ASBMR 2018 Meet-The-Professor

Handout Booklet

Extracellular Matrix and Bone

Clarissa Craft, Ph.D.

Friday, September 28

11:30 am – 12:30 pm

Room 518 B

Extracellular Matrix and Bone

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Significance:
The extracellular matrix (ECM) of the skeleton is unique. Unlike other tissues, ECM mineralization is physiological, not pathologic. Bone ECM is subject to continuous regeneration, as well as, reshaping. Further, bones are comprised of several distinct microenvironments including growth plate cartilage, marrow space, periosteum, and vasculature. These each require an ECM network with distinct physical and mechanical properties.

Learning Objectives:
As a result of participating in this session, attendees will:

(1) Be given an overview of the protein families constituting the skeletal ECM.
(2) Have an appreciation of the diverse functions of the ECM and the biochemical properties of the protein families facilitating these functions.
(3) Understand how changes in both the quantity and quality of ECM proteins lead to pathologies.
(4) Have knowledge of how ECM proteins are used to study non-ECM proteins, as well as, tools to study ECM proteins themselves.

Points of Interest:
ECM families
The skeleton, and the body in general, requires a three-dimensional network of extracellular matrix proteins to provide both mechanical support and to compartmentalize cells into distinct structures or niches. The ECM is also responsible for presenting information to cells through inherent (RGD) mechanisms, as well as, through regulation of soluble ligand delivery (growth factors). The ECM 'superfamily' can be broadly divided into collagens and non-collagenous proteins.

The predominant collagen in the skeleton is collagen-I (col-I), which provides both bone structure and a scaffold for mineral deposition. Outside the mineralized bone, the skeleton is enriched with other collagen types. For example, cartilage is enriched with col-II. Basement membranes in the marrow mileu will be enriched with col-IV. Col-X is present in the hypertrophic cartilage of the growth plate. Non-collagenous proteins can be further divided into the following groups: glycoproteins, proteoglycans, γ-carboxylated (gla) proteins, and matrix-modifying proteins.

Glycoproteins are proteins in which a protein core is linked to variable, short and branched carbohydrates. Importantly, classification into the glycoprotein category implies that

the protein core dominates over carbohydrates. Classic examples of glycoproteins found in the skeleton are alkaline phosphatase and osteonectin. Proteins in which the linked carbohydrates dominate over the core protein are considered proteoglycans. Proteoglycan-associated carbohydrates are long, linear and unbranched; giving a feather-like appearance. Classic examples of proteoglycans found in the skeleton are aggrecan and versican. Gla (γ-carboxylated) proteins are glycoproteins that have been post-translationally modified to contain dicarboxylic glutamyl, which can facilitate interactions with calcium binding. Examples of gla-proteins found in the skeleton are periostin and osteocalcin. Enzymes with the capacity to cross-link ECM proteins or cleave them are considered matrix-modifying proteins. Examples of these enzymes are lysyl oxidase which cross-links ECM structures and MMPs which cleaves ECM fibers.

ECM functions:
The most notable function of the ECM is to provide mechanical support to tissues. Collagen fibers provide tensile strength, which is further strengthened with mineralization. Elastic fibers impart recoil properties to tissues, and proteoglycans provide compression resistance. The ECM is also important to filling the extracellular space between cellular structures/niches, providing a substrate for cell migration and cell polarization. Further, the ECM is a critical regulator of cell signaling. Specifically, integrin mediated interactions with the RGD domains of ECM proteins such as fibronectin activate intracellular signaling pathways that modify cell migration, proliferation, differentiation, and gene/protein expression. Unknown to many is that soluble ligands (growth factors) interact with proteins of the ECM milieu before binding their receptors, thereby controlling the diffusion and delivery of signaling molecules, allowing gradients of signal molecules to be generated, and preventing aberrant activation of signal transduction pathways.

Quantity versus quality:
Mutations in the genes encoding ECM genes can be devastating in two main ways: a reduction in the amount of protein made and the secretion/assembly of a defective protein. The later is especially devastating as ECM proteins undergo significant post-translational modifications important to their functionality. Improper folding of ECM proteins can interfere with protein-protein interactions necessary for a properly assembled three-dimensional matrix. Further, the inability to appropriately crosslink ECM proteins can be especially detrimental to the stability of the assembled matrix and its mechanical properties.

ECM proteins as research tools, and research tools to study ECM proteins:
Diagnostic tools for monitoring bone formation and bone resorption typically quantify ECM components. For example, serum bone-specific alkaline phosphatase, osteocalcin, and collagen-C1NP/P1NP are frequently used for monitoring bone formation, and collagen-derived hydroxyproline and CTX provide information on bone loss. ECM genes are also a major class of proteins used for conditional deletion of genes within the skeleton. To target the osteoblast lineage, the following ECM-Cre's are used: col-3.6, col-1a1, col-2.3, osteocalcin, and Dmp1. To target osteoclasts, the ECM modifying enzyme cathepsin-K-Cre is used. Further, the chondrocyte lineage can be targeted using col-2a1 and col-10a1 Cre.

The ability to study ECM proteins has proven difficult because many diseases related to ECM-gene mutations can be caused not only by changes in the amount of protein, but also improper assembly of the ECM network. Further, expression of a gene does not guarantee proper assembly/incorporation of the targeted ECM protein. Thus, the usefulness of PCR and Western blots is limited. IHC is similarly limited due to its failure to show protein interactions, cross-linkage and fiber orientation. Several ECM components are insoluble and self-aggregate making Western blots challenging. Cell culture is limited by the temporal availability of the fiber proteins, as well as, the machinery required for proper assembly. Finally, genetic manipulation *in vivo* has to be carefully done as many ECM molecules are ubiquitously expressed and therefore their deletion/mutation often has multi-organ complications. Electron microscopy, mass spectroscopy and Raman spectroscopy are frequently used to assess the quality and quantity of the ECM matrices.

Cases

Time permitting, pathologic mutations associated with fibrillin-rich fibrils (Marfan syndrome) will be discussed.

Suggested References

The Composition of Bone. Adele L. Boskey and Pamela Gehron Robey. Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism, Eighth Edition. Chapter 6. 2013

Connective Tissue Pathways That Regulate Growth Factors. Gerhard Sengle and Lynn Y. Sakai. Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism, Eighth Edition. Chapter 5. 2013

The Extracellular Matrix: an Overview. Biology of the Extracellular Matrix Book Series. Robert Mecham (Editor). Springer (Publisher). ISBN 978-3-642-16555-9

Factors that Influence Mouse Model Variability

Clifford Rosen, M.D.

Friday, September 28

11:30 am – 12:30 pm

Room 521

Factors that Influence Mouse Model Phenotype Variability

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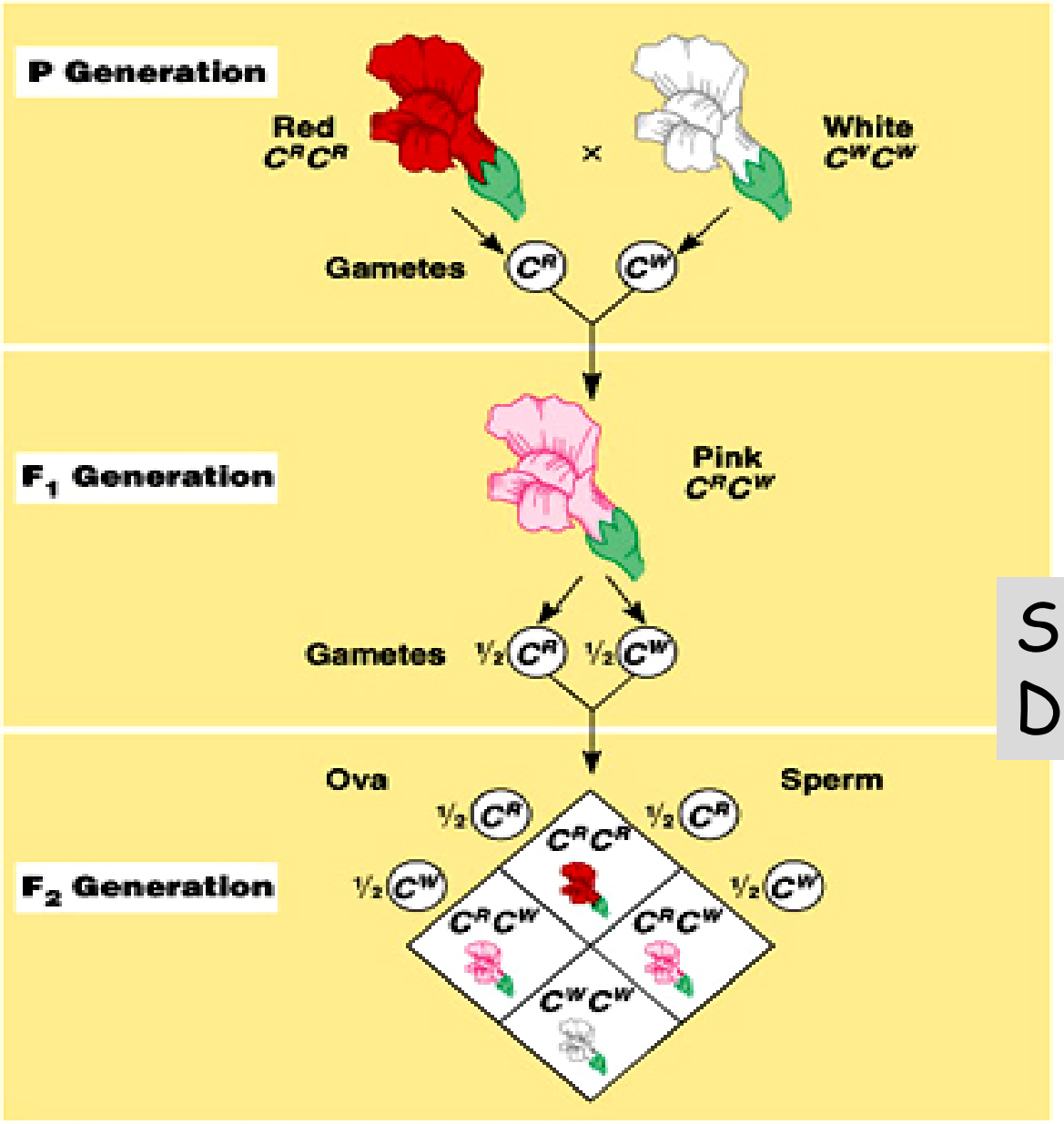
Outline

- Factors that influence phenotypic variability
  - Gene
  - Sex
  - Gene x environment
  - Nutrition-Microbiome
  - Season
  - Temperature
- Analysis of variability- MAD
  - The meaning of mean
  - Dispersion
  - Examples
- What to do??? Build your Ns and pay attention to variability

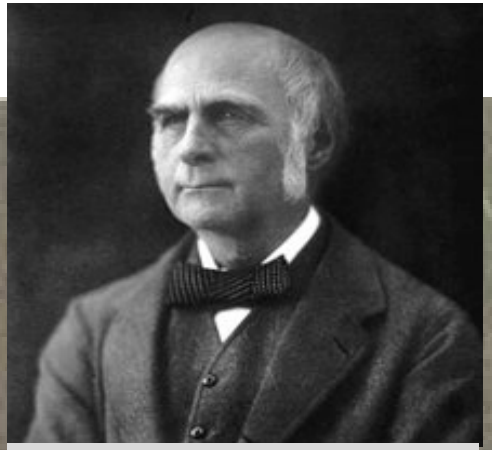


Gregor Mendel

Single genes
Discrete traits

Complex traits: Francis Galton, Karl Pearson

Karl Pearson



Francis Galton

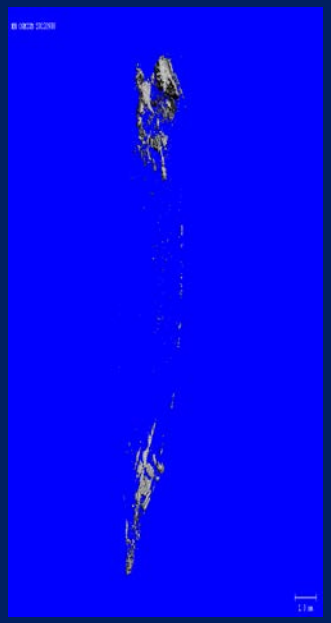
Multiple genes
Continuous traits

Genetic Determinants of Bone Mass and MAT

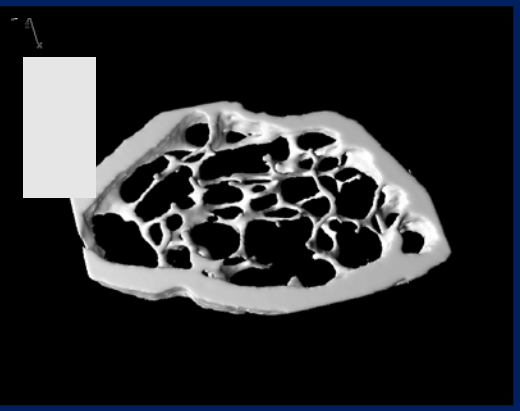
:

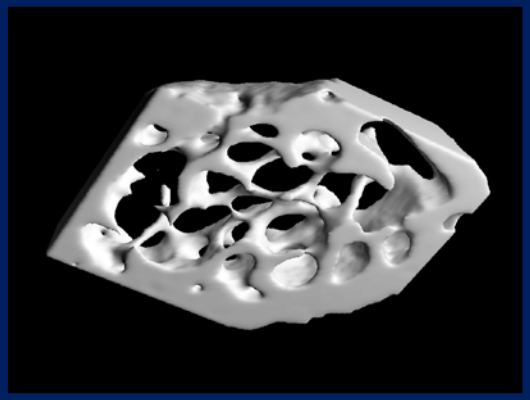
C57BL6J

C3H/HeJ



SNV in TLR4- loss of function





Guntur, Endocrinology,2014

F2 Distribution of C3H x B6 Crosses

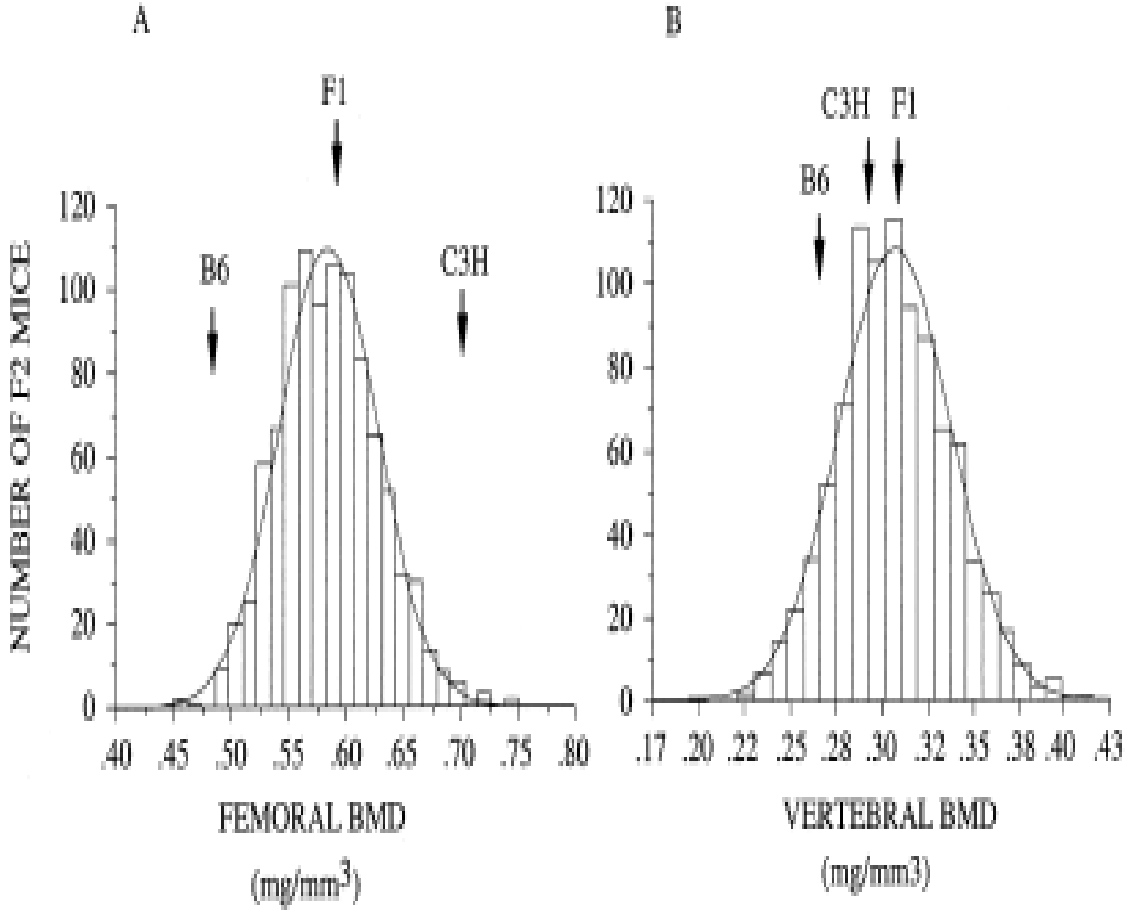
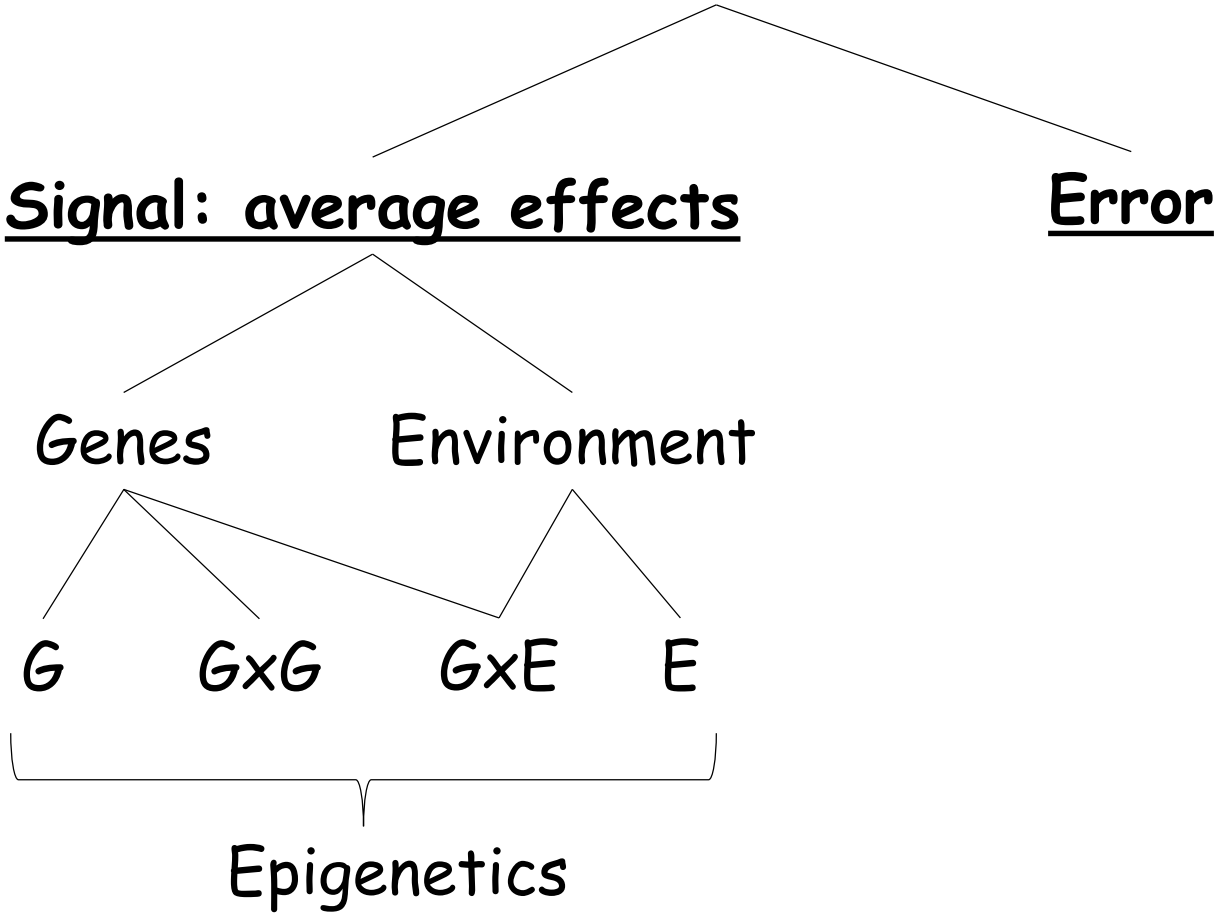


