1).

> Although the relative risks of AFFs have been reported to be very high in patients on bisphosphonates, ranging from 2.1 to 128 in the literature, the absolute risk is quite low (ranging from 3.2 to 50 cases per 100,000 person-years), especially when compared to the number of other fractures prevented by their use (2). However, there is concern that lack of awareness, lack of recognition and underreporting may be downplaying the true incidence of AFFs. These fractures cause significant anxiety amongst patients and physicians, and clinical guidelines regarding AFF screening, identification, and medical, surgical, and rehabilitative management have yet to be developed for health care professionals. Moreover, the literature is still unclear regarding a causal relationship between bisphosphonate use and AFFs, but recent observations have indicated that risk may rise with increasing duration of use (~100 per 100,000 person-years in patients who have used bisphosphonates for 8 to 9.9 years (3)). *AFFs are rare, but incomplete AFFs may be more common and may be present for a long time prior to a complete fracture.* Further information and knowledge translation is urgently required to guide clinical decision making regarding duration of bisphosphonate therapy, as well as prevention, early diagnosis, and management of complete and incomplete AFFs.

Learning Objectives:

As a result of participating in this session, attendees should be able to:

1) **Define AFFs** as per the 2014 ASBMR case definition
2) **Identify patients at risk for AFFs using** established risk factors
3) **Explain to patients the current understanding regarding pathogenetic mechanisms behind AFF development**
4) **Order the correct diagnostic tests for identification and management of AFFs**
5) **Discuss with AFF patients regarding management options**

Case definition:

- **2014 ASBMR Case Definition for AFF (2)**

  Location: Below lesser trochanter, above supracondylar flare

  Major features (4 out of 5 criteria):
  1) Little or no trauma
  2) Transverse (or mostly transverse)
  3) Non-comminuted (or minimally comminuted)
  4) Complete fractures extend through both cortices and may have a medial spike; Incomplete fractures involve only the lateral cortex
  5) Localized periosteal or endosteal reaction of the lateral cortex

  Minor features (none required):
  - Generalized increase in cortical thickness
  - Delayed healing
  - Prodromal symptoms such as dull aching pain in groin or thigh
  - Bilateral fractures and symptoms

  **An incomplete AFF has to satisfy criteria 1, 2, 4, and 5.*

Key Clinical Feature: Prodromal pain

- ASBMR Task Force review
  - 75% have prodromal pain

  These features are fundamentally different from common osteoporotic femur fractures and strongly suggest a distinct pathogenesis.

- Ontario AFF Cohort (n~180)
  - ~90% have prodromal symptoms: pain, ache, weakness, loss of function

Risk Factors:

- Younger women
- Osteopenic (can vary)
- Asian race
- Long duration of BP therapy
- Multiple anti-resorptive medications
- Glucocorticoid use
- Rheumatoid arthritis
- Varus hip angle, bow-leg deformity, small diameter

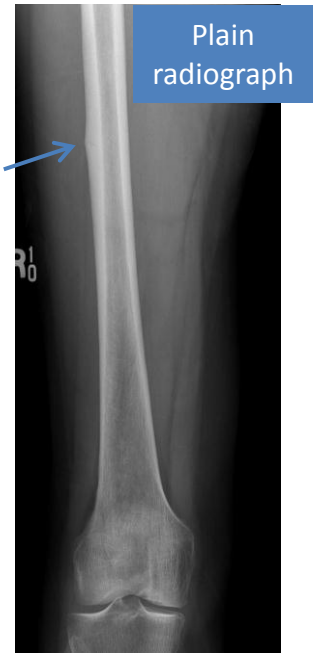
Patients taking bisphosphonates for an extended duration (typically for more than 5 years), tend to be at greater risk of AFFs, however reports of AFFs have also been found in patients who have not been treated with bisphosphonates.

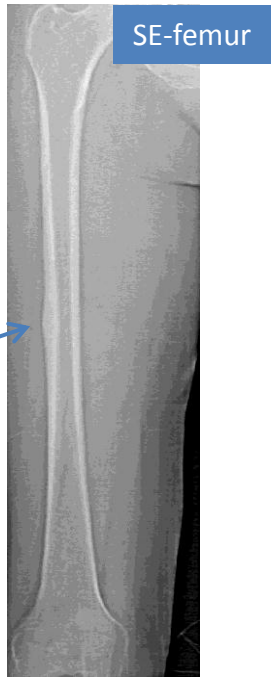
Potential Pathogenetic Mechanisms:

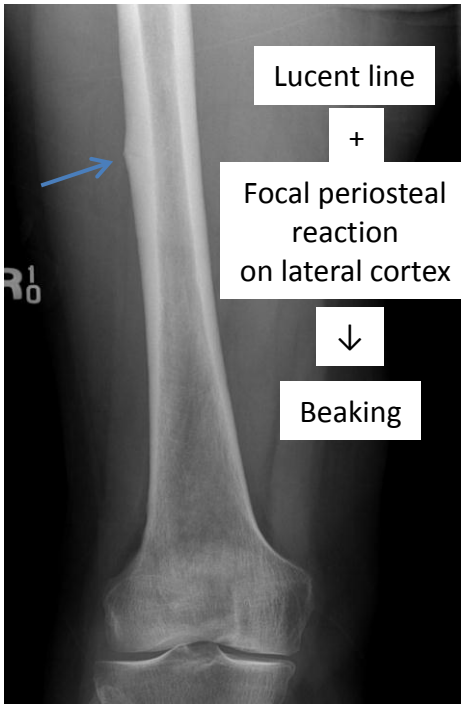
- Bisphosphonate use leads to suppression of bone remodeling. Animal studies have indicated that long term bisphosphonate use could affect AFF development via effects on:
  - Bone's material properties – alterations to collagen & AGEs (may cause reductions in post yield deformation, energy to fracture, and toughness), increase in homogeneity of the bone tissue (could permit further damage accumulation), increased tissue mineral density, increased microdamage accumulation and crack initiation, retention of bisphosphonates in bone. Clinical studies have been inconclusive.
- Healing of stress fractures
  - AFFs are stress or insufficiency fractures that develop over time.
  - Location and bisphosphonate use may impair healing of stress fractures.
- Relationship of hip and lower limb geometry
- Genetic susceptibility – collagen abnormality, low bone turnover at baseline, etc…

Tests for diagnosis of AFFs:

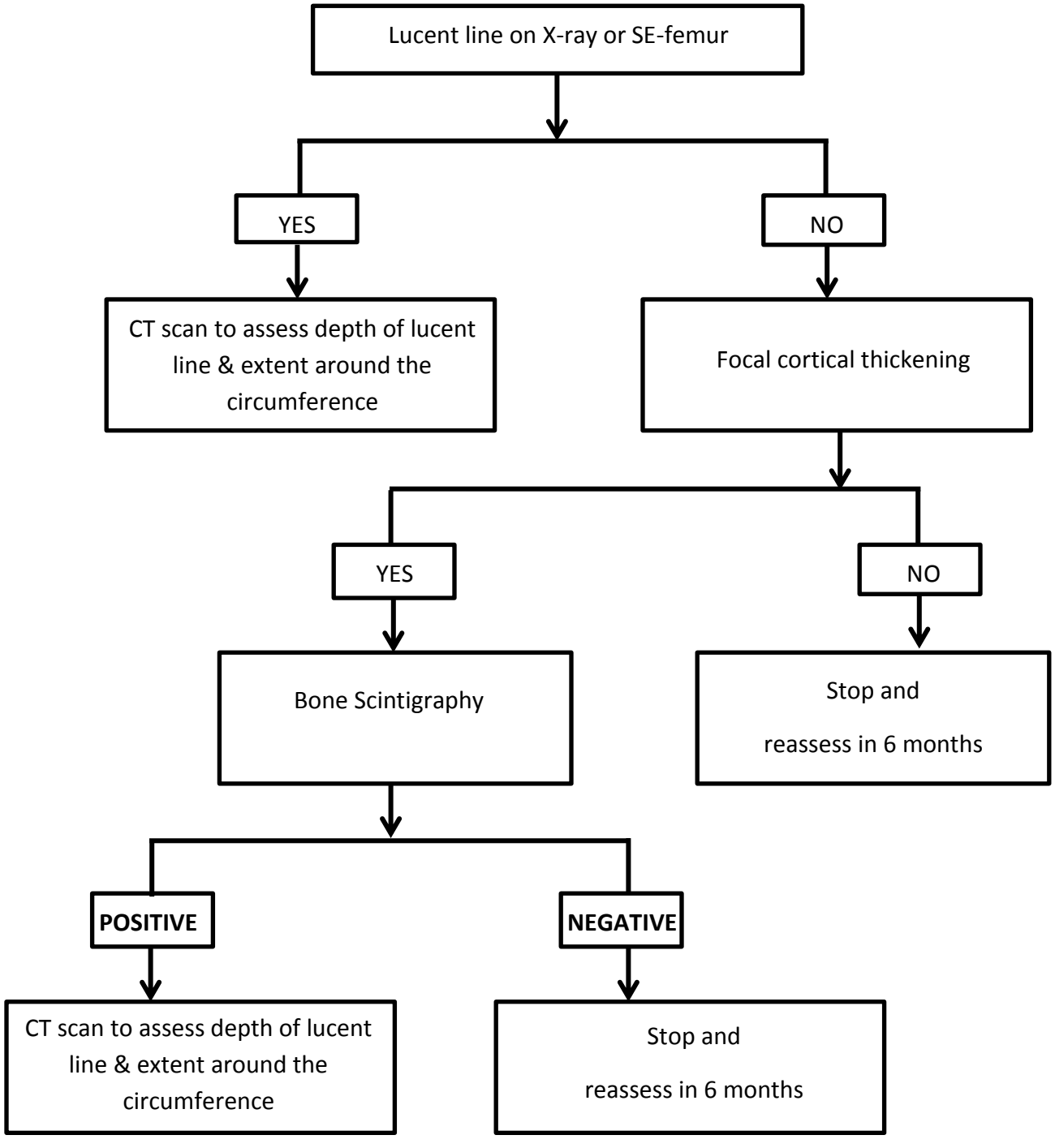
- **Complete AFFs –** complete AFFs have characteristic findings have characteristic radiographic findings as mentioned above. Often these are not difficult to diagnose.
- **Incomplete AFFs** – Symptomatic patients (those with thigh/groin pain, ache or weakness) should do an X-ray (AP view) of the whole femur (1, 2) or a SE (single energy) –femur scan using a densitometer.







My recommendations:



*****_ASBMR Task Force recommends that areas of cortical thickening **should be further evaluated with higher-order imaging** (bone scintigraphy, MRI, or CT) (2)_

Current Recommendations Regarding Management of AFFs:

- Complete AFFs:
  - Fixation with a full-length intramedullary rod (IMR) is the preferred method
  - Assess contralateral side for incomplete AFF

- Incomplete AFFs:
  - Medical Management:
    1. Reduce and limit weight bearing activities
    2. Discontinue potent antiresorptive agents
    3. Optimize calcium and vitamin D status
    4. Consider teriparitide for those who appear not to heal on conservative therapy (i.e. 1 + 2 + 3) (4)
    5. Worsening thigh or groin pain will need to be assessed for prophylactic insertion of IMR
  - Surgical management:
    - Prophylactic insertion of full-length IMR for fixation

  **TAFF is an ongoing randomized placebo-controlled trial assessing the effect of teriparatide on healing of incomplete AFFs. Without conclusive results from the TAFF trial, patient's preference needs to be considered when choosing medical versus surgical therapy. In some cases, it is reasonable to do both.*

- Prevention of AFFs in osteoporosis patients:
  - Drug treatment for those at increased fracture risk – consider benefit/risk ratio
  - Reassess drug therapies after 3-5 years, consider drug holiday for stable moderate risk patients
  - Educate physicians and patients about prodromal pain (1)

References

1. Shane E, Burr D, Ebeling PR, Abrahamsen B, Adler RA, Brown TD, Cheung AM, Cosman F, Curtis JR, Dell R, Dempster D, Einhorn TA, Genant HK, Geusens P, Klaushofer K, Koval K, Lane JM, McKiernan F, McKinney R, Ng A, Nieves J, O'Keefe R, Papapoulos S, Sen HT, van der Meulen MC, Weinstein RS, Whyte M; American Society for Bone and Mineral Research. Atypical subtrochanteric and diaphyseal femoral fractures: report of a task force of the American Society for Bone and Mineral Research. J Bone Miner Res. 2010;25:2267–94.
2. Shane E, Burr D, Ebeling PR, Abrahamsen B, Adler RA, Brown TD, Cheung AM, Cosman F, Curtis JR, Dell R, Dempster D, Einhorn TA, Genant HK, Geusens P, Klaushofer K, Koval K, Lane JM, McKiernan F, McKinney R, Ng A, Nieves J, O'Keefe R, Papapoulos S, Sen HT, van der Meulen MC, Weinstein RS, Whyte M; American Society for Bone and Mineral Research. Atypical subtrochanteric and diaphyseal femoral fractures: second report of a task force of the American Society for Bone and Mineral Research. J Bone Miner Res. 2014; 29: 1-23.
3. Dell RM, Adams AL, Greene DF, Funahashi TT, Silverman SL, Eisemon EO, Zhou H, Burchette RJ, Ott SM. Incidence of Atypical Nontraumatic Diaphyseal Fractures of the Femur. J Bone Miner Res. 2012; 27:2544-50
4. Chiang CY, Zebaze RM, Ghasem-Zadeh A, Luliano-Burns S, Hardidge A, Seeman E. Teriparatide improves bone quality and healing of atypical femoral fractures associated with bisphosphonate therapy. Bone. 2013; 52:360-5.

The NIH Geroscience Summit

Robert Jilka, Ph.D.,
Joan McGowan, Ph.D., and
John Williams, Ph.D.

Meet the Professor Session
GeroScience

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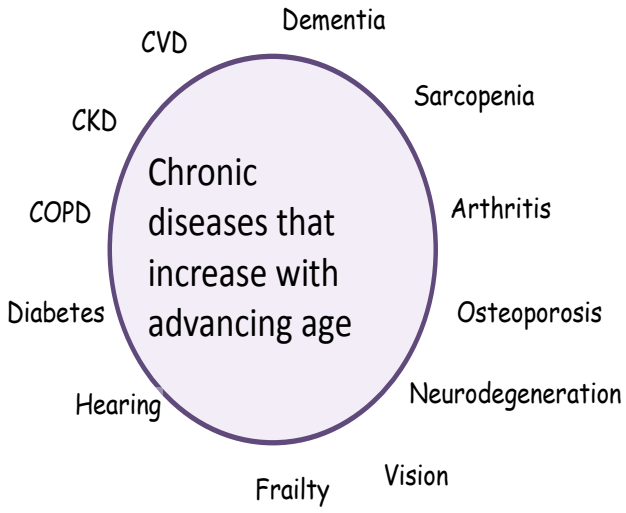
Purpose of this Meet the Professor Session: to summarize and discuss recent developments in aging research, as presented at the GeroScience Summit held at NIH in October of 2013.



GeroScience is an important trans-NIH target. Aging is the single largest risk factor for many chronic diseases. Integrated GeroScience aims at developing reliable quantitative metrics of conserved molecular and cellular pathways of aging that underlie multiple chronic diseases and conditions. The metrics will allow, in pre-clinical animal models, the identification of interventions that might affect multiple age-related diseases including comorbidities.

The overarching goal of GeroScience is to understand how perturbations of specific molecular pathways and/or cellular processes in any given tissue across the spatial dimension of lifespan affect the onset, progression, or severity of a variety of chronic diseases and to determine whether agents that extend lifespan also prevent chronic diseases leading to increases in healthspan.

Given the rapid pace at which human populations are aging, extending healthspan would have a global transformative effect on par with those of improved hygiene, the discovery of antibiotics, and immunizations against infectious agents such a polio and measles. Over the past 25 years, researchers have made impressive progress in understanding the genetics, biology and physiology of aging. The elderly comprise the fastest growing segment of our population, and a

large proportion of health resources are used to treat the elderly, who are often affected by multiple diseases / conditions.

Basic research in animal models has demonstrated the plasticity of lifespan. Most importantly, it has shown that often, extension of lifespan is accompanied by a delay in the appearance and progression of morbidity, as well as a slowing in age-related functional decline. That is, slowing the aging processes leads to an increase in healthspan, the portion of life spent in good health. Yet many fundamental issues remain to be addressed and understood, especially translational research and the application of these findings to the human population.

The **GeroScience Interest Group (GSIG) at NIH** was formed under the leadership of the NIA to provide a collaborative framework for the many NIH institutes with interests in exploring the biological mechanisms that drive the appearance of multiple diseases of the elderly. The GSIG aims to accelerate and coordinate efforts across NIH to promote further discoveries on the common risks and mechanisms behind such diseases. By pooling resources and expertise, the GSIG identifies major cross-cutting areas of research and proposes coordinated approaches to identify hurdles and envision solutions. To assist scientists interested in solving the health problems of our burgeoning elderly population, the GSIG supports the development of new tools, models and paradigms that address the basic biological underpinnings of multiple diseases.

The GSIG organized the Advances in GeroScience Summit held at NIH, Oct 30-31, 2013 "Advances in GeroScience: Impact on Healthspan and Chronic Disease "

The Trans-NIH GeroScience Interest Group was formed in 2011 and had an inaugural seminar in March of 2012. To date, 20 NIH Institutes/Centers have joined GSIG. The purpose of this Summit was to outline the current status of science in the field and identify the most promising research opportunities for progress in understanding the biology of aging and its relationship to the emergence of the diseases of aging.

The topics addressed by the Summit speakers and attendees were:

Adaptation to Stress - Both physiological and psychological stressors are linked to aging and chronic disease states, but it has been difficult to pin down precisely how specific stressors interact with molecular drivers of pathology and how age impacts these interactions. Despite the difficulty, this is a critical field of endeavor since many forms of stress can be modified by behavioral change, opening a potentially rapid path by which people might prevent or delay chronic diseases.

Epigenetics - Widespread epigenetic changes are evident in a number of chronic diseases, including but not limited to cancer. Recently, epigenetic changes have been linked to the aging process directly. Described in briefest terms, age-associated changes drive developmentally organized epigenomes toward entropy, challenging the ability of cells to maintain normal function.

Inflammation - Acute inflammation is an important adaptive response to mediate tissue repair in response to a range of insults. However, recent research led to the discovery that low grade chronic inflammation is a contributing factor to aging and chronic disease states.

Macromolecular Damage - One of the oldest theories of aging is that cumulative damage contributes to aging phenotypes, since many molecules exhibit increasing damage with age. It remains unclear which of these events promote aging and to what extent. Strong evidence has emerged for macromolecular damage as a driver of chronic diseases (e.g. DNA damage in cancer; oxidative damage in cardiovascular disease). A systematic understanding of types and levels of macromolecular damage in a wide range of tissues chronic diseases may help to identify the common features associated with aging and underlie the effects of aging on disease.

Metabolism - Widespread metabolic changes occur during aging that could underlie part of the increased incidence of chronic diseases, including type II diabetes, cardiovascular disease, neurodegenerative disease, osteoporosis and cancer. However, the specific metabolic changes during aging that underlie disease are still a matter of extensive debate.

Proteostasis - Altered proteostasis is increasingly associated with aging and interventions improving proteostatic mechanisms including autophagy, proteasome function and unfolded protein responses are all linked to longevity in animal models of aging. Increasingly, these interventions are showing promise for disease states as well. Thus, strategies to enhance proteostasis could have wide therapeutic advantages across the spectrum of chronic diseases.

Stem Cells and Regeneration - Aging is accompanied by intrinsic changes to adult stem cells from many tissues and the niches they inhabit. Recent evidence indicates that these changes may underlie several aspects of aging, but the extent to which these changes promote age-related diseases remains poorly understood.

GSIG Summary of the Meeting and Recommendations (October 2013):
http://www.nia.nih.gov/sites/default/files/geroscience_summit_2013_outcomes-recommendations_v2.pdf

Highlights of the GSIG Recommendations

- Foster studies aimed at identifying how our current knowledge of the biology of aging can be applied to study the impact of aging on individual (and multiple) age-related diseases/conditions.

- How are the seven research areas outlined above connected with each other and with chronic diseases? The seven discussion areas were treated separately during the meeting, but are likely inter-related. Certainly they influence each other, raising questions as to whether early changes in one or more of these processes drive maladaptive changes in others.

- Moreover, the connections likely overlap but differ across chronic disease states. Studies that focus on the connections between different aspects of aging and their relationship to disease should be encouraged. The NIA has published a notice in the Guide advising the community of our interests, using epigenetics as a starting point.

Funding Opportunity Announcement for Epigenetic Analysis of Aging as a Risk Factor for Chronic Disease and Degenerative Conditions (U34)

Funding Number:NOT-AG-14-012
Funding Type: NOT
Release Date: March 21, 2014
Expiration Date: January 1, 2017
Web Site: http://grants.nih.gov/grants/guide/notice-files/NOT-AG-14-012.html

Issues and questions raised during the GeoScience Summit of potential interest to the musculoskeletal research community.

- With increased life expectancy, people live more years with disability; and musculoskeletal disorders are a leading cause of disability. http://www.thelancet.com/themed/global-burden-of-disease

- The "sterile" inflammation associated with chronic diseases of aging differs from the inflammation caused by acute insults and infection.

- Practical considerations lead many to use rodent models for investigating the aging musculoskeletal system. However, vivarium conditions, for example lack of environmental enrichment, can increase levels of stress in mice, leading to chronic disease.

Reference Materials

Abstracts of GeroScience Summit presentations:
http://sigs.nih.gov/geroscience/Documents/NIH%20GeroScience%20Summit%20(2013)%20Abstracts.pdf

Articles published Journals of Gerontology Series A summarizing the presentations
http://biomedgerontology.oxfordjournals.org/content/69/Suppl_1.toc

Public Policy and Aging Report: "The longevity dividend: Geroscience meets Geropolitics:
http://www.afar.org/docs/Public_Policy__Aging_Report_-_Fall_2013.pdf

Additional publications of interest

Lim et al, A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. The Lancet Volume 380, Issue 9859, 2224–2260.
http://www.sciencedirect.com/science/article/pii/S0140673612617668

Forum on aging and skeletal health: summary of the proceedings of an ASBMR workshop. J Bone Miner Res. 2011 Volume 26:2565-78.
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3625440/pdf/nihms-456848.pdf

RL Jilka, The Relevance of Mouse Models for Investigating Age-Related Bone Loss in Humans. *J Gerontol.A Biol Sci.Med Sci.* 2013 Journals of Gerontology: Biologic Sciences. Volume 68(10):1209–1217.
http://biomedgerontology.oxfordjournals.org/content/68/10/1209.full.pdf+html

Using Large Databases for Osteoporosis Research

Jeffery Curtis, M.D., M.S., MPH

Jeffrey R Curtis, MD MS MPH
ASBMR 2014, Meet The Professor Session
Using Large Databases for Osteoporosis Research