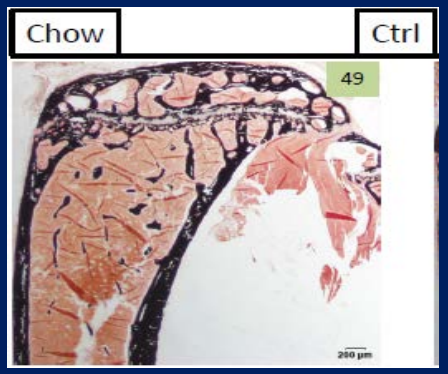
FIG. 2. Distributions of femoral and vertebral BMD obtained from B6C3F2 progeny. (A) Femoral BMD in female mice at 4 months of age. Positions of mean BMDs for the inbred progenitors and F1 parentals are indicated by arrows, while the bell-shaped line depicts a normal distribution of data. (B) L5 vertebral BMD from the same F2 mice. Location of mean values for the progenitors are markedly different than for the femoral BMD shown in panel A.

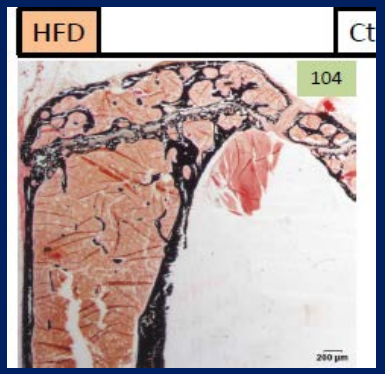
Environmental Interactions

Unaccounted phenotypic variation



DIO: Strain by Diet by Drug Interactions





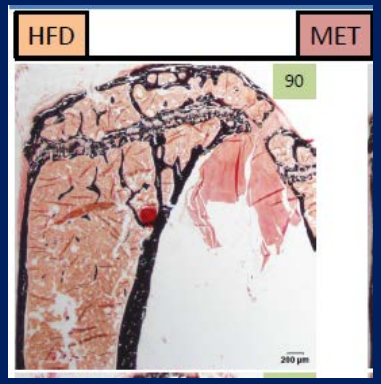
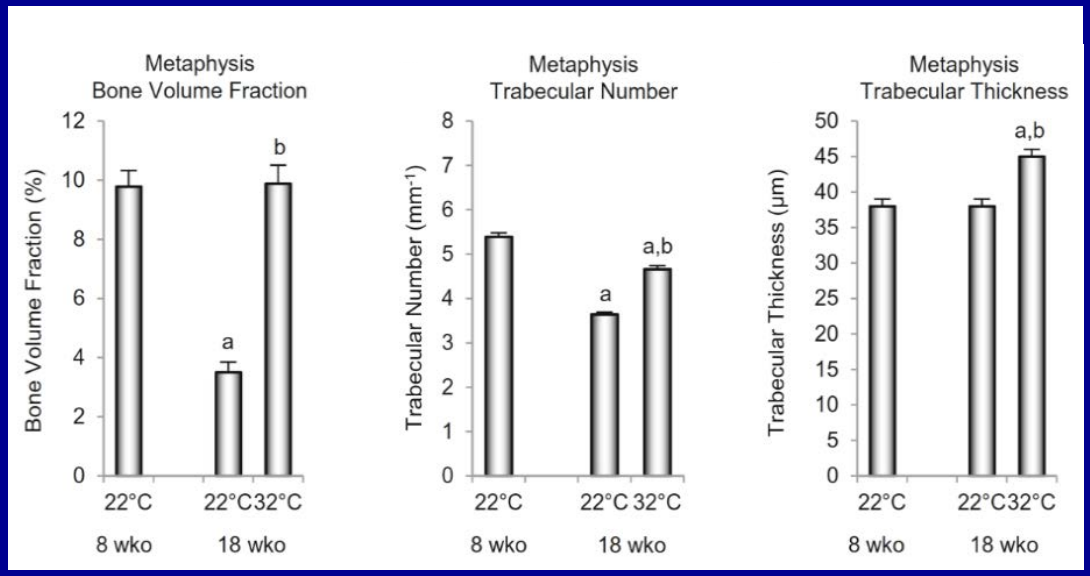
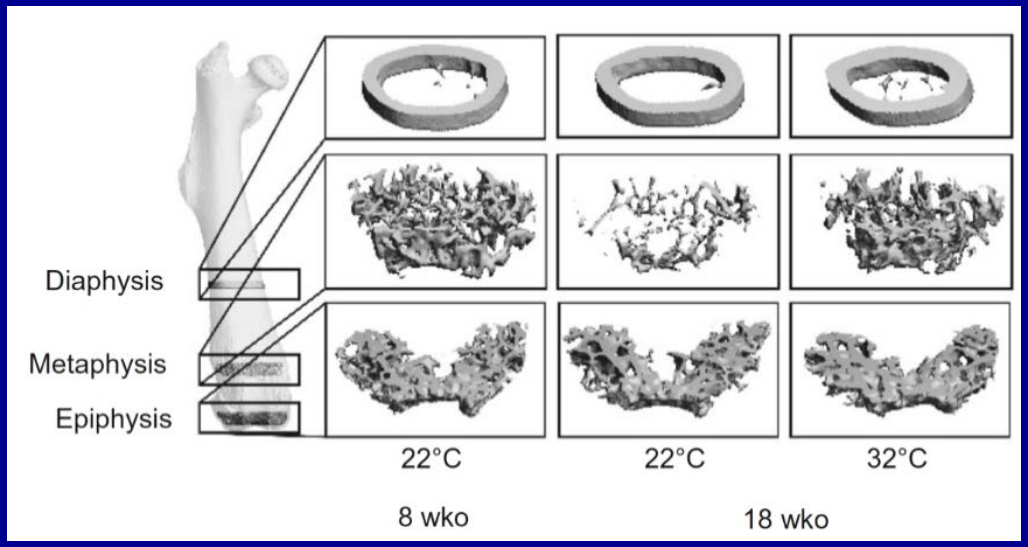


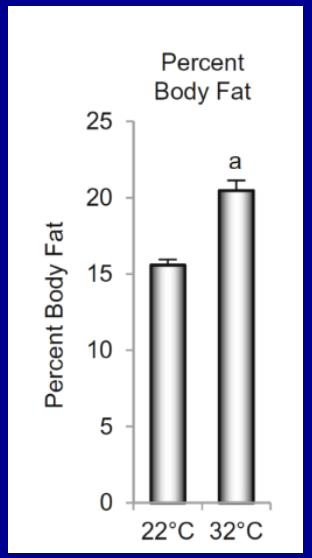
Table Parameters	Chow Control (n=5)	HFD Control (n=5)	HFD Metformin (n=5)	Statistics One-way ANOVA
BV/TV (%)	10.8±3.94	4.92±1.82 *	6.23±2.28	p=0.0158
Tb.Th (um)	41.8±10.2	31.0±4.68	31.1±6.58	p=0.0665
Tb.N (/mm)	2.52±0.55	1.54±0.38 *	1.97±0.45	p=0.0190
Tb.Sp (um)	377±131	666±186 *	499±121	p=0.0307
MAR (um/day)	0.79±0.34	0.86±0.26	1.16±0.20	p=0.1224
MS/BS (%)	23.7±7.43	31.0±3.44	44.5±2.15 **##	p<0.0001
BFR/TV (%/day)	0.10±0.07	0.09±0.05	0.21±0.08 ##	p=0.0331
BFR/BV (%/day)	0.95±0.50	1.81±0.70	3.35±0.95 **##	p=0.0018
BFR/BS (um^3/um^2/day)	0.20±0.11	0.27±0.10	0.51±0.07 **##	p=0.0007
N.Ob/B.Pm (/mm)	7.35±1.70	4.80±1.52	7.09±3.54	p=0.2264
N.Ob/T.Ar (/mm^2)	38.2±12.1	15.9±6.24	28.9±18.2	p=0.0579
Ob.S/B.Pm (%)	9.74±3.42	5.62±1.85	8.36±4.66	p=0.2078
OS/BS (%)	5.06±3.69	6.23±2.73	5.85±5.42	p=0.8998
O.Th (um)	2.36±0.92	2.25±0.40	2.73±1.54	p=0.7596
N.Oc/B.Pm (/mm)	2.97±0.67	3.99±0.76	3.23±0.98	p=0.1607
N.Oc/T.Ar (/mm^2)	15.1±4.07	13.0±1.64		
Oc.S/B.Pm (%)	7.52±1.18	9.77±1.82	8.07±2.54	p=0.1976
ES/BS (%)	2.77±0.45	3.18±0.79	2.51±0.80	p=0.3462
N.Ad/T.Ar (#/mm^2)	2.40±2.12	19.5±15.9	11.8±13.1	p=0.1183
Ad.V/TV (%)	0.21±0.17	3.36±2.95	1.56±2.38	p=0.1149

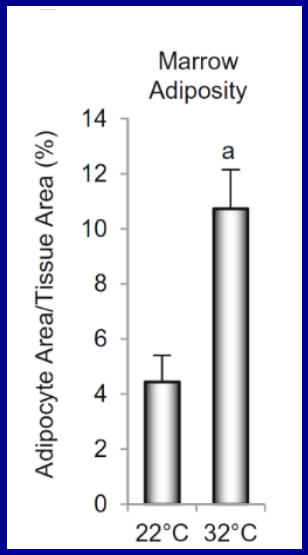
Temperature as an important environmental variable

Cold Induced Thermogenesis – SNS Activation in B6 Mice at 22°C Induces Bone and Fat Mass Loss



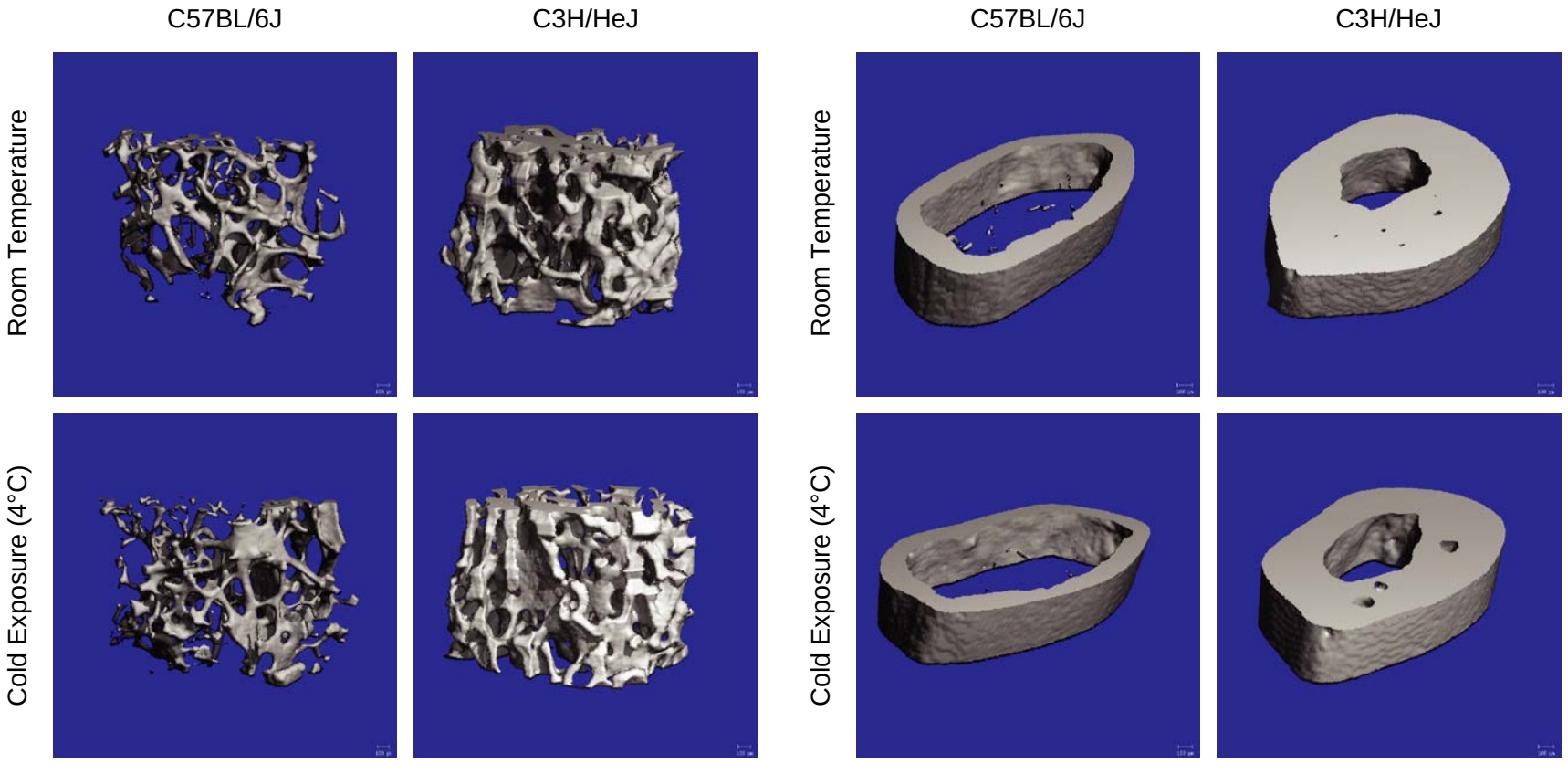




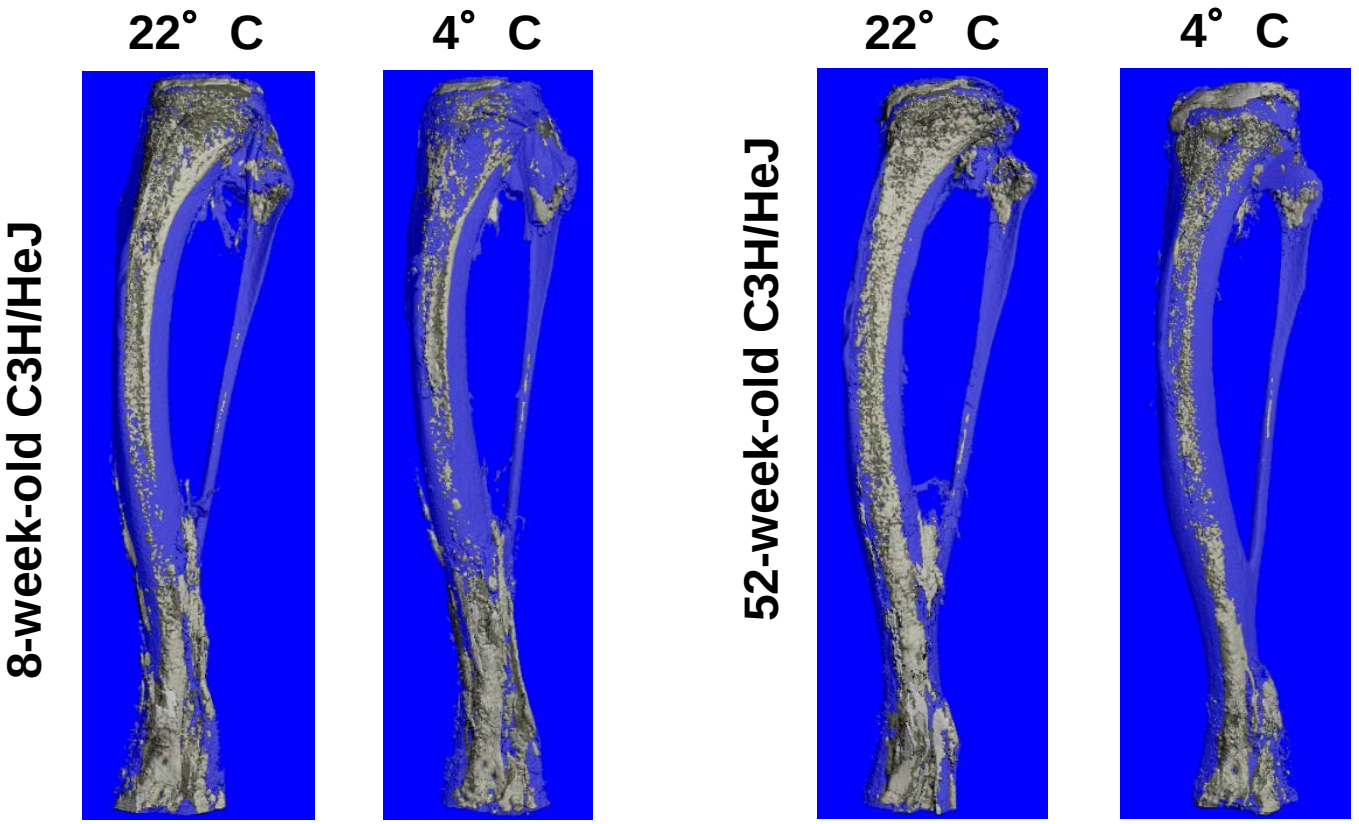


Iwaniec U et al Osteoporos Int 2016;27:3091-101.