Learning Objectives

1. Review different types of data available for clinical osteoporosis research and the strengths and weaknesses of each
2. Evaluate the types of research questions most suitable to each type of data source
3. Recognize opportunities and pitfalls in the use of large databases for osteoporosis research

Data Sources Available for Clinical Research in Osteoporosis & Mechanisms for Data Collection

- Clinical Trials and Long Term Extensions of Trials
- Cohort Study (e.g. SOF, MrOS)
- Registry (e.g. NBHA Quality Improvement Registry, GLOW, Creakybones.org) [mail, CATI, HIT]
- Administrative Claims Data (e.g. Medicare, commercial insurance companies like United Healthcare, Aetna; data aggregators like IMS Health, Truven Marketscan)
- Electronic Health Record Data (e.g. single health systems, health information exchange, PCORI-funded Clinical Data Research Network)
- Closed health systems (e.g. VHA, Kaiser)
- Linkages between data sources

Pros and Cons of Different Data Sources

	RCT	Observational Cohort/Registry	EHR Data	Claims Data
Typical Questions the Data Is Optimized to Address	Efficacy	Variable	± Effectiveness Practice Patterns	± Effectiveness Safety Practice Patterns
Data Capture	+++ phenotype ++ completeness	+++ phenotype + completeness	++ phenotype ++ completeness	+ phenotype +++ completeness
Internal Validity	+++	++	++	±±
External Validity	-	±±	±±	+++
Data Acquisition Costs	+	++	++	+++

Key: + = fair; ++ = good; +++ = very good; ±± = variable

Research questions that might be asked using Administrative Data

- Incidence and risk factors for outcomes of interest (e.g. fracture)
- Medication use & adherence
- Healthcare utilization (e.g. ED visits, rehabilitation visits)
- Cost & health economics

Reasons to Consider Using Large Databases for Osteoporosis-Related Research

- Long term follow-up
- Rare outcomes
- Novel exposures (e.g. newly approved drug)
- Efficiency & cost
- Assessment of additional covariates
- Assess Quality of Care

Validity of Administrative Data

- Medications (oral & parenteral)
- Diagnoses (e.g. osteoporosis)
- Medical events (e.g. fractures)
- Other health-related procedures

Important Questions and Caveats when Working with Administrative Data

- What qualifies people for enrollment?
- Does the reason for qualification of enrollment potentially impact access to certain services (e.g. parenteral OP drugs)
- Extent of follow-up and reasons for dis-enrollment
- Can patients re-enroll and maintain the same patient ID?
- Ability to assess mortality
- Missing-ness of potentially important data (e.g. physician speciality; prescriber / provider IDs)
- Availability of lab results for common labs
- Ability to externally link to other information sources
- Cost
- Terms of Engagement (e.g. Data Use Agreement)
- Identifiability of individuals (e.g. research-identifiable, limited dataset, fully de-identified)

Selected Citations

Curtis JR et. al. Linkage of a de-identified United States rheumatoid arthritis registry with administrative data to facilitate comparative effectiveness research. Arthritis Care Res (Hoboken). 2014 Jun 6 (epub).

Schousboe JT et. al. Magnitude and consequences of misclassification of incident hip fractures in large cohort studies: the Study of Osteoporotic Fractures and Medicare claims data. Osteoporos Int. 2013 Mar;24(3):801-10.

Virnig B et. al. Linking the Iowa Women's Health Study cohort to Medicare data: linkage results and application to hip fracture. Am J Epidemiol. 2010 Aug 1;172(3):327-33.

Xue F et. al. Design and methods of a postmarketing pharmacoepidemiology study assessing long-term safety of Prolia® (denosumab) for the treatment of postmenopausal osteoporosis. Pharmacoepidemiol Drug Saf. 2013 Oct;22(10):1107-14.

What is a Mesenchymal Stem Cell?

Pamela Robey, Ph.D.

What is a Mesenchymal Stem Cell?
Pamela Gehron Robey, Ph.D., CSDB/NIDCR/NIH/DHHS, USA

Significance of the Topic: Bone marrow stromal cells (BMSCs) contain a subset of skeletal stem cells (SSCs) that are capable of recreating skeletal tissues (cartilage, bone, hematopoiesis-supportive stroma, marrow adipocytes). Based on the fact that these cells not only form bone, but also regulate osteoclast formation, BMSCs and the subset of SSCs are central mediators in skeletal homeostasis. Consequently, any intrinsic change (mutation) or extrinsic change (change in their microenvironment) that alters their normal biological activity will result in a skeletal derangement.

While this was the original concept (put forward by Friedenstein and Owen), BMSCs/SSCs were subsequently termed "mesenchymal stem cells," What followed was a long list of publications proclaiming "trans-differentiation" of the cells outside of their normal lineage, and that cells identical to BMSCs/SSCs could be found in virtually all tissues. However, this is NOT the case. Consequently, it is of significance for the field to recognize the differences between "MSCs" from different tissues, and more importantly, what BMSCs/SSCs can and cannot do.

Learning Objectives: As a result of participating in this session, attendees should be able to:
1) understand the biological properties of BMSCs/SSCs
2) be aware of appropriate assays by which to assess BMSCs/SSCs
3) realize that while similar in cell surface character, "MSCs" from different tissues are not the same, and are tissue-specific

Outline:
1. History
2. The classic experiment
3. Development
4. Assays (see Robey et al, Meth Mol Biol 1130:279-294, 2014.
5. Evolution of pericytes (see Sacchetti et al, Cell, 2007; Bianco et al, Cell Stem Cell, 2008)
6. "Mesenchymal stem cell" – What's in a name?

Bone marrow stromal stem cells

Owen, M.E., Friedenstein, A.J., Stromal stem cells: marrow-derived osteogenic progenitors. Ciba Foundation Symp 136:42-60, 1988 – clonal strains and in vivo transplantation

↓

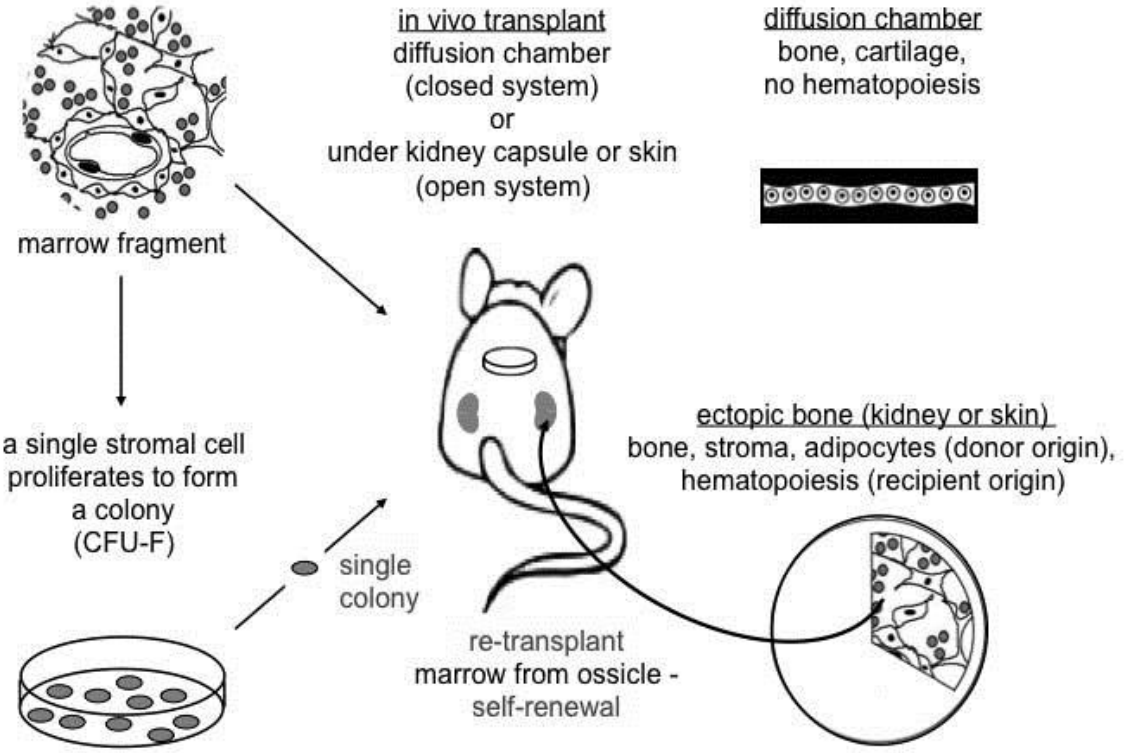
"Mesenchymal stem cells"