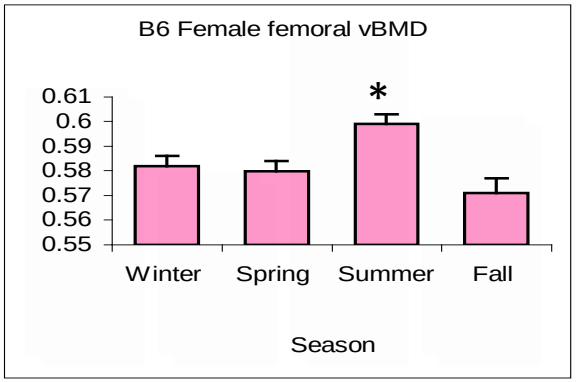
Cortical BA/TA Reduced in C3H Mice, Trabecular BV/TV Lower in B6 Mice with Long-Term Cold Exposure

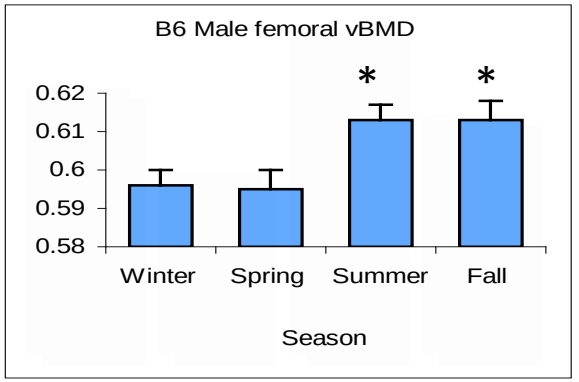


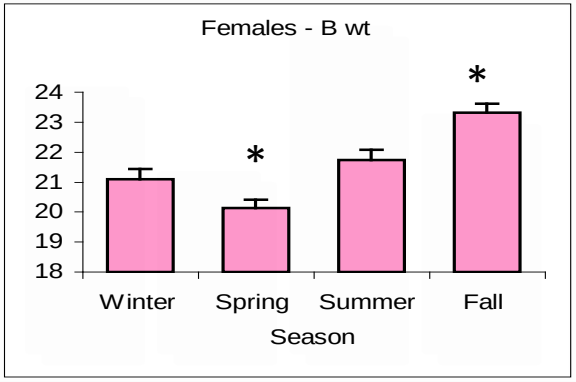
Decreased MAT with Long-Term Cold Exposure in C3H Mice – β3 AR

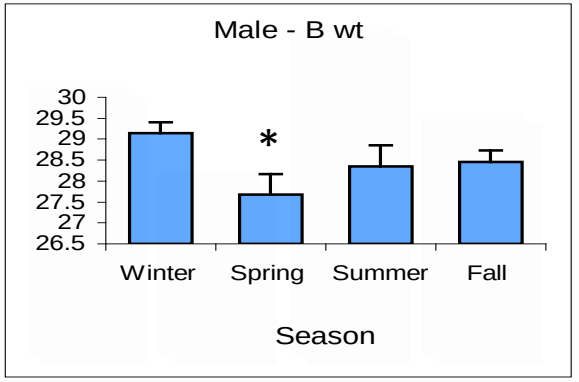


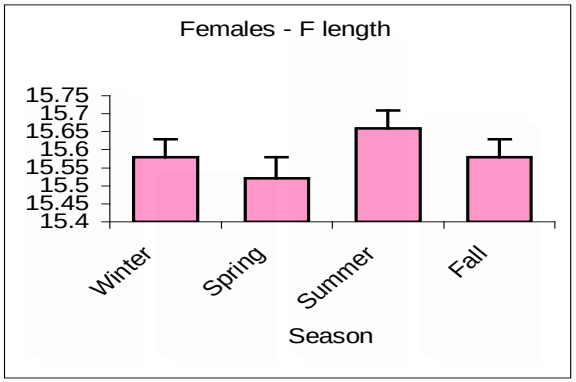
Seasonal Differences in pQCT: B6 Progenitor colony at 16 wks

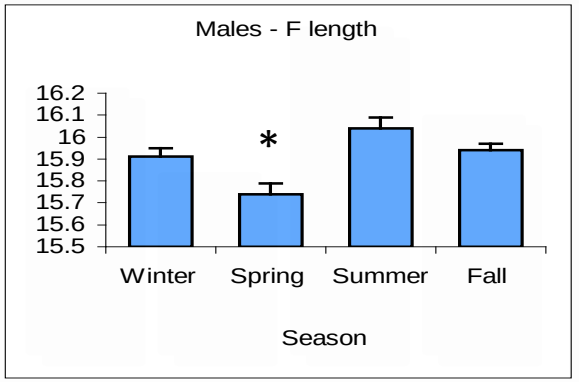












Microbiome Influences on the Metabolic and Skeletal Response

Review

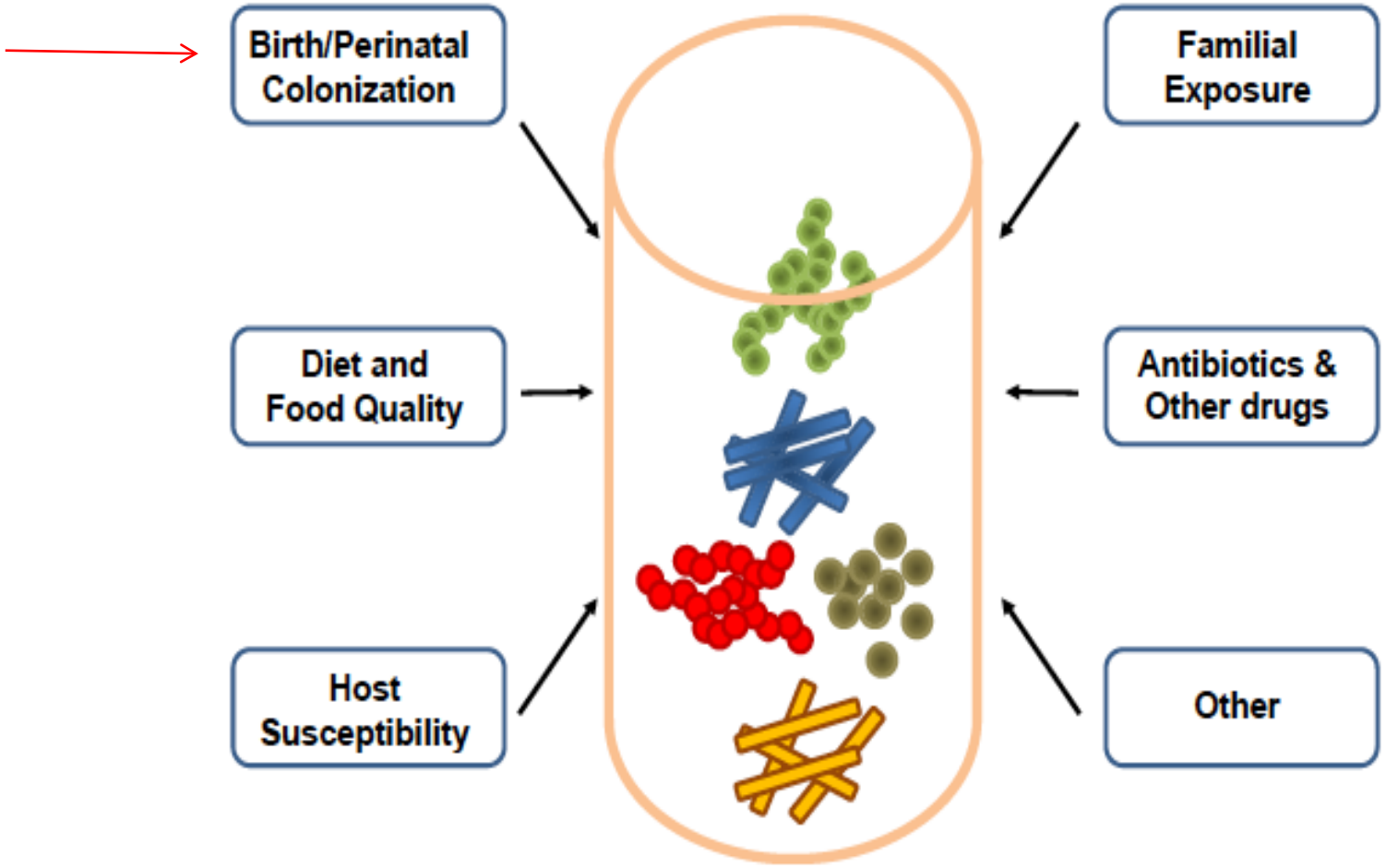
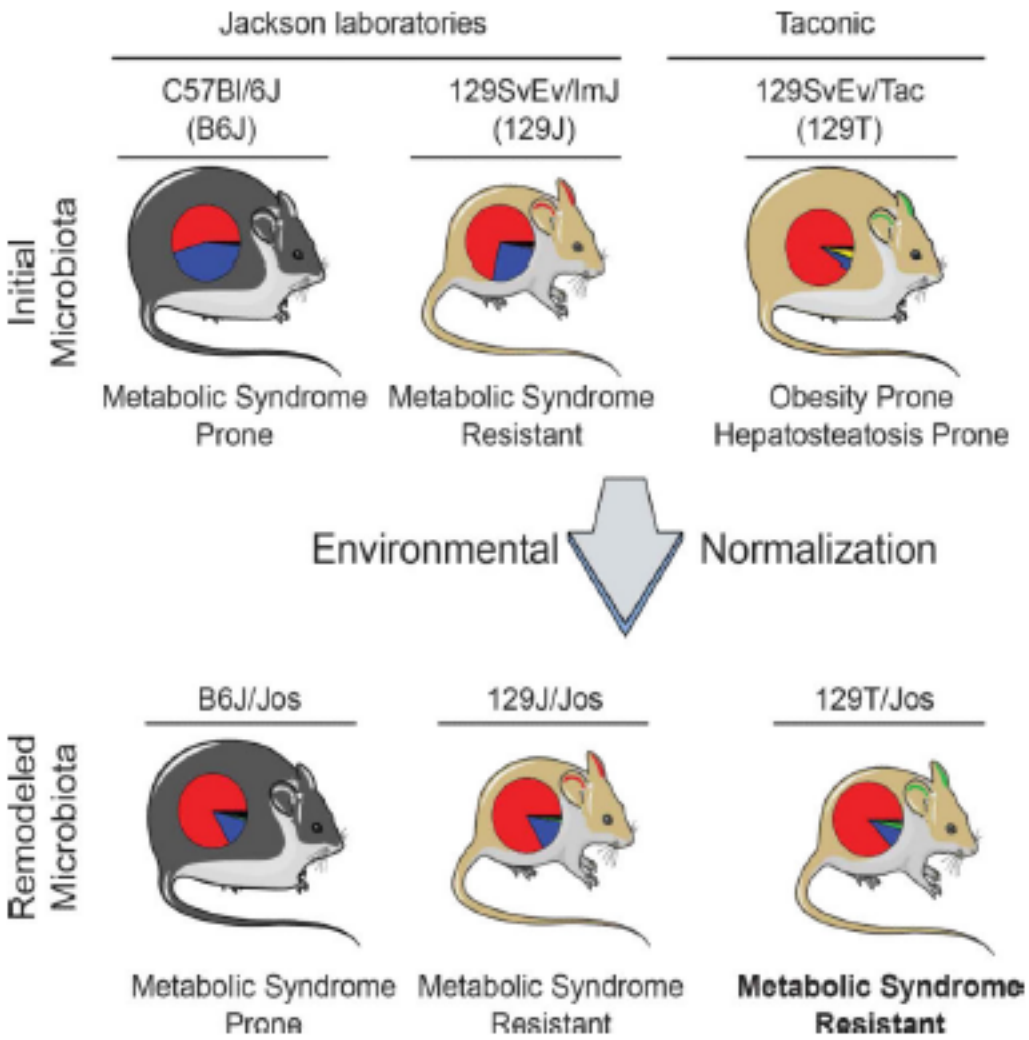
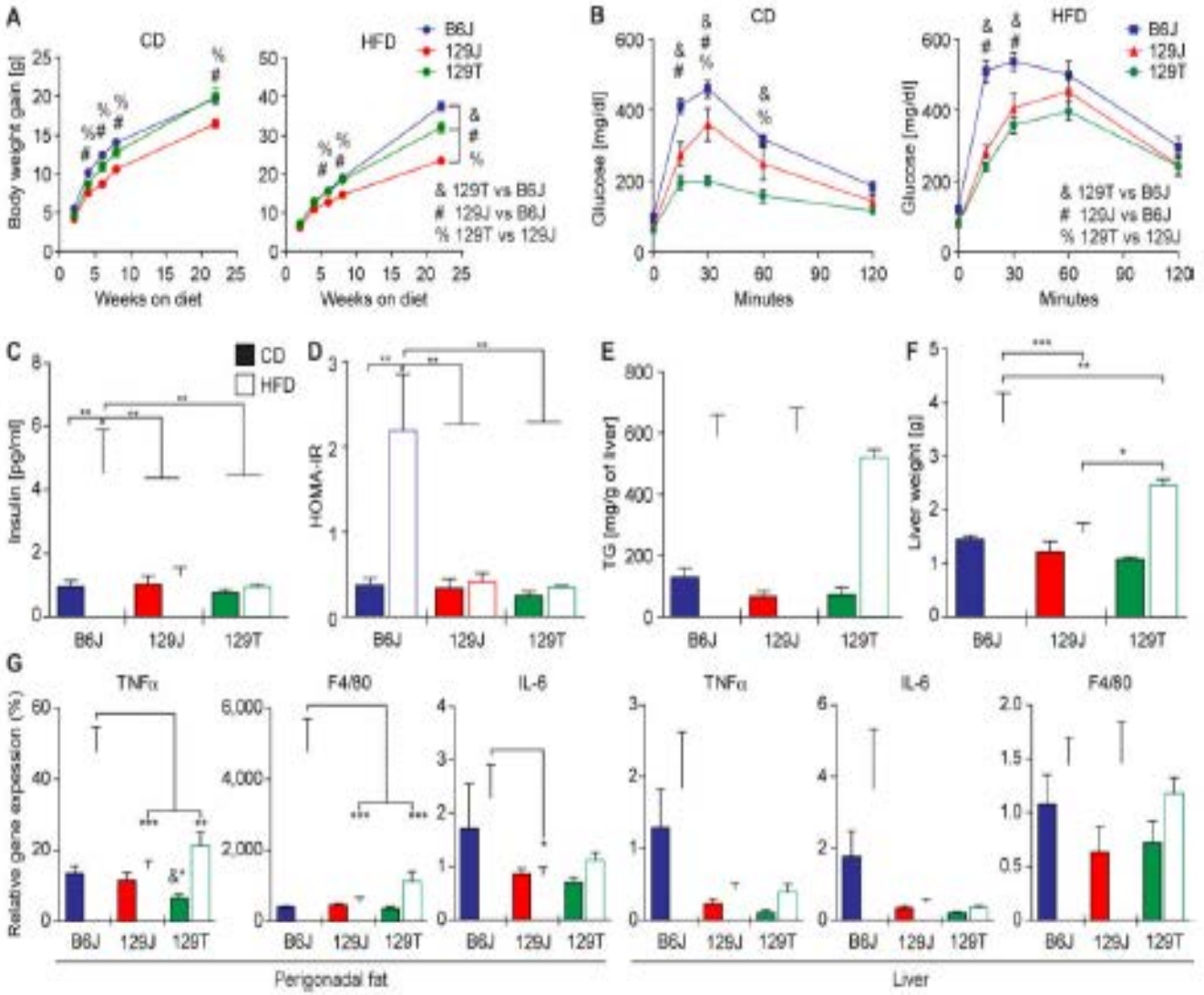


Figure 1: Factors contributing to the development of the microbiome. The development and composition of the gut microbiome is highly dependent on a multitude of


Jackson laboratories
Taconic
C57Bl/6J
(B6J)
129SvEv/ImJ
(129J)
129SvEv/Tac
(129T)
Initial Microbiota
Metabolic Syndrome Prone
Metabolic Syndrome Resistant
Obesity Prone
Hepatosteatosis Prone
Environmental
Normalization
B6J/Jos
129J/Jos
129T/Jos
Remodeled Microbiota
Metabolic Syndrome Prone
Metabolic Syndrome Resistant
Metabolic Syndrome Resistant

Differences in Metabolic Function within Strain



Strain and Environment Changes in Metabolic Status

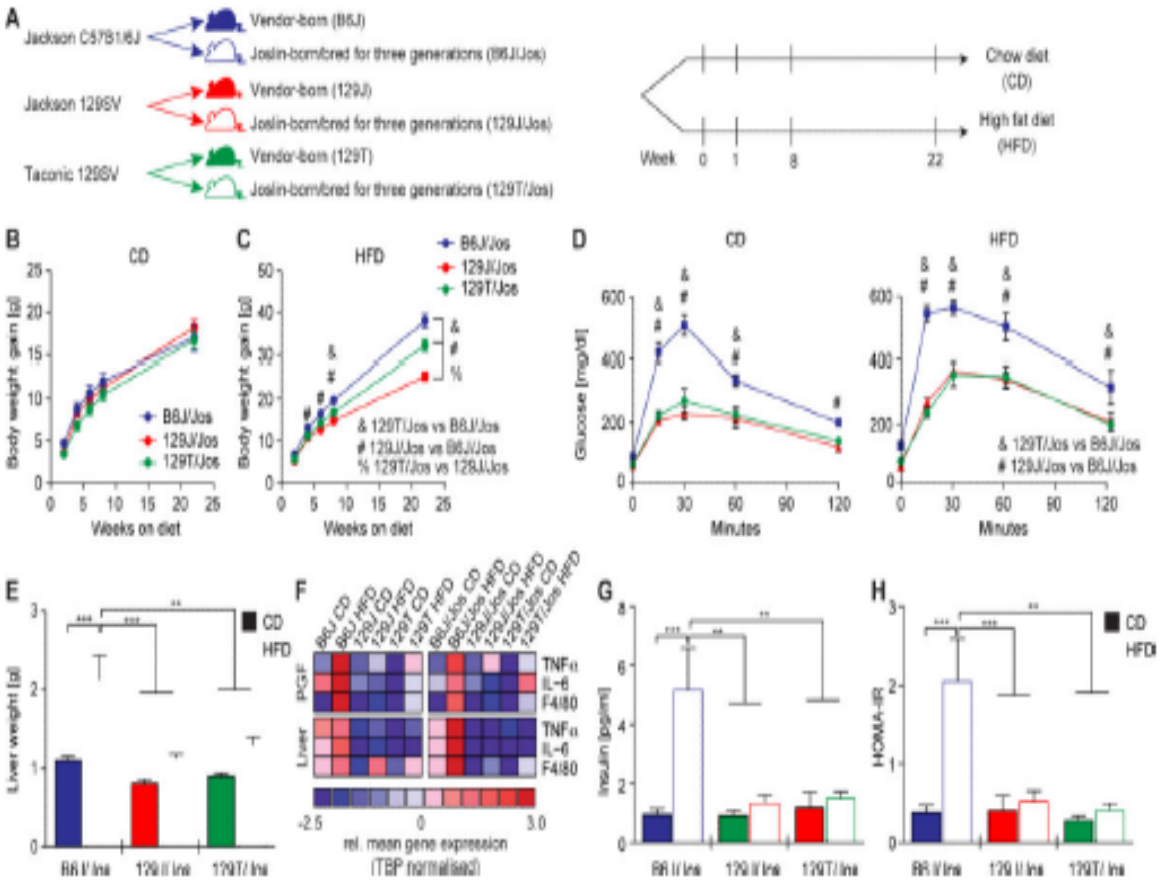
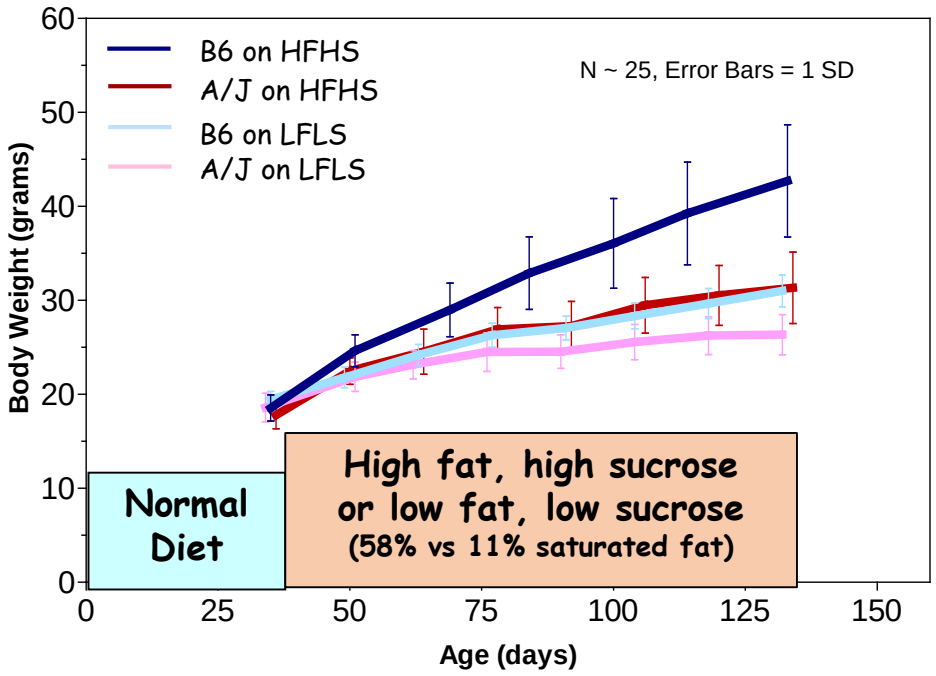


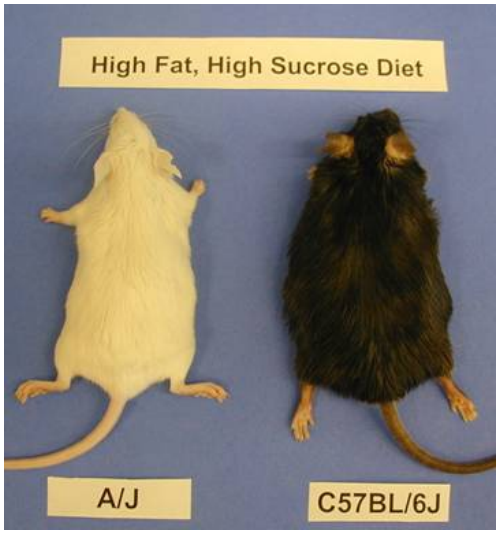
Figure 2. Phenotypic changes associated with breeding at Joslin

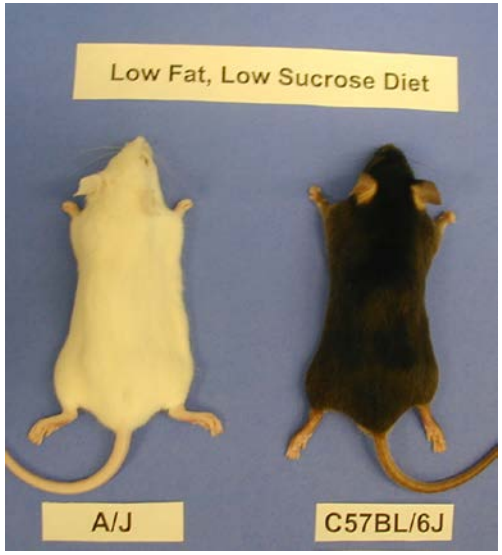
Kahn et al, Cell Met 2015

A Closer Look at Within Strain Variation in Response to Diet

Diet-induced obesity



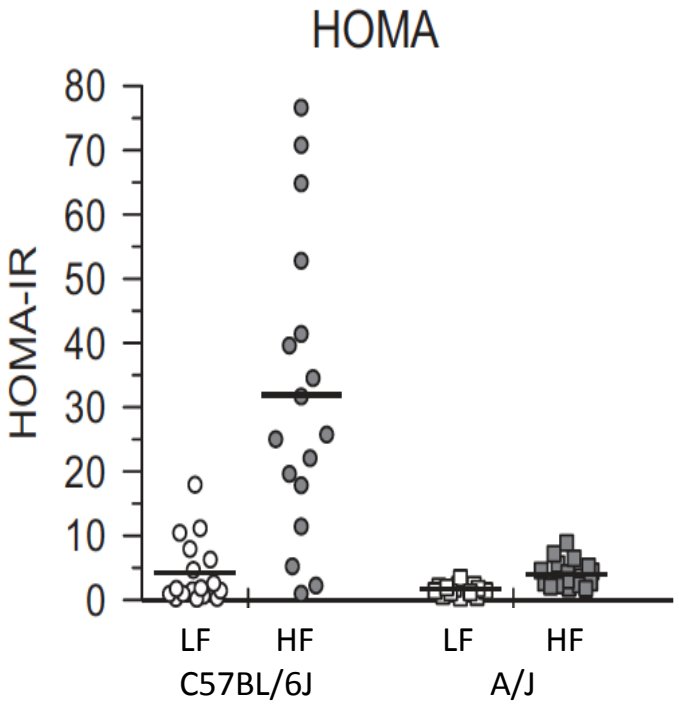


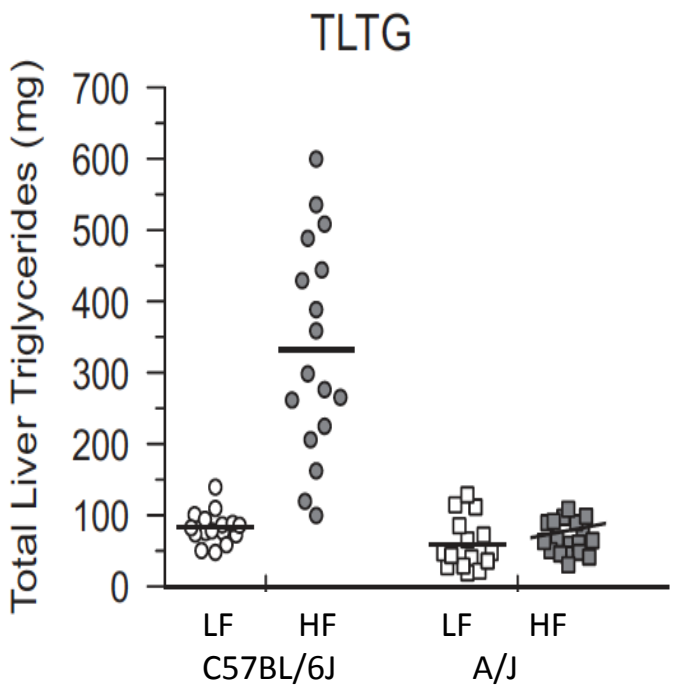


B6, obese only with a HFHS diet

A/J, lean regardless of diet

Examples of diet-induced metabolic changes in two inbred strains



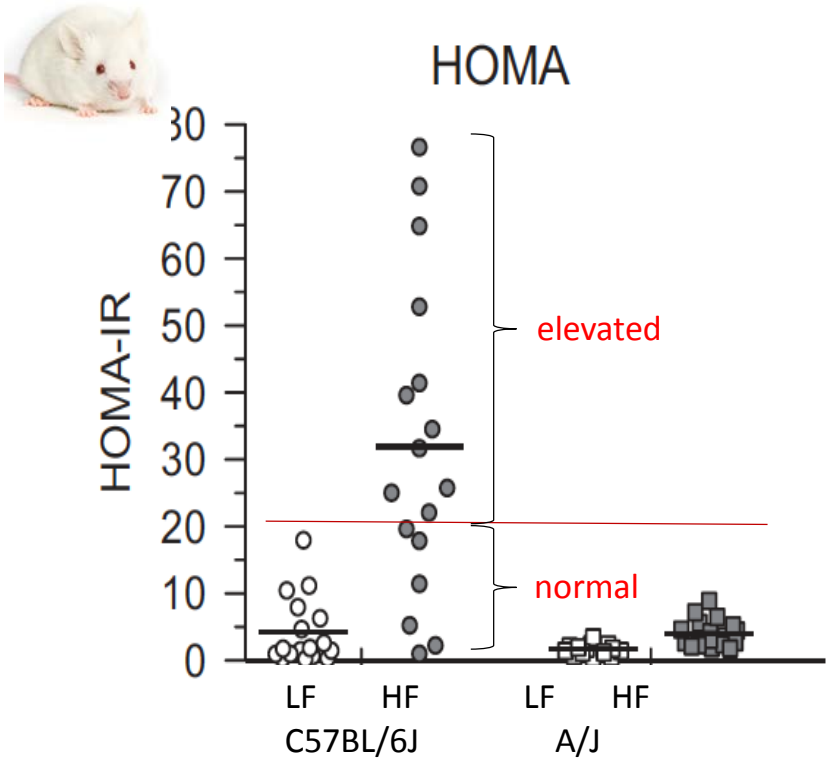


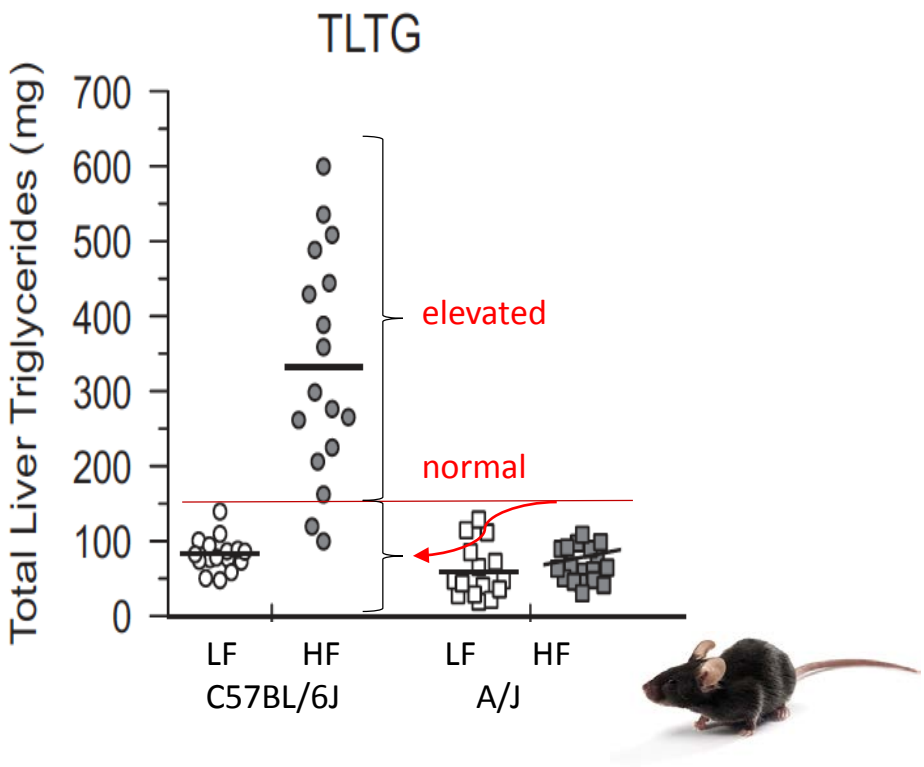
C57BL/6J and A/J males on HFHS vs control diet for 100 days

HOMA – homeostatic model assessment
TLTG – total liver triglycerides

Sinasac, Riordan, et al, Int J Obesity, 2016

Even with increased noise, some mice remain 'normal'





C57BL/6J and A/J males on HFHS vs control diet for 100 days

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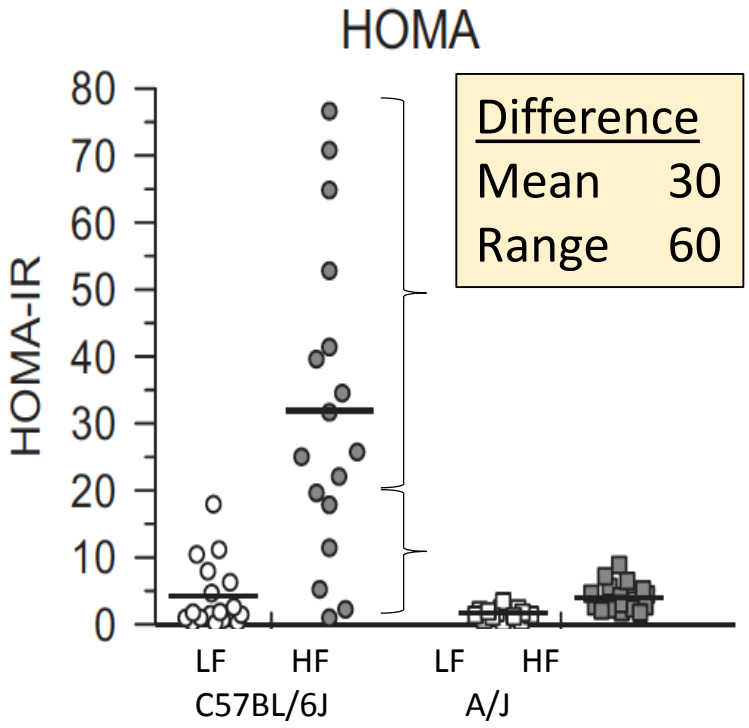
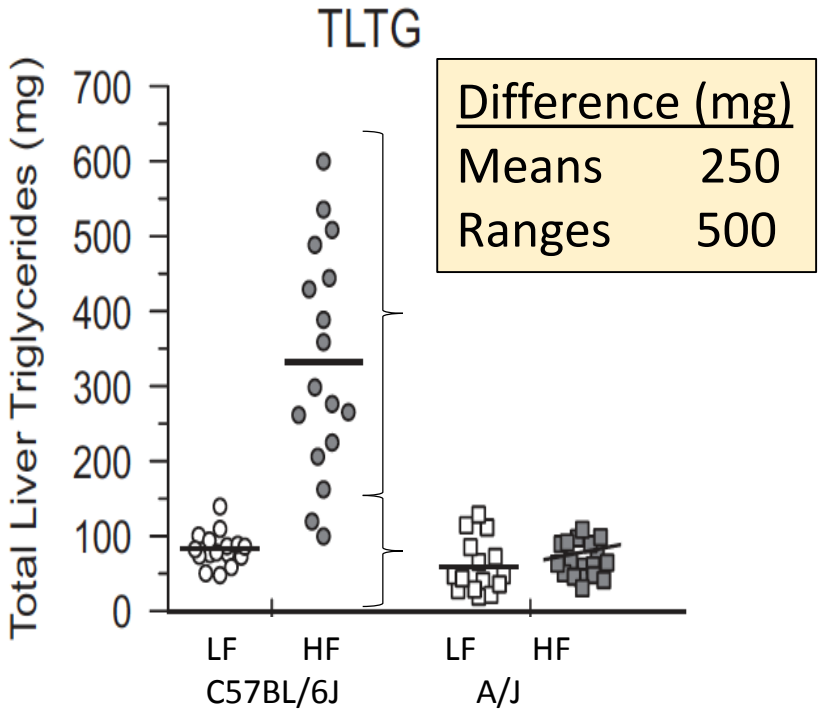
Sinasac, Riordan, et al, Int J Obesity, 2016

Difference in range > mean





C57BL/6J and A/J males on HFHS vs control diet for 100 days

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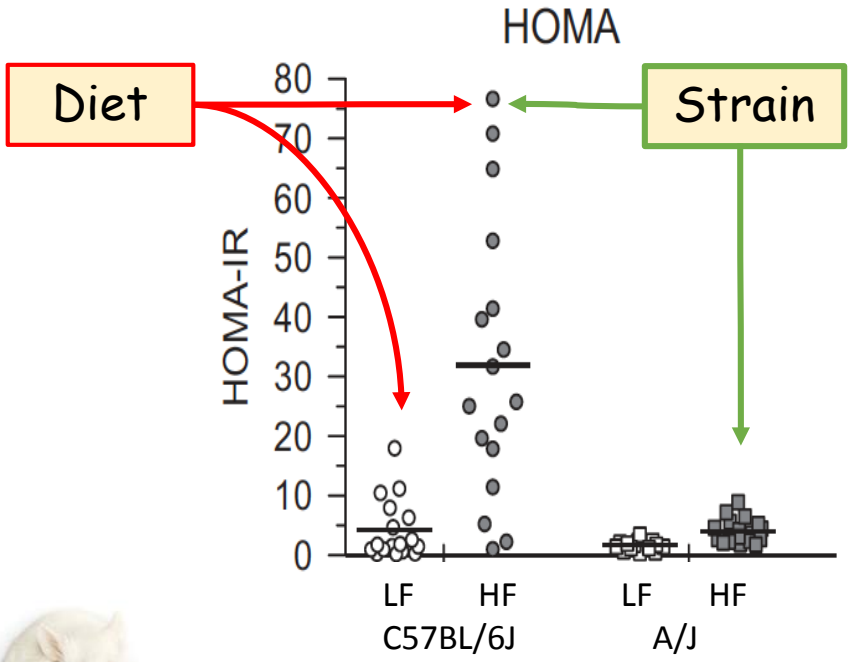
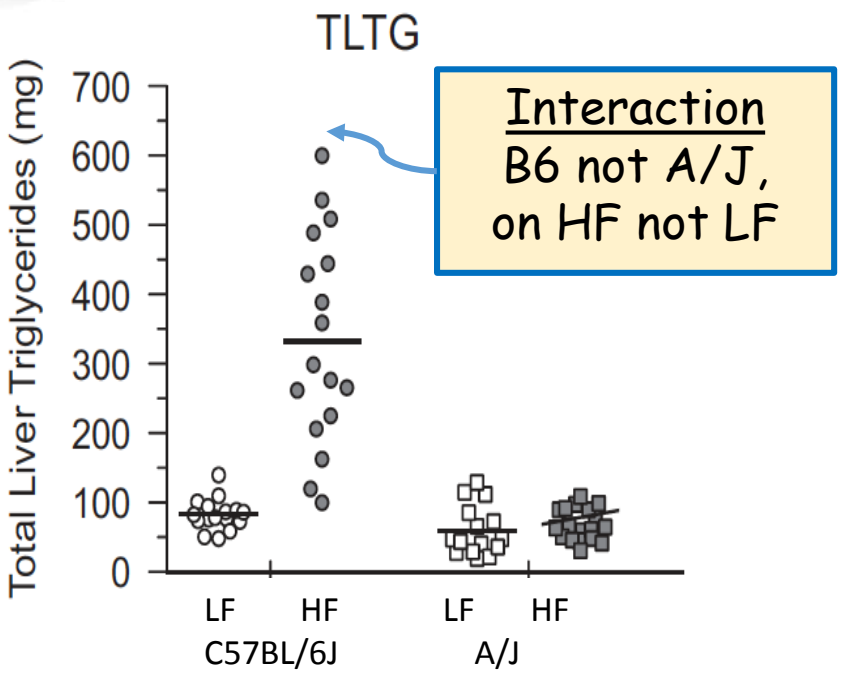
Sinasac, Riordan, et al, Int J Obesity, 2016