Caplan, A.I., Mesenchymal stem cells. J Ortho Res 9, 641,1991 – the "mesengenic process"

Pittenger et al, Multilineage potential of adult human mesenchymal stem cells. Science 284:143-147, 1999 – in vitro assays

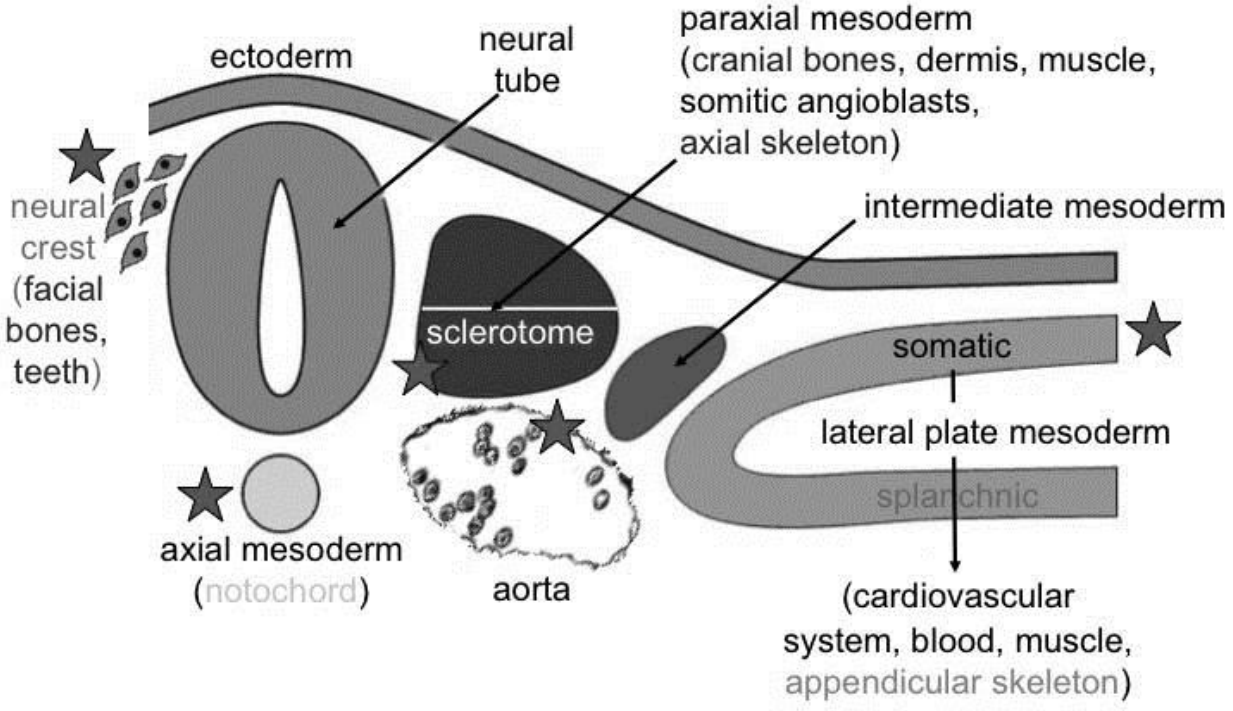
Dominici et al, Cytotherapy 8:315-317, 2006
"First, MSC must be plastic-adherent when maintained in standard culture conditions.
Second, MSC must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules.
Third, MSC must differentiate to osteoblasts, adipocytes and chondroblasts in vitro."

The classic experiment



Friedenstein, A. (1990). Osteogenic stem cells in bone marrow. In Bone and Mineral Research. (Heersche and Kanis, eds), Amsterdam: Elsevier, 7: 243–272

Development



There is no common “mesenchymal stem cell” during development

Markers of "mesenchymal" stem cells: cell sorting for enrichment

$CD34^-/CD45^-/CD14^-$ (blood markers)

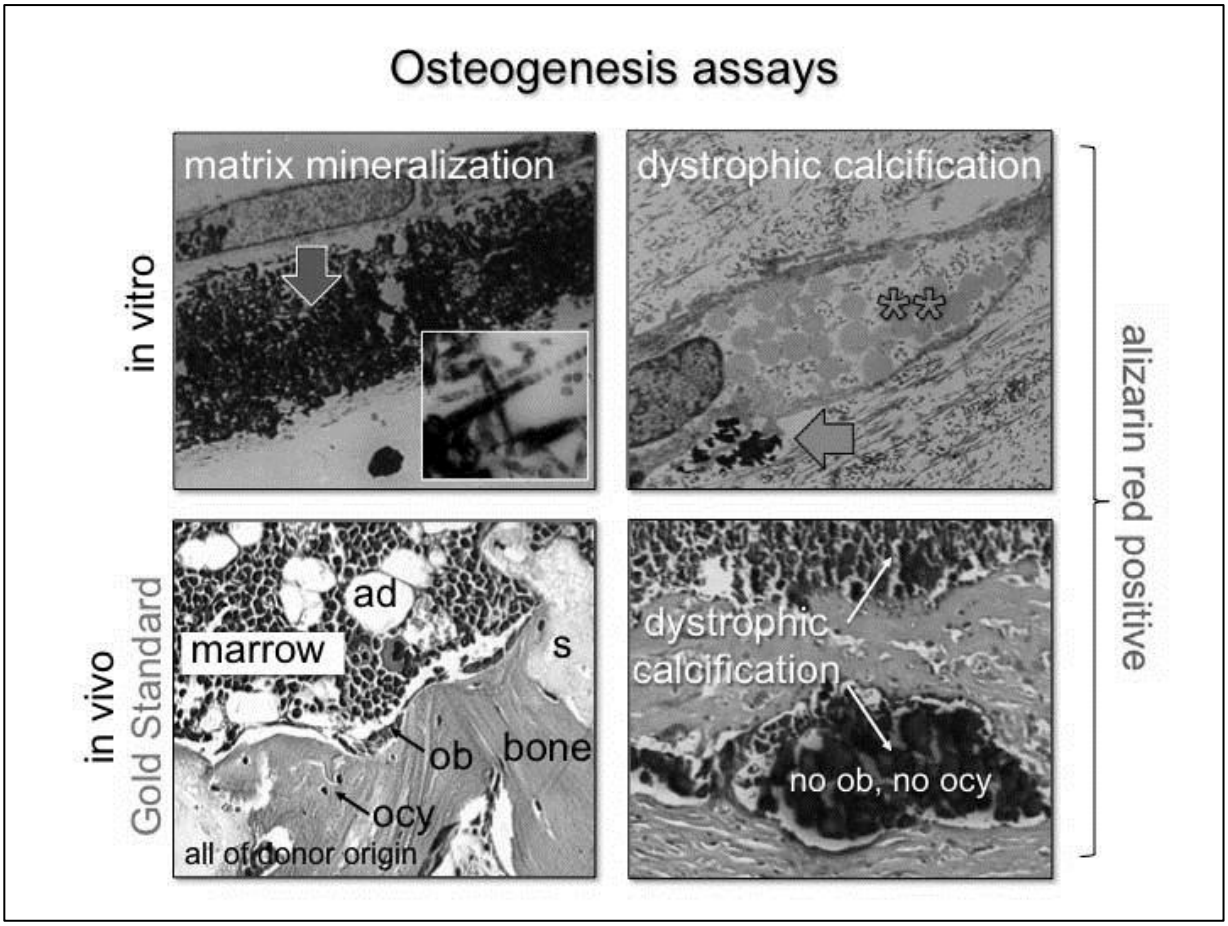
$CD31^-$ (endothelial marker)