Diet, strain, and their interactions



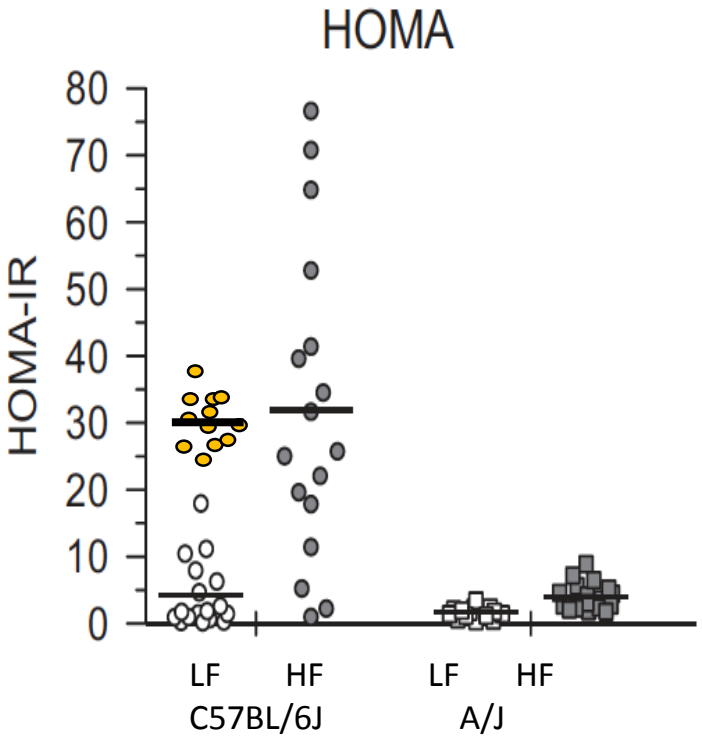


C57BL/6J and A/J males on HFHS vs control diet for 100 days

HOMA – homeostatic model assessment
TLTG – total liver triglycerides

Sinasac, Riordan, et al, Int J Obesity, 2016

Do mean effects drive response to perturbation? Or does variability?



If a change in mean is all that is involved,
why does variability change?

But if diet drives a change in variability, then the mean must increase, as a secondary consequence.

C57BL/6J and A/J males on HFHS vs control diet for 100 days

HOMA – homeostatic model assessment
TLTG – total liver triglycerides

Sinasac, Riordan, et al, Int J Obesity, 2016

Our preoccupation with means

Our perspective and methods focus on mean effects

Why means? Test for differences; predict next observations

Statistics - most tests assess mean differences (central tendencies)

t-tests, ANOVA, correlation, regression
variance (std dev) estimates confidence

We usually ignore 'error' (residual noise)

means effects are tested before residuals,
which assumes means are more important

Information science shows that signals are often embedded in noise

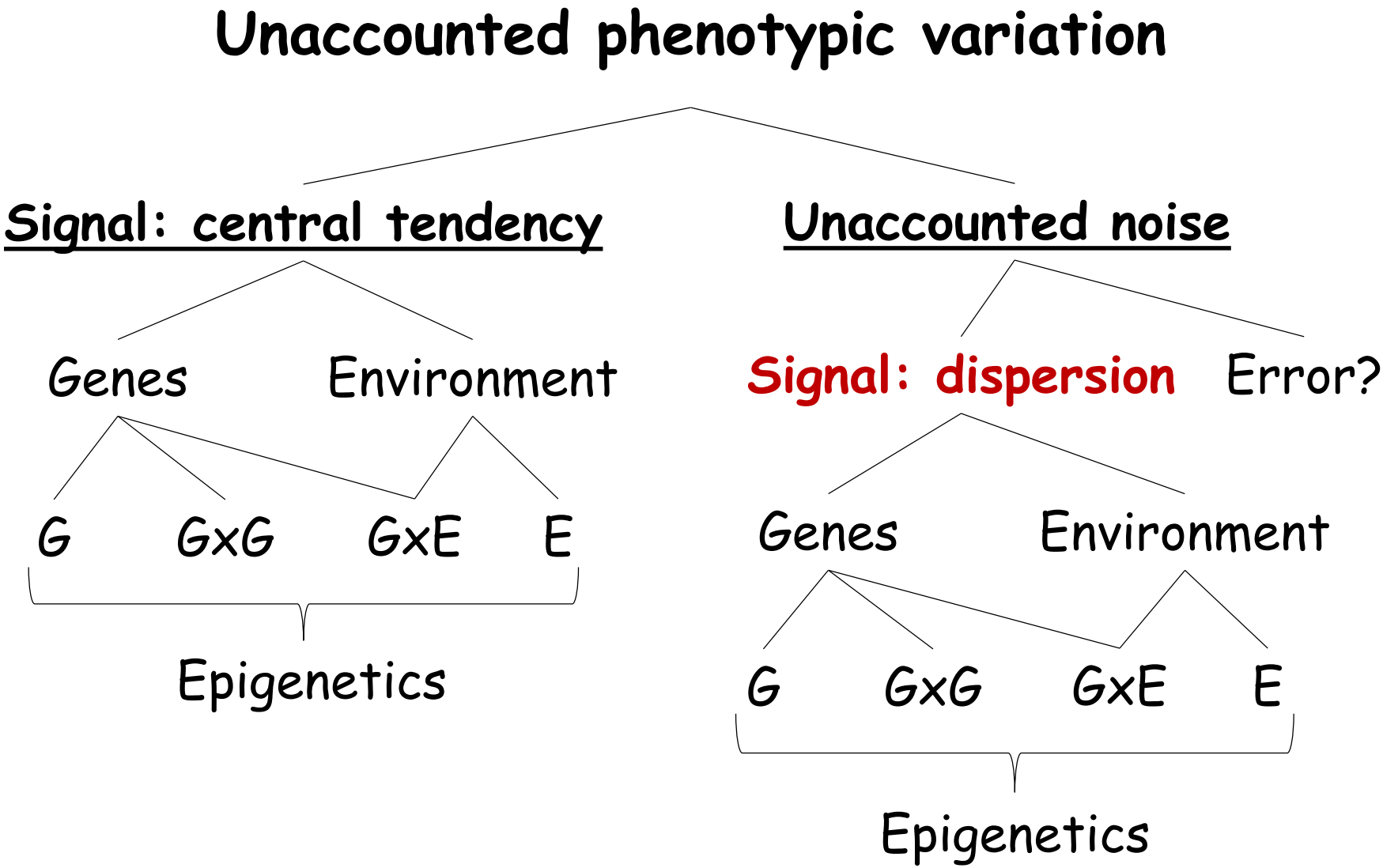
"*When we perform an operation with clear consciousness of what we are aiming at, we may correctly speak of every deviation as being an error; but when Nature presents us with a group of observations, it is a rather bold metaphor to speak of error, as if She had been aiming at something all the time, but missed her mark more or less in every instance*".

(*paraphrased from Venn 1888*)

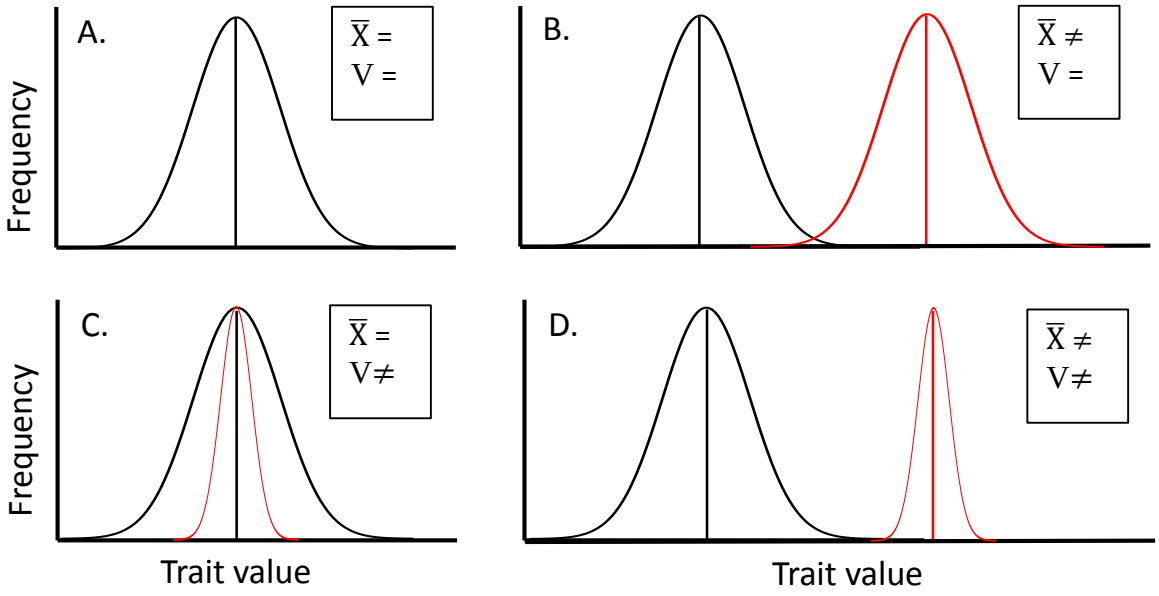
John Venn (1834-1923)

But since we rarely analyze Nature's 'noise', we don't know what we are missing……..

Venn J. 1888. *The Logic of Chance.* MacMillan, London. p42


Unaccounted phenotypic variation
Signal: central tendency
Unaccounted noise
Genes
Environment
Signal: dispersion
Error?
G
GxG
GxE
E
Genes
Environment
G
GxG
GxE
E
Epigenetics
Epigenetics

Means and dispersion



Meaningful noise in unaccounted signal is possible
when differences in phenotypic variation are found

- between genotypes in the same environment (*genetic effect*)
- between exposure groups sharing the same genotype (*environmental effect*)
- depending on genotypes *and* exposures (*gene-environment interaction*)

What are the proximate sources of phenotypic 'noise'?

- **unaccounted gene interactions** *(O. Carlborg et al.)*

  there is no epistatic 'noise' with fully defined genetics

- **epigenetics and transgenerational effects** (*Nadeau*)

  where phenotypes in the present generation result from genetics and environments among ancestors

- **precision of molecular actions** (G. Yvert et al., J. Ayroles et al.)

  resulting from selection for precision versus variability

- **limiting reagents in molecular and biochemical actions**

  a systems consequence of rate-limiting reagents

Phenotypic 'noise' in diet-induced metabolic conditions

How much genetics, mechanisms, systems biology are we missing?

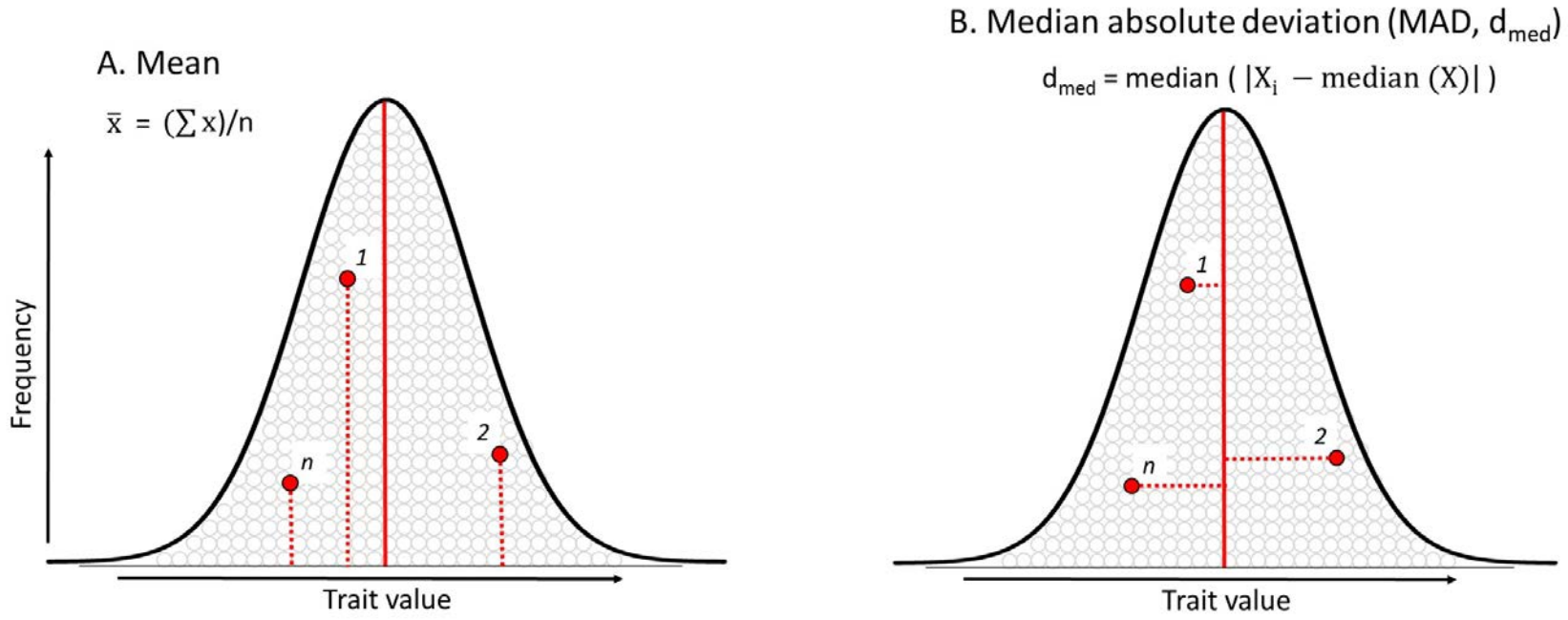
Is there evidence for phenotypic 'noise', and its genetic and environmental control?

What are the implications of 'noise'?

Molecular mechanisms?

Focus: control of diet-induced metabolic conditions in mouse models

Measures: means and median absolute deviation (MAD)



- Median is a more robust measure of central tendency than mean.
- d_{med} is a more robust measure of spread than Std Dev.
- Same sample size, distribution

Features of variance versus MAD

$$\text{Var}(X) = [X_i - \text{mean}(X)]^2$$

- Outliers are removed
- The difference of each observation from the sample mean is squared, giving extra weight to observations far from the mean
- Tests based on variance are sensitive to deviations from normality

$$\text{MAD}(X) = \text{median}\,[|X_i - \text{median}(X)|]$$

- Not sensitive to outliers; each observation is a single case
- Biological outliers are retained; measurement outliers are removed
- Less sensitive to sample size
- Not sensitive to the distribution of observations
- Permutation tests for differences between groups; non-normal data ok

Evidence for phenotypic noise?

1. Response to challenge
2. Genetic control
3. Gene interactions
4. Transgenerational epigenetic inheritance
5. Candidate genes
6. Developmental origins - volatility

Mean and MAD changes – difference in measured units

final BW, B6 HFHS – B6 chow

Mean: 41.14 – 26.39 = 14.75 g

MAD: 5.11 – 0.75 = 4.36 g

change test vs ref	
up	
down	

B6 HFHS vs control		Final BW	BMI	GLU	INS	HOMA	CHOL	TG	Liver Wt	Liv TG	Total Liv TG	Ave % mean
difference	MEAN	14.75	0.12	5.48	235.08	27.75	2.35	-0.08	0.39	133.65	249.73	
	MAD	4.36	0.03	1.22	120.13	12.86	0.21	-0.02	0.25	31.00	122.20	
% mean		29.6	24.3	22.3	51.1	46.4	8.8	29.6	63.3	23.2	48.9	34.7
AJ HFHS vs control												
difference	MEAN	5.73	0.04	2.73	25.37	2.36	1.39	0.11	-0.05	16.07	9.10	
	MAD	-1.04	-0.01	0.00	4.99	0.59	0.06	-0.11	-0.02	-1.50	-0.79	
% mean		18.1	13.7	0.0	19.7	24.9	4.6	98.8	40.8	9.3	8.7	23.9
B6 control vs AJ control												
difference	MEAN	2.30	0.03	0.86	32.11	2.54	0.47	-0.27	0.24	9.95	23.24	
	MAD	-1.15	-0.01	0.22	11.70	0.86	0.05	-0.18	-0.03	-4.00	-10.61	
% mean		50.1	29.3	25.8	36.4	34.1	11.1	66.1	13.0	40.2	45.7	35.2
B6 HFHS vs AJ HFHS												
difference	MEAN	11.32	0.10	3.61	241.82	27.92	1.43	-0.46	0.68	127.53	263.87	
	MAD	4.25	0.03	1.44	126.84	13.14	0.19	-0.10	0.24	28.50	112.38	
% mean		37.5	26.9	40.0	52.5	47.1	13.6	20.9	34.8	22.3	42.6	33.8
Units		g	cm–2	nmol l–1	pmol l–1	na	nmol l–1	nmol l–1	g	liver	mg	

1. MAD differences are ~1/3 mean differences
2. MAD effects are comparable to many reported mQTLs
3. Largest strain, diet - B6 HFHS vs B6 chow, AJ HFHS
4. Largest MAD (% mean) - insulin and liver triglycerides

Mean and MAD changes – magnitude (fold-change)

Fold-change, B6 HFHS / B6 chow

Mean: 41.14 / 26.39 = 1.56 fold

MAD: 5.11 / 0.75 = 6.81 fold

Change	
up 2x	
down 50%	
bold	4x

test vs reference			Final BW	BMI	GLU	INS	HOMA	CHOL	TG	Liver Wt	Liv TG	Total Liv TG	Ave %
B6 HFHS vs control													
	MEAN	%	1.56	1.46	1.55	**4.87**	**7.66**	2.17	0.87	1.34	2.83	**4.03**	**2.83**
	MAD	%	**6.81**	**5.06**	2.00	**7.02**	**9.91**	2.14	0.75	7.33	2.94	**15.11**	**5.91**
MAD vs MEAN			>	>		>	>			>		>	
AJ HFHS v control													
	MEAN	%	1.24	1.18	1.30	1.89	2.45	1.90	1.13	0.94	1.26	1.15	**1.44**
	MAD	%	0.46	0.60	1.00	1.60	2.01	1.50	0.60	0.69	0.93	0.96	**1.03**
B6 control vs AJ control													
	MEAN	%	1.10	1.11	1.09	2.12	2.56	1.30	0.67	1.26	1.16	1.39	**1.38**
	MAD	%	0.39	0.49	1.22	2.42	2.49	1.40	0.33	0.56	0.80	0.45	**1.06**
B6 HFHS vs AJ HFHS													
	MEAN	%	1.38	1.37	1.31	**5.48**	**8.00**	1.49	0.52	1.80	2.61	**4.86**	**2.88**
	MAD	%	**5.91**	**4.13**	2.44	**10.57**	**12.26**	2.00	0.41	**5.96**	2.54	**7.08**	**5.33**
MAD vs MEAN			>	>		>	>			>	eq	>	
Units			g	; cm–2	nmol l–1	pmol l–1	na	nmol l–1	nmol l–1	g	liver	mg	

B6 on HFHS has biggest effect

~3x for mean

~5x – 6x for MAD

Longterm HFHS exposure increases dispersion

Evidence for phenotypic noise?