$CD13^+/CD29^+/CD44^+/CD49a^+/CD63^+/CD90^+$

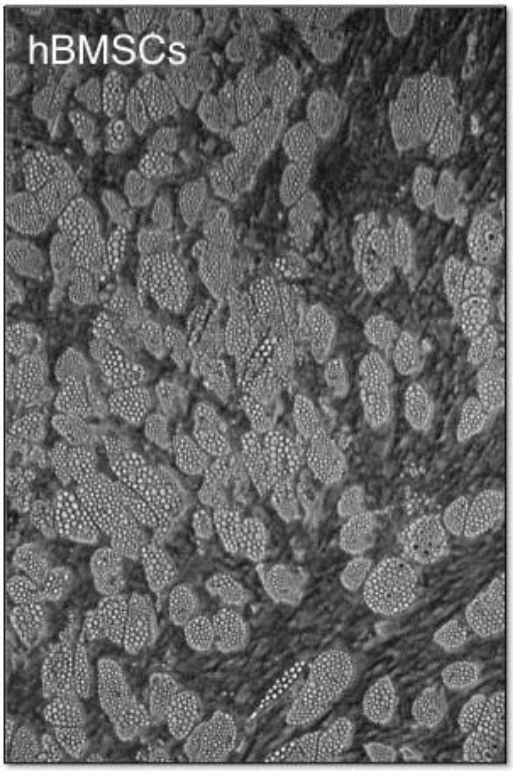
$CD105^+/CD106^+/CD166^+/Stro1^+$ /etc.

$CD146^+$

Not specific – markers of adherent, connective tissue (CT) stromal cells and fibroblasts



Adipogenesis assay



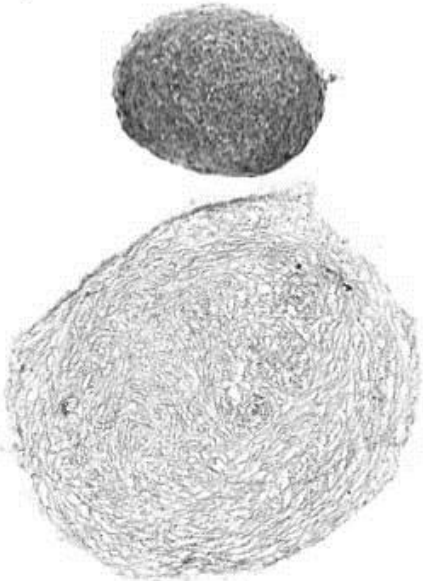
Chondrogenesis assay

P1 human BMSCs

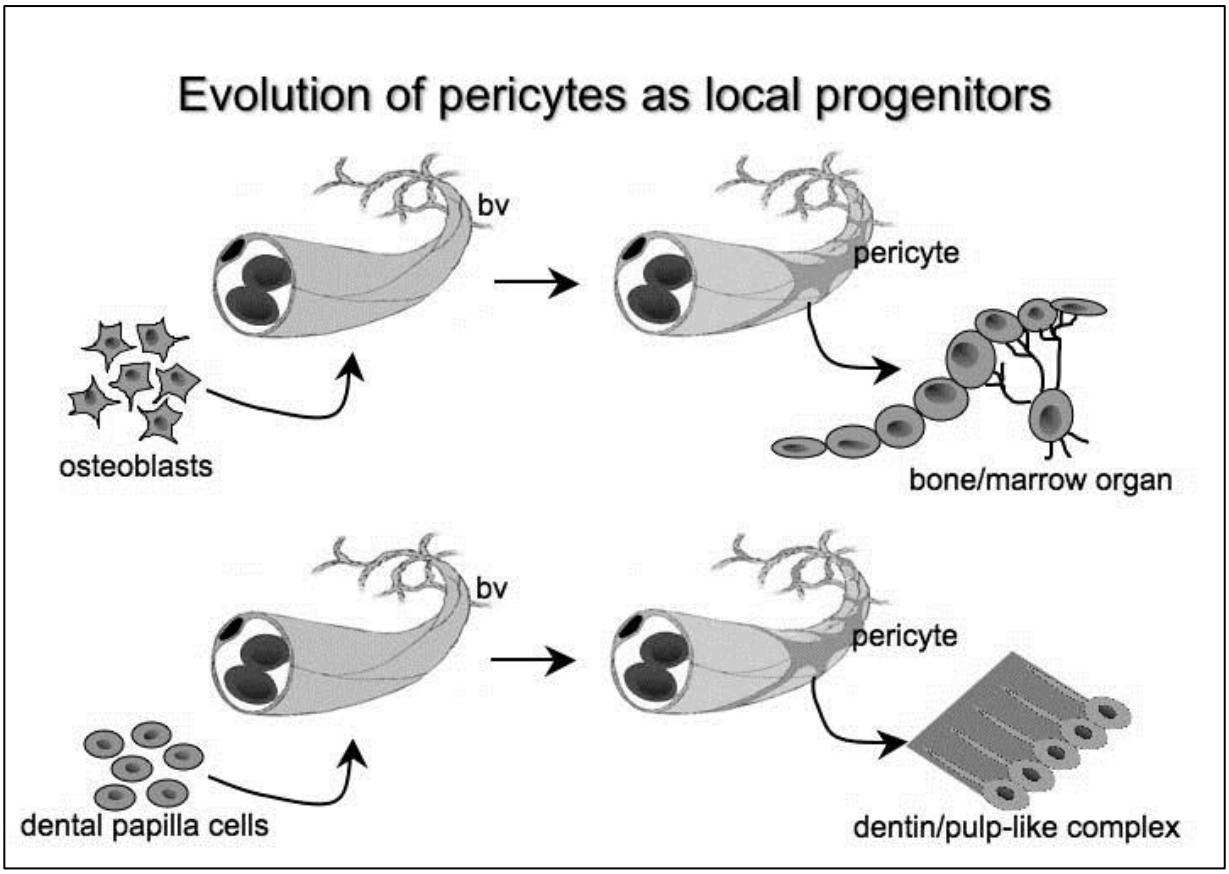
metachromatic staining with toluidine blue, chondrocytes in lacunae

hypertrophy or bone/marrow organ

adipose-derived stromal cells



alcian blue^{+}, no metachromasia with toluidine blue, no chondrocytes in lacunae



(modified from Bianco et al, Cell Stem Cell, 2008)

"Mesenchymal stem cell"
what's in a name?

- Mesenchyme is an embryonic connective tissue that gives rise to not only connective tissues, but also blood and blood vessels (there is no post-natal stem cell that can do that)
- during development, there is no one single mesenchymal stem cell that gives rise to all connective tissues.
- there is no common post-natal "mesenchymal" stem cell distributed throughout the body.
- while clonogenic cells from different tissues are $CD146^{+}$ pericytes, they are not equivalent in their differentiation potential in vivo.
- terminology should be based on tissue of origin.

Bianco et al, Cell Stem Cell, 2008; Robey, Tiss Eng Part B, 2011; Bianco et al, Nature Medicine, 2013

RNA Sequencing

Matthew Warman, M.D. and

Ugur Ayturk, Ph.D.