1. Response to challenge
2. Genetic control
3. Gene interactions
4. Transgenerational epigenetic inheritance
5. Candidate genes
6. Developmental origins - volatility

Mouse Phenome Database and MMPC

Evidence for dispersion found for every trait,
though not necessarily for every assay

Exercise and endurance

Behavior
- anxiety
- balance, coordination
- depression
- exploration
- fear-conditioning
- impulsivity
- involuntary movement
- learning, memory
- locomotion
- parental nurturing
- sleep
- social
- wildness

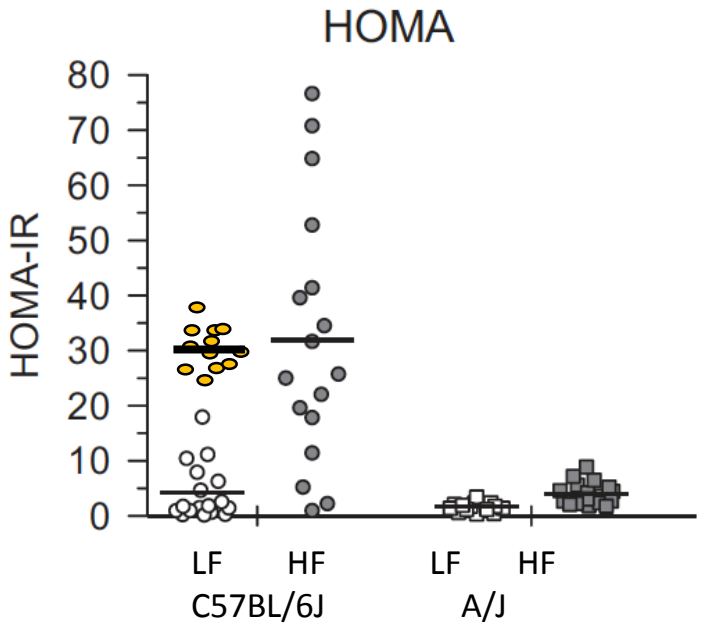
Metabolism
- body temperature
- energy
- food intake
- respiration
- sucrose
- water intake

Brain
- cerebral cortex – analytes
- electroconvulsive threshold
- hippocampus – miRNAs
- neurotransmitters
- pathology
- xenobiotics

Implications of phenotypic noise

1. Driver – mean or dispersion?
2. Dysfunction or adaptation?
3. Meaning of distributions
4. Noise in networks and pathways
5. Adaptation in fluctuation environments
6. Precision medicine

What is the driver of metabolic change?

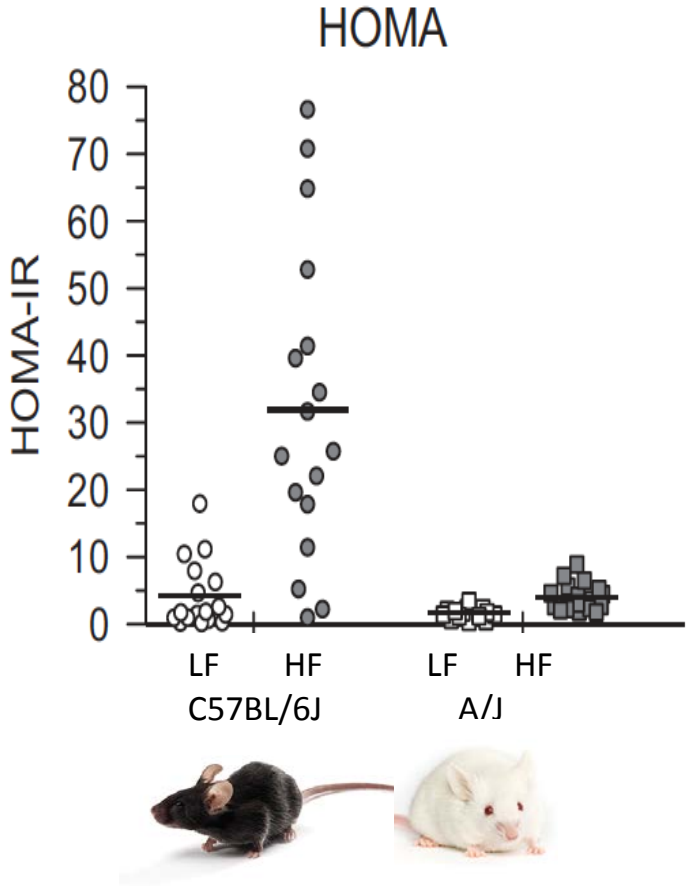


'Mean effect' is the usual explanation,

but might dispersion be the primary driver,

with mean differences as a secondary effect?

Dysfunction or adaptation?



Loss of control,

or adaptive strategy?

(bet-hedging, plasticity)

C57BL/6J and A/J males on HFHS vs control diet for 100 days

HOMA – homeostatic model assessment
TLTG – total liver triglycerides

Sinasac, Riordan, et al, Int J Obesity, 2016

Summary

- Genotypic differences across strains is very meaningful
- There is significant gene x environment interactions in every mouse experiment
- Variation in mean values for a particular phenotype should not be discounted solely as a measure of the means
  - Much of what variation means is related to compensatory responses, which are in themselves important
- Median Absolute Deviation should also be considered

Diabetes and Skeletal Health

Ann Schwartz

Friday, September 28

11:30 am – 12:30 pm

Room 518 C

Diabetes and Skeletal Health
Ann Schwartz PhD
University of California San Francisco, USA
ASBMR MTP 2018

SIGNIFICANCE OF THE CLINICAL PROBLEM

Diabetes is associated with higher fracture risk. In type 1 diabetes, hip fracture risk is about 4-5 times higher than for non-diabetic patients [1, 2]. In type 2 diabetes, the increased risk is more modest, about 1.3-1.7 times higher [3, 4]. However, type 2 diabetes affects over a quarter of older adults in the US, resulting in a substantial absolute increase in fracture risk. While fracture risk is increased, bone mineral density in type 2 patients tends to be higher than in those without diabetes. Diabetic patients are less likely to be screened and treated for osteoporosis, in spite of their higher risk. Possible reasons include the difficulties of fracture risk assessment along with the challenges of identifying optimal pharmacological therapy for osteoporosis in diabetic patients.

BARRIERS TO OPTIMAL PRACTICE

Obtaining an accurate assessment of fracture risk in diabetic patients is a challenge. The standard tools, BMD T-score and FRAX, tend to under-estimate risk in this population. Another challenge is identifying the potential impact of specific diabetic medications and of glycemic control on fracture risk. Finally, there are challenges in determining the optimal pharmacological therapy for osteoporosis when this level of treatment is warranted in a diabetic patient.

LEARNING OBJECTIVES:

As a result of participating in this session, learners should be able to:
Identify under-estimation of fracture risk with BMD T-score or FRAX in diabetic patients
Discuss effects of diabetes medications on skeletal health
Describe evidence for optimal pharmacological osteoporosis therapy in diabetic patients

STRATEGIES FOR DIAGNOSIS, THERAPY, AND/OR MANAGEMENT

ASSESSMENT OF FRACTURE RISK

BMD T-score does predict fracture in type 2 diabetes. As shown in Figure 1, among diabetic patients, those with lower BMD have greater fracture risk. However, BMD T-score under estimates absolute fracture risk in diabetic patients compared with non-diabetic patients [5]. As a rough estimate, one can subtract 0.5 from the measured femoral neck BMD T-score to identify the “fracture risk equivalent” T-score in a diabetic patient. For example, an older diabetic woman with femoral neck BMD T-score of -2.0 would have a hip fracture risk similar to an older non-diabetic woman with T-score of -2.5.

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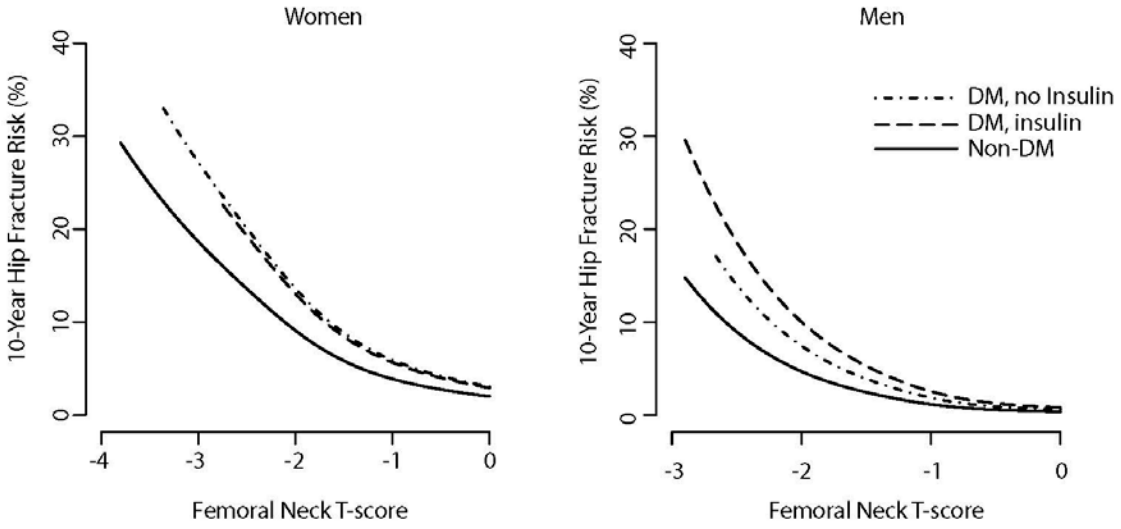

Figure 1. Femoral Neck BMD T Score and 10-Year Fracture Risk at Age 75 Years by DM and Insulin Use Status
Adapted with permission from Schwartz, et. al. [5].

The standard risk factors for fracture that are incorporated into FRAX are also predictive of fracture risk in diabetic patients, such as age, gender and BMI [6]. However, as with T-score, FRAX tends to under-estimate risk in diabetic patients [5, 7]. Diabetes is not currently included in the FRAX algorithm. It may be incorporated into the algorithm in the future but, meanwhile, one can make a crude compensation by reducing the BMD T-score by 0.5 or by adding 10 years to the patient's age in the FRAX estimator for a diabetic patient.

Similar studies of fracture risk assessment have not been carried out in type 1 diabetes. A meta-analysis of type 1 diabetes, BMD and fracture risk found that the lower BMD associated with type 1 diabetes does not fully account for the substantially increased hip fracture risk in these patients [8]. Based on this finding, it is reasonable to assume that BMD T-score and FRAX will also under-estimate fracture risk in type 1 diabetes. However, without additional studies, it is not known by how much T-score or FRAX might underestimate risk.

FRAX provides a useful method to incorporate traditional risk factors for fracture (age, gender, BMI, etc.) into one score for a patient. But, notably, fall history is not included in the FRAX algorithm. Falls are more common in diabetic patients, and this aspect of patient history should be considered. There are also diabetes-specific factors that are not part of FRAX but could help with a clinical assessment of risk. Key factors to consider: Longer duration of diabetes, Presence of microvascular complications, Insulin therapy, Hypoglycemic episodes, Poor glycemic control.

DIABETES MEDICATIONS

Diabetes medications may affect bone health and fracture risk. Increased fracture risk has been identified with use of thiazolidinediones (TZDs), most definitively in women [9] but also recently in men [10]. One consequence has been greater attention to fracture outcomes in trials of new diabetes medications. The table below summarizes currently available evidence regarding the skeletal effects of different classes of diabetes medications.

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Insulin is associated with increased fracture risk which is surprising given evidence that insulin is anabolic for bone. However, insulin use is associated with longer duration of diabetes and higher prevalence of complications. It may therefore be a marker for increased fracture risk rather than a causal factor. Other diabetes medications appear to have a neutral effect on fracture risk with the exception of sodium-glucose cotransporter 2 (SGLT2) inhibitors. The evidence for this class of medications is mixed. Based on analysis of combined smaller RCT's, canagliflozin treatment was associated with higher fracture risk (HR=1.32) compared with placebo/comparator [11]. Updated results for the ADVANCE trial confirmed this modest increased fracture risk with canagliflozin [12]. However, an analysis of trials of empagliflozin found no evidence of increased fracture risk [13].

Diabetes Medication	Bone turnover markers	BMD	Fracture risk
Insulin	??	↑ (a)	↑ (a)
Sulfonylureas	??	??	↔ (b*)/ ↑(a)
Metformin	↓ (a)	↔ (a)	↔ (b*)
Thiazolidinediones	↓/↔ formation (b); ↑/↔ resorption (b)	↓ (b)	↑ (b)
GLP-1 receptor agonists	↔ (b)	??	??
DPP-4 inhibitors	↔ (b)	??	↔ (b)
SGLT2 inhibitors	↑ /↔ (b)	↓/↔ (b)	↑/↔ (b)

a = prospective cohort or nested case control studies
b = randomized controlled trials (AE's for fractures)

American Diabetes Association in the Standards of Medical Care in Diabetes (2018) recommends: "For patients with type 2 diabetes with fracture risk factors, thiazolidinediones and sodium–glucose cotransporter 2 inhibitors should be used with caution."

PHARMACOLOGICAL THERAPY FOR OSTEOPOROSIS IN DIABETIC PATIENTS

Bone turnover markers tend to be lower in type 1 and type 2 diabetes {Hygum, 2017 #21094}, leading to concerns that anti-resorptive therapy may not be effective for fracture prevention in these patients. Evidence to date remains limited, but generally indicates that anti-fracture efficacy is similar in diabetic and non-diabetic patients. Studies include subgroup analyses of results from randomized trials of osteoporosis therapies and large observational studies using registry data. A subgroup analysis of the Fracture Intervention Trial found that alendronate increases BMD in diabetic women, similar to its effects in non-diabetic women [16]. Subgroups analyses of the RUTH trial found reduced risk of vertebral fracture in diabetic as well as non-diabetic women [17]. An observational study, using Danish registry data, also found no differences in fracture efficacy for bisphosphonates or raloxifene comparing diabetic and non-diabetic patients [18]. A small observational study of teriparatide found BMD and fracture effects were similar in diabetic and non-diabetic patients [19]. Data are not currently available for strontium or denosumab.

Nutrition and Fragility

Marian Hannan, Ph.D. and Shivani Sahni, Ph.D.

Saturday, September 29

11:00 am – 12:00 pm

Room 519 B

Mechanosensitive Osteocytes: Insights into How the Osteocytes Control the Bone Response to Bone Loading and Unloading

Jean X. Jiang, Ph.D.

Department of Biochemistry and Structural Biology, University of Texas Health Science Center at San Antonio, Texas, USA

Significance of the topic:

The skeleton adapts to mechanical usage and mechanical loading promotes bone formation and remodeling. Although most bone cells are involved in mechanosensing, it is well accepted that osteocytes are the principal mechanosensory cells. Osteocytes are embedded inside the bone mineral matrix and have stellate morphology with small cell body and long dendritic processes. The long dendritic processes of osteocytes form a network not only connecting the neighboring osteocytes, but also the cells on the bone surface, such as osteoblasts and osteoclasts. Recently, morphological studies also show the connection of osteocytes with bone marrow and blood vessels. The osteocyte has been perceived as the center of bone remodeling by coordinating both osteoblast and osteoclast function, and also as the initiator of bone remodeling by sensing the bone matrix. Osteocyte cell body and processes are surrounded by fluid-filled space, forming an extensive lacuno-canalicular network. The osteocyte dendritic processes and the cell body are surrounded by fluid filled spaces termed as canaliculi and lacuna, respectively. The canaliculi around the dendrites are narrow when compared to that of the lacunar space surrounding osteocyte cell body. Various studies suggest that flow of interstitial fluid driven by extravascular pressure is a likely stress-related factor that transmits mechanical stimulation to bone cells. Dendritic processes of osteocytes are postulated as the mechanical sensory region on osteocytes. The mechanisms by which osteocytes sense and respond to mechanical loading and unloading in osteocytes are active research focuses in many laboratories.

Learning Objectives:

As a result of participating in this session, attendees should be able to understand the current knowledge and research in

(1) Current models of mechanical stimulation on osteocytes.

(2) Mechanosensory areas of osteocytes and primary approaches in vivo and in vitro being used.

(3) Critical mechanosensory molecules involved

(4) Roles of osteocytic connexin and pannexin channels in mechanostransduction.

(5) Signaling mechanisms activated by mechano-stimulation.

(6) Relevance to physiology and pathology of the bone tissues.

(7) Challenges and future research directions.

An Outline/Points of Interest

1. Major types of mechanical stimulation on osteocytes.
   - Fluid flow shear stress.

2. Major mechanosensory areas of osteocytes
   - Dendritic processes and cilia

3. Mechanosensory molecules involved
   - Integrins, connexin, pannexin, ion channels, glycocalyx, etc.

4. Osteocytic connexin, pannexin, P2X7 channels and Ca^{2+} channels in mechanotransduction
   - Transmit signals between cells through gap junction channels
   - Activation of connexin or pannexin hemichannels and release factors, such as prostaglandins and ATP.
   - P2X7 and its association with pannexin channels.
   - T-type voltage-sensitive calcium channels

5. Signaling mechanisms
   - Ca^{2+}
   - LRP/Wnt, sclerostin,
   - PGE_2, ATP
   - PI3K-Akt, β-catenin
   - IGF-1
   - MAPK

6. Physiology and Pathology
   - Force-bearing exercise, disuse and lack of gravity.

7. Challenges
   - Translate into therapeutic strategies without pharmaceutical drugs.

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AFF, Drug Holiday

Bo Abrahamsen, M.D., Ph.D.

Saturday, September 29

11:00 am – 12:00 pm

Room 521

AFF AND DRUG HOLIDAY

Bo Abrahamsen
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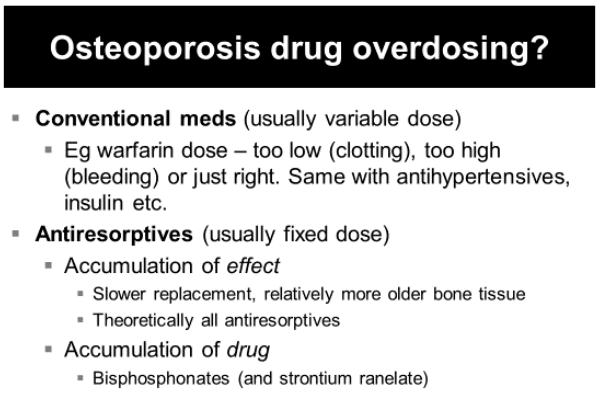
University of Southern Denmark and
Holbæk Hospital Dept of Medicine, DK-4300 Holbæk, Denmark

SIGNIFICANCE OF THE TOPIC

The rationale behind drug holidays in osteoporosis management is an expectation that the risk of adverse events will decline very rapidly and the risk of osteoporotic fractures increase only slowly.



Despite of a low incidence rate compared with osteoporotic fractures, atypical femur fractures (AFF) have attracted much attention and highlighted the need for good long term, evidence based treatment strategies. For most osteoporosis drugs, the number of patients in placebo controlled trials beyond 4-5 years has been very small. Unfortunately the evidence supporting drug holidays is sparse as is the evidence supporting time unlimited treatment. As with other chronic diseases, the absolute risk of complications and the pros and cons of continued or changed treatment should be assessed periodically.

Most clinical guidelines advocate a pause in treatment after 3 to 5 years of bisphosphonate treatment with the exception of patients at the highest risk of fracture. For non-bisphophonate antiresorptives such as SERMs and denosumab, it is dubious if drug holidays can be recommended at all due to a rapid onset of bone loss. Though the drug holiday and AFF scenario is confined mostly to long term treatment, many countries have seen a large widening of the treatment gap for osteoporosis due to concerns among patients and their physicians.

LEARNING OBJECTIVES

Following the sessions, participants will

- Understand the knowledge gaps regarding drug holidays in the prevention of Atypical Femur Fractures.
- Be aware of current guidelines and recommendations regarding the duration of antiresorptive treatment.
- Be able to diagnose AFFs and take appropriate steps to manage this outcome in collaboration with colleagues and relevant services /specialities.
- Be able to advise patients on absolute risks and benefits of long term osteoporosis therapy.
- Recommend steps to monitor patients during bisphosphonate drug holidays.

CASE BASED DISCUSSION

Drug holidays and hip fractures

- Medicare data US 2006-2014: Identified 156,236 women who used BPs with at least 80% adherence for at least three years. A total of 3,745 hip fractures occurred.

Current user	Ref 1
Stop < 3 mo	1.29 (1.17-1.42)
Stop 3-12 mo	1.12 (1.02-1.24)
Stop 1-2y	1.21 (1.09-1.35)
Stop 2-3y	1.39 (1.21-1.59)

Curtis JR, Abstract 4953 EULAR 2018

Case 1

70-year old woman, currently taking weekly alendronate, completed three years of treatment. Never experienced fractures. T-score of the spine increased from -3.0 to now -2.3. Femoral neck T-score unchanged -2.4. Drug holiday and monitoring? Would the plan change if the patient had experienced a humerus fracture last year? Any change to plan if patient has type II diabetes?

Case 2

65-year old woman who presents with a new grade II vertebral fracture five years after her alendronate treatment was stopped due to an atypical femur fracture. Prior to her AFF she had been on alendronate for eight years due to a mild (grade I) wedge deformity with femoral neck T-score -2.6. Spine BMD normal but pronounced degenerative changes leading to potentially falsely elevated BMD. Now, following 8 years of alendronate and five years of no treatment her spine T-score is -0.5 and femoral neck T-score is -1.7. Plan?

ASBMR 2014 CRITERIA FOR DIAGNOSIS OF AFF

- Femur fracture located along the diaphysis from just distal to the lesser trochanter to just proximal to the supracondylar flare.

Minor or inconstant features:

1. Generalized increase in cortical thickness of the femoral diaphyses
2. Prodromal symptoms such as dull or aching pain in the groin or thigh
3. Bilaterality
4. Delayed fracture healing

- **Four of these met**:
- Minimal or no trauma, as in a fall from a standing height or less
- Fracture line originates at the lateral cortex and is substantially transverse in its orientation, although it may become oblique as it progresses medially across the femur.
- Complete fractures extend through both cortices and may be associated with a medial spike; incomplete fractures involve only the lateral cortex
- The fracture is non-comminuted or minimally comminuted
- Localized periosteal or endosteal thickening of the lateral cortex is present at the fracture site ("beaking" or "flaring")

Adapted from Shane, E., Burr, D., Abrahamsen, B., Adler, R. A., Brown, T. D., Cheung, A. M., … Whyte, M. P. (2014). Atypical subtrochanteric and diaphyseal femoral fractures: Second report of a task force of the American society for bone and mineral research. Journal of Bone and Mineral Research, 29(1), 1–23.

DELL – PROPOSED MANAGEMENT OF AFF

Proposed Management Based on Presentation in BP users

Thigh pain with stress reaction	Incomplete AFF	Complete AFF
Consider prophylactic rodding		Surgery as below:
1) The antiresorptive should be stopped immediately. 2) Protected limited weight-bearing should be advised. 3) Check calcium, vitamin D, and other metabolic factors and correct them as needed. 4) Warn the patient that symptoms might progress and an AFF is still possible even with precautions. 5) A course of teriparatide may be considered but the results have been mixed. 6) If pain is not decreasing by 2–3 months or if a dreaded black line develops, then consider prophylactic rodding.		• Surgical considerations • Avoid plating and short rodding • Check for bowing of the femur • Check canal thickness/over ream 1.5–2 mm • Check for stress reaction in the other femur • Watch for delayed healing • Consider excision of the dreaded black line

Table based on: Dell, R.A proposal for an atypical femur fracture treatment and prevention clinical practice guideline. Osteoporosis International, 29(6), 1277–1283.

Dell and Greene, Osteoporosis International (2018) 29:1277–1283

Managing a drug holiday

Information	Relevance
BMD status	Low T-score? Rate of loss? Patient now at high risk of fracture?
New fractures	Indicator of high risk of fracture
Change in clinical risk factors e.g. • Patient now has recurrent falls • Began GCs • Developed diabetes	Indicator of high risk of fracture
Bone Turnover Markers (optional)	Though no strong data on best use in drug holidays, BTMs in premenopausal range suggest patient still covered by rx given in the past
Other	Review any new pharmaceutical and non-pharmaceutical options that have become available or have become relevant due to change in the health or daily functioning of the patient.

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DRUG HOLIDAYS

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Reversal Phase in Bone Remodeling

Jean-Marie Delaisse, Ph.D.

Saturday, September 29

11:00 am – 12:00 pm

Room 519 A

Meet-the-Professor session – ASBMR 2018
Jean-Marie Delaisse, University of Southern Denmark, Denmark

Reversal Phase in Bone Remodeling

Significance

Bone remodeling replaces existing bone matrix by new bone matrix. Malfunction of the remodeling process leads to bone loss and increased fracture risk.
Remodeling involves (i) local cell teams (called BMUs) which consist of osteoclasts resorbing the bone and osteoblasts re-forming the bone, and (ii) a mechanism coupling resorption and formation.
During many years, the main research focus has been on resorption and formation. However, coupling is more and more regarded as a major component of the remodeling cycle as it is obligatory for preserving bone architecture and strength throughout life. Importantly, understanding coupling requires attention for the biological events occurring between resorption and initiation of bone formation at a remodeling site. These events are commonly defined as the **reversal phase**. What are these events? How do they contribute to reverse resorption to formation? We start understanding this mechanism (1-4). A failure at this level may significantly contribute to bone loss(1;2;5;6).

Learning objectives

Basic questions concerning the role played by the reversal phase in the mechanism of the bone remodeling cycle, remained unresolved for more than 30 years(2). As a result of participating in this session, attendees should receive an answer to these questions (see below). They should understand the importance of the reversal phase in relation with bone loss – and its implications when considering histomorphometric assessments and treatment strategies.
More broadly, attendees should become aware of the need of "functional histology" in order to fully understand biological processes such as bone remodeling. It is not enough to identify the elements that a machine needs to work. Full understanding of function requires knowledge of the spatiotemporal relationship between these elements (engineers have to draw plans of their machines…).

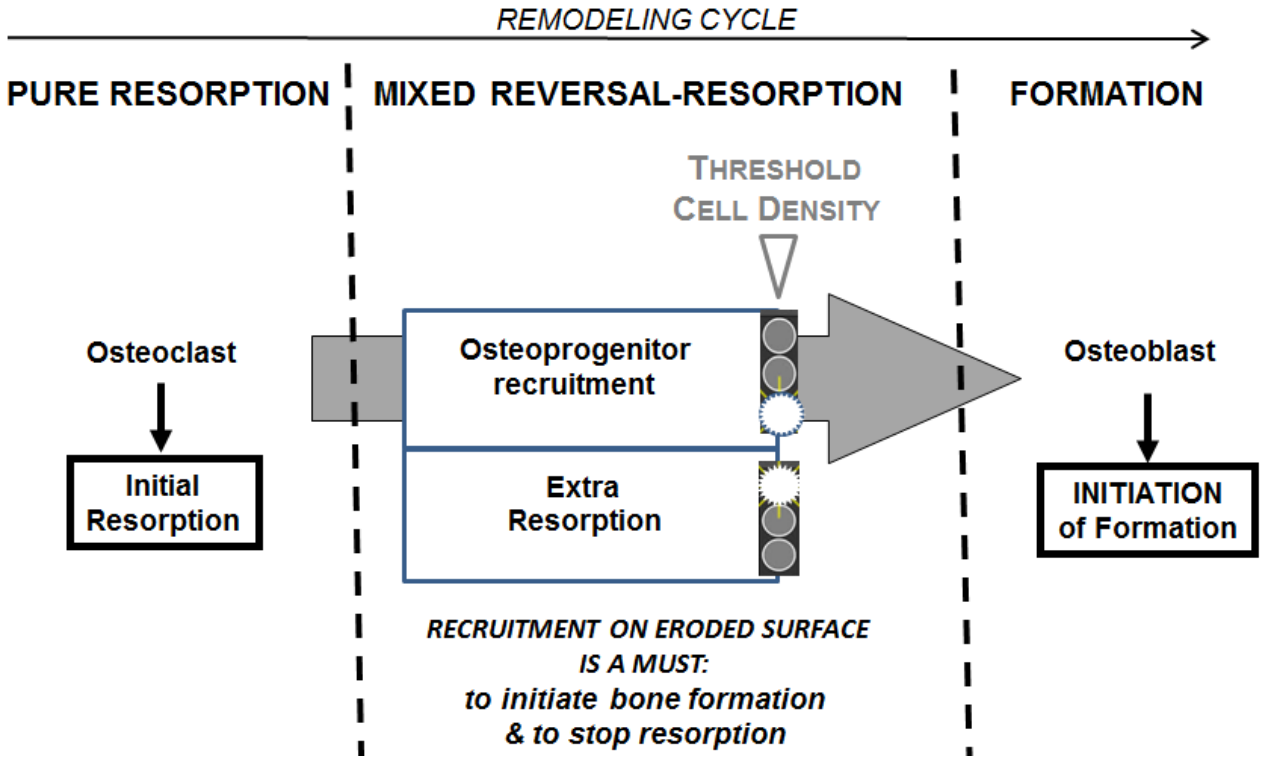
Comparing the common view on the reversal phase with the new one.

Common view	New view
Bone remodeling is essentially seen as a two-step process: bone resorption by osteoclasts and bone formation by osteoblasts.	How sure is it that bone loss originates only from a failure at the level of the resorption or formation phase(1;5;6)? The "reversal phase" is gaining attention(3): it is the step where it is decided that the bone resorbed at a given site should either undergo further resorption, or be left unreconstructed, or be replaced by new bone formation. Ongoing research investigates the determinants of this decision, at the level of coupling molecules(7) and of the dynamics of osteoclast and osteoblast lineage cell populations on the bone surfaces(4;5;8;9).
Accordingly, bone resorption and formation have been the main focus of the research aiming at prevent bone loss and thereby reducing fracture risk.	
The "reversal phase" is usually defined as the "transition" between resorption and formation (without clear functional content).	The "reversal phase" is the "coupling phase". The reversal cells are actually **osteoprogenitors** colonizing the eroded surfaces as soon as the osteoclast has moved away(1;4;5;8;9). Their cell density increases up to a threshold permissive for bone formation(4). Formation is not initiated in situations where the threshold is not reached(1;5;6). Recruitment of osteoprogenitors on eroded surfaces is thus an essential activity involved in coupling resorption and formation.
This transition corresponds with the appearance of mononucleated cells on the eroded surface(10;11). They are described as reversal cells on reversal surfaces. Their nature and role within the remodeling cycle were unknown until recently.	

Common view (continued)	New view (continued)
The current model of the remodeling unit is presented as composed of 3 single successive periods aligned according to their theoretical sequence: pure resorption, pure reversal, and formation(12). However, this is only a model and not a real picture of a remodeling site, since standard histological sections do not hit remodeling sites along their operational axis and cannot capture the remodeling events as a continuum.	Appropriate sections show that a remodeling unit involves repeated alternations of resorption and reversal before formation starts(4). This leads to the concept of a **mixed** "**reversal-resorption**" phase occurring between the initial bone resorption episode and initiation of bone formation. Osteoclastic resorption and osteoprogenitor recruitment appear thus as an intimately integrated process.
This current model does not allow understanding how the putative osteogenic signals released during resorption may affect distant/later bone formation sites(7).	This integration opens the way for understanding how osteoblast lineage cells are exposed to the osteogenic signals released by the osteoclasts, thereby leading to maturation of bone forming osteoblasts.
Bone loss is commonly ascribed to insufficient bone formation on eroded surfaces. Hence much attention is given to measurement of bone formation levels – but of note, these levels are assessed at bone formation sites: i.e. where bone formation has started, and not taking into account complete absence of bone formation(1;5). Common analyses thus consider only possible failures at bone formation sites and overlook a possible failure of the reversal phase, such as prevention of initiation of formation(1;5).	Bone loss may also arise from complete absence of bone formation in some remodeling units(1;5). This occurs when not enough osteoprogenitors are recruited on eroded surfaces(1;4-6). This is then a failure of the reversal phase – not of the formation phase itself. Note that lack of recruitment also leads to a risk of new resorption episodes, as resorption may occur as long as bone formation has not started(4). =>Osteoprogenitor recruitment on eroded surfaces is of interest in histomorphometric assessments: it directly relates to bone formation.
The immediate source of osteoprogenitors is ascribed (i) to the layer of elongated cells lining the mature osteoblasts at bone formation sites and (ii) to the bone lining cells of quiescent surfaces(1;13). However, source "i" (and its proliferation rate) is not abundant enough to build up the threshold cell density on eroded surfaces, as required for initiation of bone formation, whereas source "ii" delivers osteoprogenitors at bone formation sites(1;13).	The layer of osteoprogenitors at the osteoblast-bone marrow interface forms a continuum with cells covering the whole remodeling site, thereby generating a "canopy" (= part of the bone marrow envelope)(1;13;14). Proliferation in this canopy allows delivery of osteoprogenitors not only to bone forming surfaces but also to eroded surfaces(1;13). This "canopy-source" of osteoprogenitors complements the "bone lining cell source". This double source of recruitment on eroded surfaces makes it possible to reach the threshold cell density that is necessary to initiate bone formation(1;6;13;14). =>Analysis of the bone marrow close to the bone marrow along the bone surface is of interest in histomorphometric assessments of bone remodeling.

The new view supports a model where a mixed reversal-resorption phase drives a mechanism that links osteoprogenitor recruitment and the resorption-formation switch: the faster osteoprogenitors are recruited in a remodeling unit, the faster bone formation is initiated, and the faster bone resorption stops – and conversely(4).
Targeting specifically osteoprogenitor recruitment appears an interesting approach to prevent bone loss (especially in situations like aging).

Cartoon showing the critical events occurring between initiation of resorption and initiation of formation during bone remodeling. The identification of these events has clarified how resorption is reversed to formation thereby inducing "coupling".



Issues of special interest for discussion

Methodological considerations

Much of the upcoming view is due (i) to the use of markers revealing relevant features and specific cell activities in histological sections; (ii) to the attention for tissue areas that are usually not taken into consideration (including the bone marrow neighbouring the bone surfaces); (iii) to the choice of histological sections that are relevant to the questions to be answered.

For example, if the question is the sequence of events during the reversal phase(4), one should be aware that standard histological sections hit randomly remodeling events occurring in distinct BMUs. Thus they are not appropriate for showing the sequence of events occurring in a BMU. Instead, one should take advantage of the known orientation of the operational axis of the remodeling events in cortical bone, and make sections along this axis: it is then possible to capture in a continuum the whole range of events occurring between the initial resorption episode up to the initiation of bone formation.

If the question is the relation between bone formation and reversal phase status and canopy status(1), one can learn from comparing pathophysiological situations where bone formation is differently affected compared with healthy controls: such as hyperparathyroidism, osteoporosis (induced by age, menopause, glucocorticoids), multiple myeloma, …

Interest of cortical bone vs. cancellous bone to identify which biological activities determine bone loss

Identifying which biological activities determine bone loss is a key objective of bone research. An obvious approach is to analyze the local association between bone loss and biological activities. This association cannot be analyzed in a strict way in cancellous bone because lost bone is not visible any longer[1]. In contrast it can be analyzed in a strict way in cortical bone, where local bone loss results in empty spaces

[1] However, "average" assessments of reversal surfaces in situations where bone formation is well-known to be deficient (aging, unloading, periodontitis, glucocorticoid- and menopausal-induced osteoporosis) led to the hypothesis that remodeling cycles may abort during the reversal phase (1).

(pores) whose size can be measured, and where the critical biological activities can be identified on their walls(15). Cortical bone is thus top research-material to investigate how the bone remodeling process impacts on the bone matrix(15).