Meet-the-Professor Session: RNA sequencing
September 14, 2014

Matthew Warman, MD

Ugur Ayturk, PhD

Boston Children's Hospital, USA

Significance of the Topic

Next generation sequencing of mRNA (RNA-seq) is a new and powerful method of gene expression analysis. Owing to its large dynamic range, the ability to simultaneously assess the entire transcriptome without *a priori* target specification, the ability to detect alternate splice-forms and transcription start sites, and the ability to provide single-nucleotide resolution, RNA-seq is emerging as a powerhouse for performing gene expression analyses. In this session, we will discuss some established and potential applications of RNA-seq in skeletal biology. We will specifically highlight challenges in dealing with complex skeletal tissues such as bone and possible solutions to increase the robustness and accuracy of RNA seq.

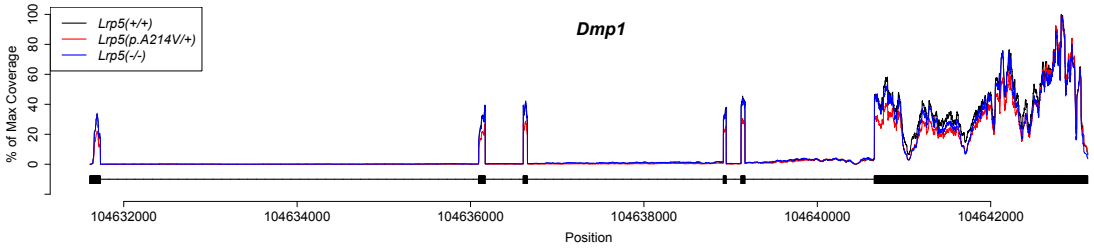
Learning Objectives

As a result of participating in this session, attendees should be able to:

- Explain the fundamentals of RNA-seq and how it differs from other methods of gene expression measurement such as qRT-PCR or microarrays.
- Describe issues that arise when RNA-seq is applied to organs rather than cells.
- Appreciate the need to include quality control metrics at several stages when generating RNA-seq libraries, performing massively parallel sequencing, and analyzing data.

Outline

- Basic description of workflow and issues involved with dealing with skeletal tissues
    - RNA extraction: *A potentially critical step based on the type of samples involved. The effects of the primary source of RNA (e.g. cultured cells vs. fresh tissue) and the complexity of the cell population involved (e.g. osteoblasts, osteoclasts and osteocytes in a single bone sample) will be discussed.*
    - Library preparation: *Several kits are commercially available for preparing sequencing-ready libraries from good quality total RNA. Basic components of a typical workflow (e.g. enrichment for polyA+ mRNA, reverse transcription, barcoded adapter ligation, PCR-based amplification) will be discussed.*
    - Sequencing: *An increasing number of companies and core facilities offer next generation sequencing services. It is possible to pool samples (e.g. n=6-10 per lane) to reduce cost. Different options available for sequencing RNA-seq libraries (e.g. paired end vs. single end) will be briefly discussed.*

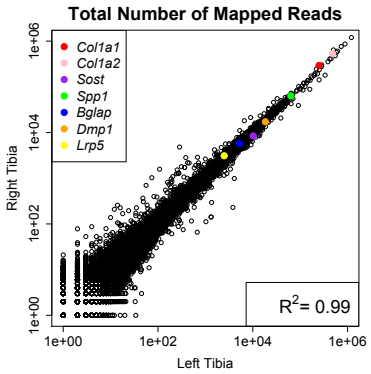


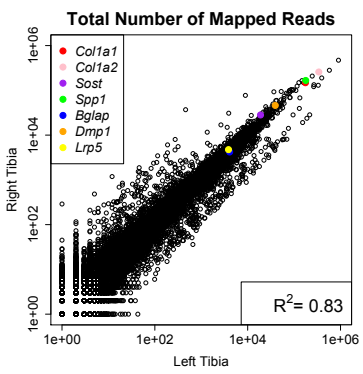
- Data analysis: *Computational steps involved in a typical study will be described, along with available software for transforming raw data into statistically meaningful expression data. Alignment, annotation, quantification, normalization, and statistical comparison steps will be discussed.*

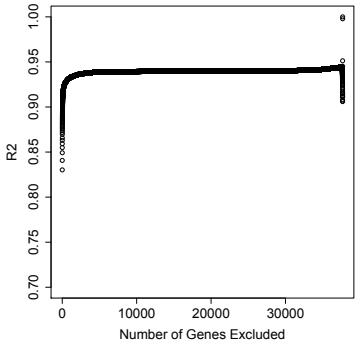
- Having the appropriate skillset in the lab
  - Required wet-lab and computer skills will be discussed.

Examples

- Gene expression changes as a result of a genetic/environmental/pharmacologic manipulation: Mutations in *Lrp5* and pharmaceutical neutralization of sclerostin[1, 2]
  - Identifying novel targets potentially modulated by Lrp5 signaling in bone
  - Do genetic manipulations generate the desired effect? Specifically:
    - Does the loss of function mutation eliminate expression?
    - Is a knock-in allele expressed at a similar level to the wild-type allele?
  - Abundance and expression are not the same:

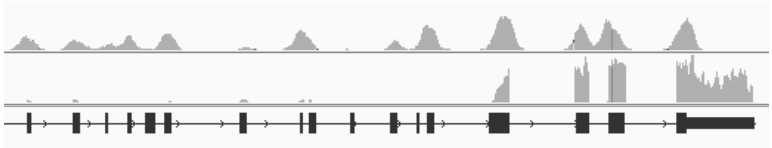






GENE	R^2
None	*0.830*
Atp2a1	0.841
Myh4	0.849
Tnnt3	0.855
Scd1	0.859
Gsn	0.863
Hbb-b1	0..866
mt-Nd2	0.870
S100a9	0.872
Ngp	0.874
Tpm1	0.875

- Searching for disease-causing mutations at the RNA level (e.g. single nucleotide polymorphisms, small insertions/deletions) detection in tissues:
  - Non-uniform 5'-3' read-depth (Figure 1)
  - The mutated gene may not be highly expressed



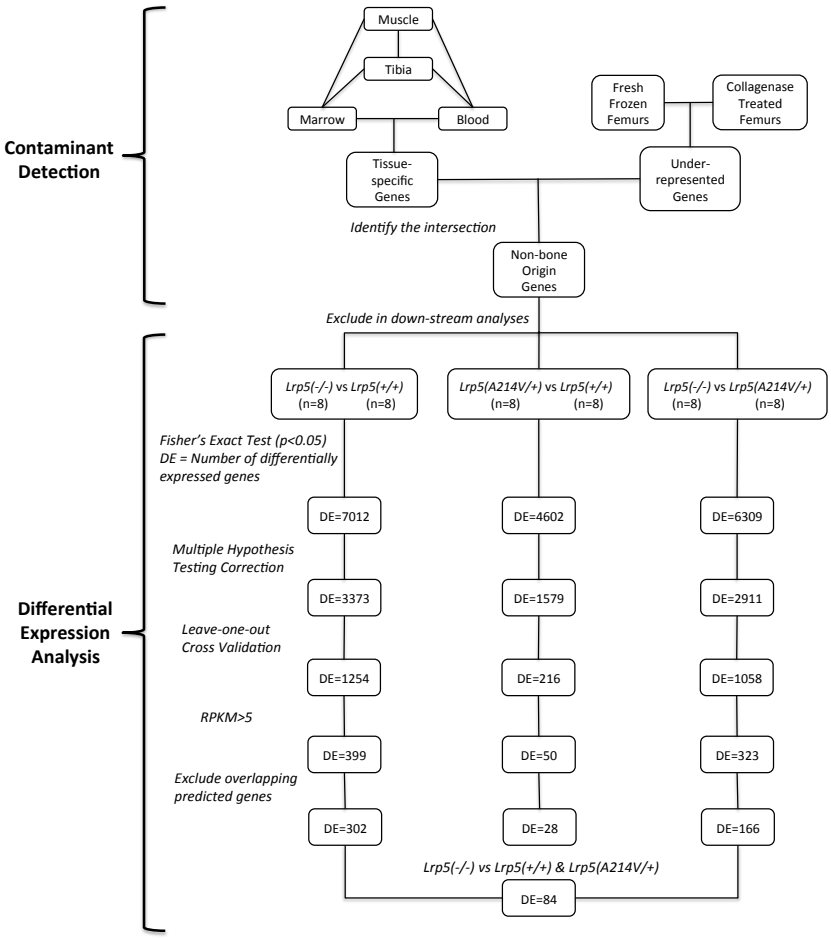
Whole Exome
RNA-seq
COL3A1 Annotation Track

Figure 1: Read depth distribution over the 3' UTR and neighboring exons of *COL3A1* in whole exome sequencing and RNA-seq data. Note that RNA-seq read depth is significantly enriched towards the 3' end of the gene, whereas no such effect is observed in whole exome data. DNA and RNA samples used in library preparation were collected from the same source.

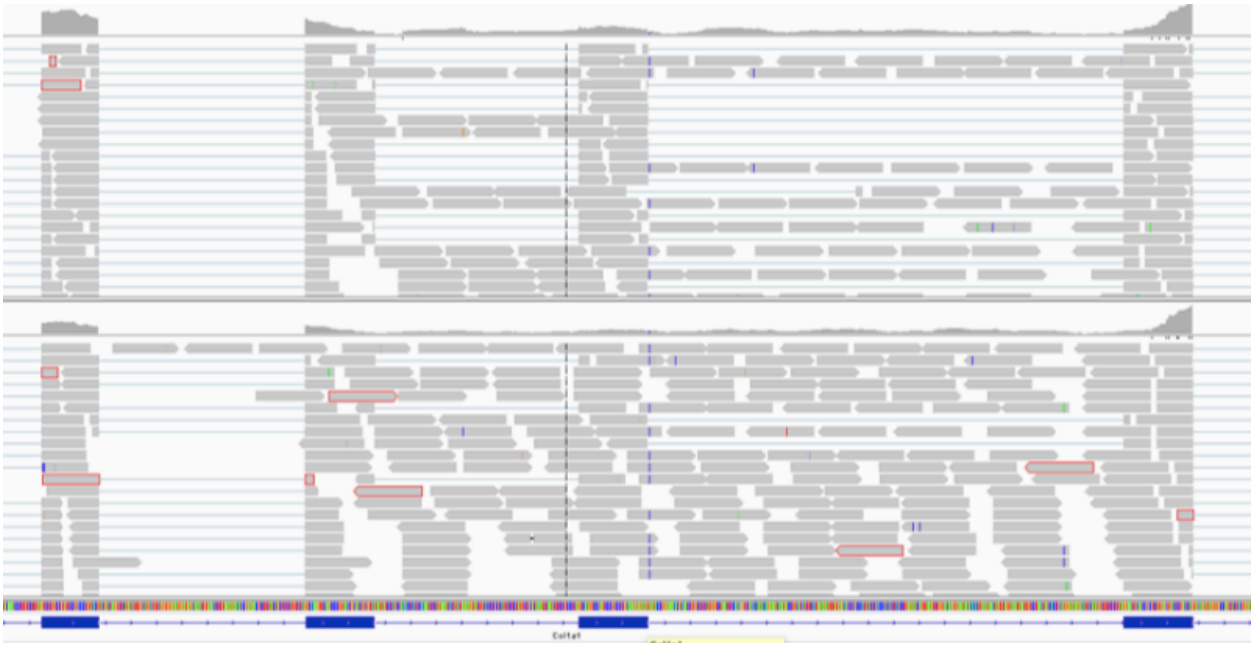
- Assessing the strength of expression of Cre-drivers and GFP reporters in knocking or transgenic mice
    - Is a Cre allele expressed at the same level as the endogenous transcript in $Prg4^{+/CreERt2}$ mice?

	Normalized w.r.t.	*Col2a1*	*Acan*	*Comp*	*Col9a2*	Total Number of Reads
CRE/WT	Exon 2	7.1%	3.3%	4.9%	8.3%	4.3%
	Exon 4	3.4%	1.6%	2.4%	3.9%	2.1%

Considerations when ssessing the reliability of RNA-seq data



- **Identifying mechanisms of mutational effect**



Exciting new developments in RNA-seq

- **Single cell RNA-seq** Macaulay and Voet. Single Cell Genomics: Advances and future perspectives. *PLOS Genetics e1004126, 2014*
- **In situ RNA-seq (FISSEQ)** Lee et al. Highly multiplexed subcellular RNA sequencing in situ. *Science*, 343:1360-3, 2014

A few Ayturk/Warman RNA-seq references

1. Ayturk UM, Jacobsen CM, Christodoulou DC, Gorham J, Seidman JG, Seidman CE, Robling AG and Warman ML. An RNA-seq Protocol to Identify mRNA Expression Changes in Mouse Diaphyseal Bone: Applications in Mice with Bone Property Altering Lrp5 Mutations. *Journal of Bone and Mineral Research,* 28: 2081-93, 2013.
2. Kedlaya R, Veera S, Horan DJ, Moss RE, Ayturk UM, Jacobsen CM, Bowen ME, Pazsty C, Warman ML and Robling AG. Sclerostin inhibition reverses skeletal fragility in an Lrp5 deficient mouse model of OPPG syndrome. *Science Translational Medicine,* 5:211ra518, 2013.
3. Bennike T, Ayturk U, Haslauer CM, Froehlich JW, Proffen BL, Barnaby O, Birkelund S, Murray MM, Warman ML, Stensballe A, and Steen H. A normative study of the synovial fluid proteome from healthy porcine knee joints. *Journal of Proteome Research (in press).*

A non-comprehensive list of resources for data analysis

RNA extraction and library preparation

- Kelly NH, Schimenti JC, Patrick Ross F, van der Meulen MC. A method for isolating high quality RNA from mouse cortical and cancellous bone. *Bone*, 68:1-5, 2014
- Ayturk UM, Jacobsen CM, Christodoulou DC, Gorham J, Seidman JG, Seidman CE, Robling AG and Warman ML. An RNA-seq Protocol to Identify mRNA Expression Changes in Mouse Diaphyseal Bone: Applications in Mice with Bone Property Altering Lrp5 Mutations. *Journal of Bone and Mineral Research,* 28: 2081-93, 2013.
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Raw read alignment

- Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biology*, 14:R36, 2013.
- Grant GR, Farkas MH, Pizarro AD, Lahens NF, Schug J, Brunk BP, Stoeckert CJ, Hogenesch JB, Pierce EA. Comparative analysis of RNA-Seq alignment algorithms and the RNA-Seq unified mapper (RUM). *Bioinformatics*, 27:2518-28, 2011.
- Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, Chen Z, Mauceli E, Hacohen N, Gnirke A, Rhind N, di Palma F, Birren BW, Nusbaum C, Lindblad-Toh K, Friedman N, Regev A. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology*, 29:644-52, 2011.

Differential expression analysis

- Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics, 26:139-40, 2010.
- Anders S and Huber W. Differential expression analysis for sequence count data. *Genome Biology*, 11:R106, 2010.
- Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature Biotechnology*, 28:511-5, 2010.

A very good tutorial for getting started:

- http://en.wikibooks.org/wiki/Next_Generation_Sequencing_(NGS)/RNA

For several useful R packages and manuals:

- http://www.bioconductor.org

Great online forums for next generation sequencing and general coding questions:

- http://www.seqanswers.com
- http://www.stackoverflow.com