A new view of the spatiotemporal dynamics of the osteoblast recruitment?
The recent observations support that bone forming osteoblasts originate from "local" osteoprogenitors, and that the latter are triggered to differentiate into mature osteoblasts upon passage of a resorbing osteoclast on the bone surface. According to this view, osteoclasts are the main traveling cells(16) that meet (i) local bone lining cells (retracting upon arrival of the osteoclast and spreading over the eroded surface after its passage) and (ii) local bone marrow envelope/canopy cells (lifted upon arrival of the osteoclast, proliferating, and delivering osteoprogenitors to the eroded surface)(1).
Of note, the osteoprogenitors/reversal cells on the eroded surfaces represent by definition a heterogenous cell population on the way of differentiation and involved in diverse reversal tasks (including cleaning of resorption lacunae(8;9;17)). Accordingly, the reversal cells next to osteoclasts show different markers compared to those sitting on eroded surfaces next to osteoid(1;5;8).

Possible involvement of other physiological entities in the reversal phase mechanism?
- Involvement of vasculature in osteoprogenitor recruitment/osteoblastogenesis at the level of eroded surfaces? Assessment of the presence of capillaries along bone surfaces show the highest values at eroded surfaces, and in close association with canopies(18). These regions of convergence coincide with a higher prevalence of proliferation and markers of osteoblastogenesis. These observations support the possible contribution of vasculature in the reversal phase activities.
- Involvement of the newly generated epitopes on the eroded surface/cement line (compared with the quiescent surfaces)? For example, collagen and fibronectin were shown to be strongly haptotactic for osteoblast lineage cells(17).
- Involvement of osteocytes? The osteogenic effects of cardiotrophin and LIF originating from osteoclasts were proposed to be mediated by downregulation of osteocytic schlerostin(7;19).
- Involvement of neurons? There is an increased presence of nerve profiles at remodeling sites(20)

Questions to be addressed in relation with the clinic
- Effect of treatment on the reversal phase, i.e. at the level of the eroded surfaces and the associated canopy: effect of bisphosphonates(21)? PTH(22)? anti-sclerostin antibody?
- Effect of aging on the reversal phase? (observations obtained so far indicate a prolonged reversal-resorption phase (1;15) as well as impoverishment in canopies(13) with aging. Possible involvement of cell senescence-associated processes(23)?…

Models mimicking human bone remodeling and the reversal phase?

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miRNAs and Bone

Anne Delaney, Ph.D.

Saturday, September 29

11:00 am – 12:00 pm

Room 525

ASBMR Meet the Professor Session: miRNAs and Bone

Saturday September 29, 2018
Anne M. Delany, PhD
Center for Molecular Oncology, UConn Health, Farmington CT

Significance of the Topic:

miRNAs are key post-transcriptional regulators of gene expression. Their importance in controlling the differentiation and function of skeletal cells is now appreciated. However, the complexities and subtly of miRNA-mediated gene regulation can make it challenging to study. In this session, we will present information on miRNA biogenesis and function, and discuss some trending research questions and strategies for understanding the role and regulation of miRNAs in the skeleton.

Learning Objectives:

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

- Appreciate the modular nature of miRNA-target interactions and function
- Appreciate some of the factors regulating miRNA sorting and transfer via exosomes
- Learn some strategies for predicting and studying miRNA-target interactions
- Learn some approaches for studying miRNA function in vivo

Outline:

I. **miRNA biogenesis**
   i. potentially complex genomic organization
   ii. processing steps in nucleus and cytoplasm represent potential for regulation

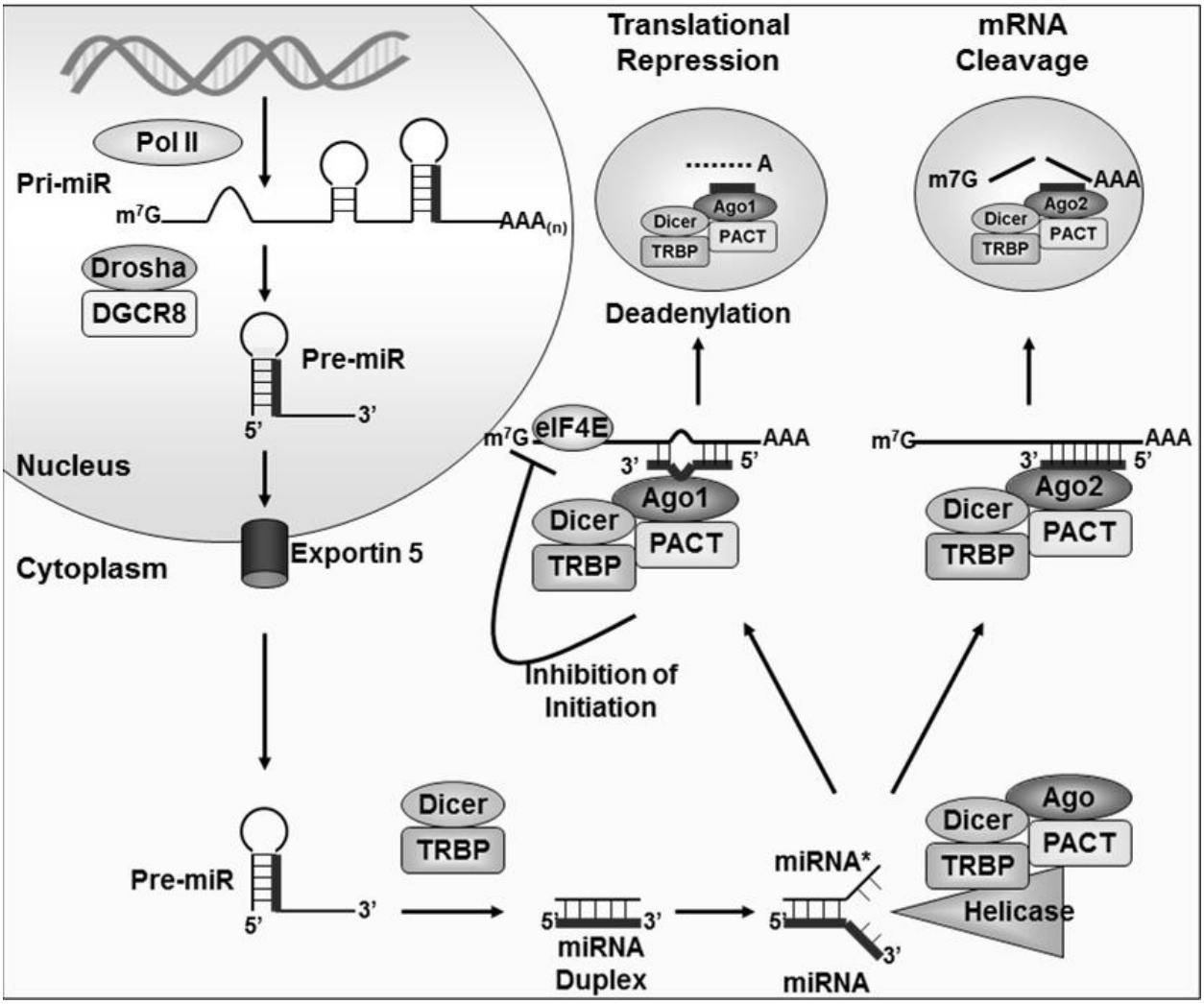
II. **miRNA function**
   i. translational repression and deadenylation
   ii. miRNAs have many mRNA targets; mRNAs are targeted by multiple miRNA
   iii. mRNA isoforms

III. **miRNA-target prediction and validation**
   i. free websites
   ii. non-biased approaches
   iii. target validation

IV. **miRNA transfer via exosomes**
   i. miRNA sorting into exosomes - not random
   ii. factors regulating exosome content

V. **Animal models**
   i. Genome modification
   ii. Ectopic bone formation assay
   iii. Systemic administration vs targeted delivery
   iv. Biomaterial-mediated delivery

miRNA Biogenesis



Kapinas & Delany, Arthritis Res Therapy. 13(3):220, 2011

Predicting miRNA-target interactions

Potentially effective miRNA binding sites:

- **Good seed match (miRNA bases 2-8)**
- **Conservation**
- **Complementarity at other miRNA regions, especially miRNA bases 13, 14 or 18, 19**
- **Near proximal or distal end of 3' UTR**
- **Flanking regions rich in A or U**
- **Multiple sites**
- **Site not involved in secondary structure**

miRNA-target prediction tools

Site		Features
Pictar		Predictions based primarily on evolutionary conservation
TargetScan		
miRanda		Support vector regression (SVR) takes into account miRNA and target features (including site accessibility, conservation)
PITA		Energy of miRNA-target site interaction, site accessibility
RNAhybrid		
Diana Tools	Micro-CDS	Trained on positive and negative sets of miRNA Recognition Elements (MREs) located in both the 3'-UTR and CDS regions.
	TarBase	A manually curated target database. Includes targets from high throughput experiments, such as microarrays, proteomics, and sequencing (HITS-CLIP and PAR-CLIP) experiments.
	miR-Path	Performs miRNA pathway analysis. Can utilize predicted miRNA targets and/or experimentally validated miRNA interactions
	Diana-mirExTra	Estimates miRNA effects on expression protein-coding RNAs based on the frequency of hexamers in the 3'UTR sequences of genes.

Some articles of interest:

A nice review of miRNA molecular mechanisms:
Gebert LFR, MacRae IJ. **Regulation of miRNA function in mammals**. Nat Rev Mol Cell Biol. 2018 Aug 14.

Example of how miRNA content in vesicles changes with differentiation state:
Lin Z, McClure MJ, Zhao J, Ramey AN, Asmussen N, Hyzy SL, Schwartz Z, Boyan BD **MicroRNA contents in matrix vesicles produced by growth plate chondrocytes are cell maturation dependent**. Sci Rep. 2018 Feb 26;8(1):3609.

A recent review on miRNAs and bone:
Gennari L, Bianciardi S, Merlotti D. **MicroRNAs in bone diseases**. Osteoporos Int. 2017 Apr;28(4):1191-1213.

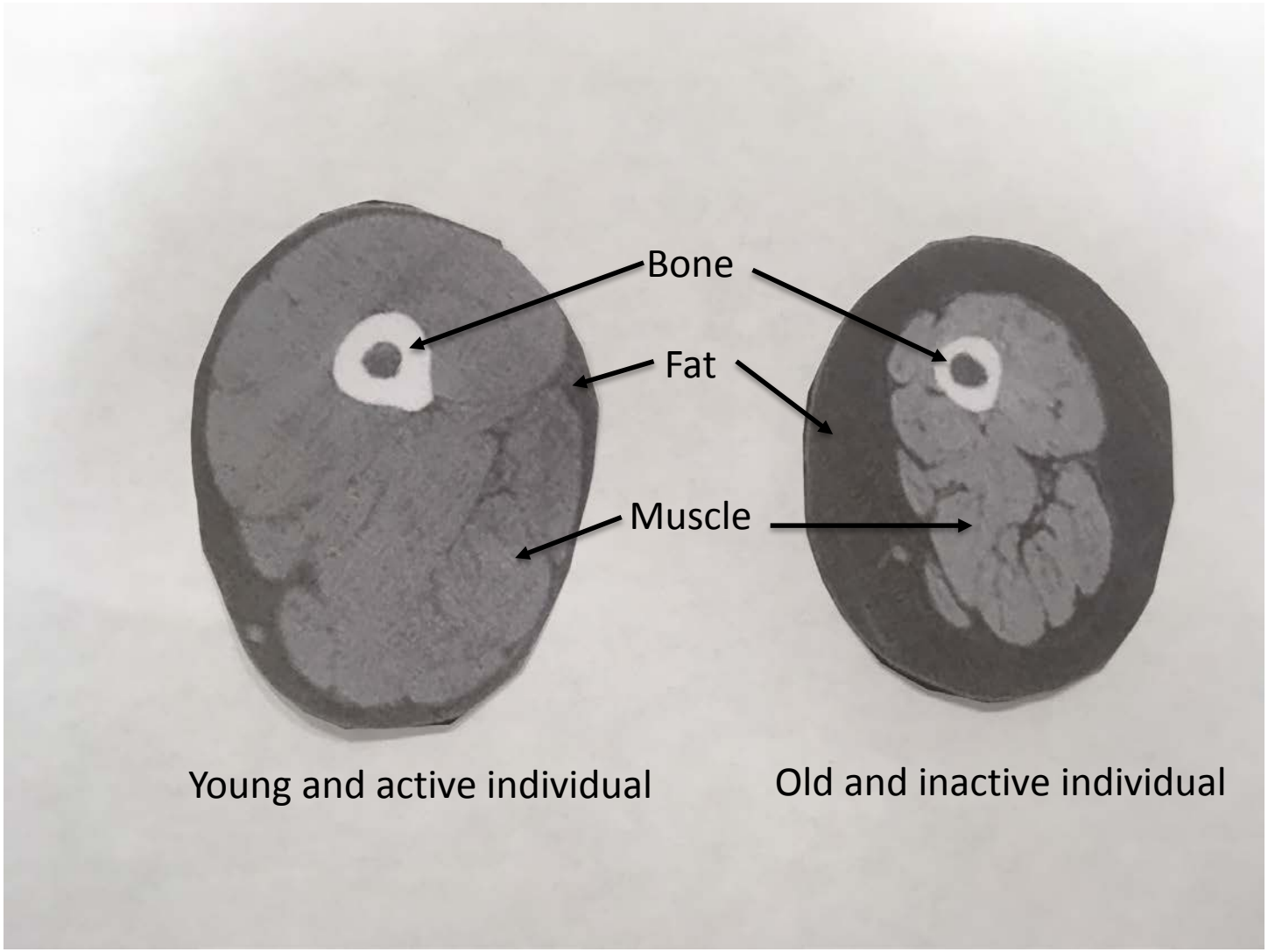
Bone Muscle Interactions

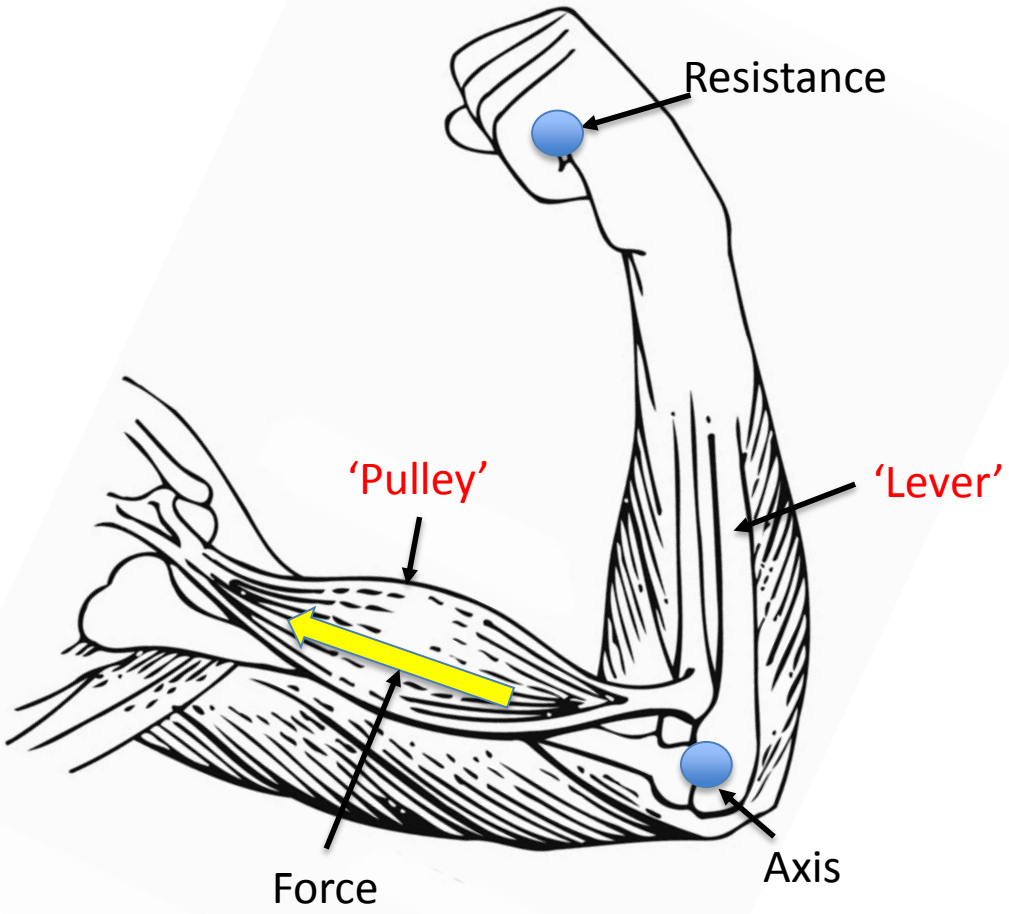
Lynda Bonewald, Ph.D.

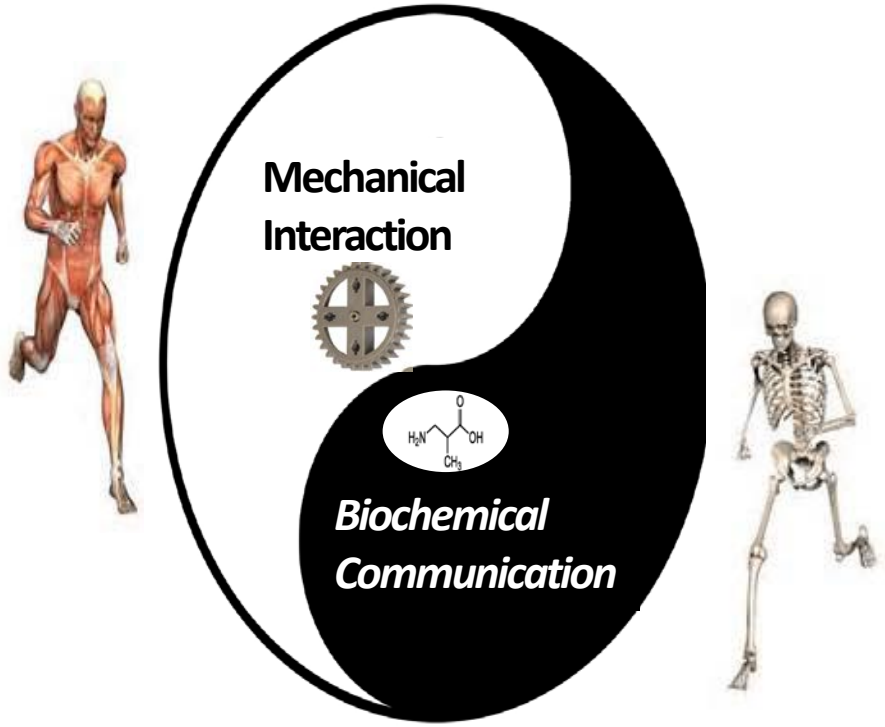
September 29

11:00 am – 12:00 pm

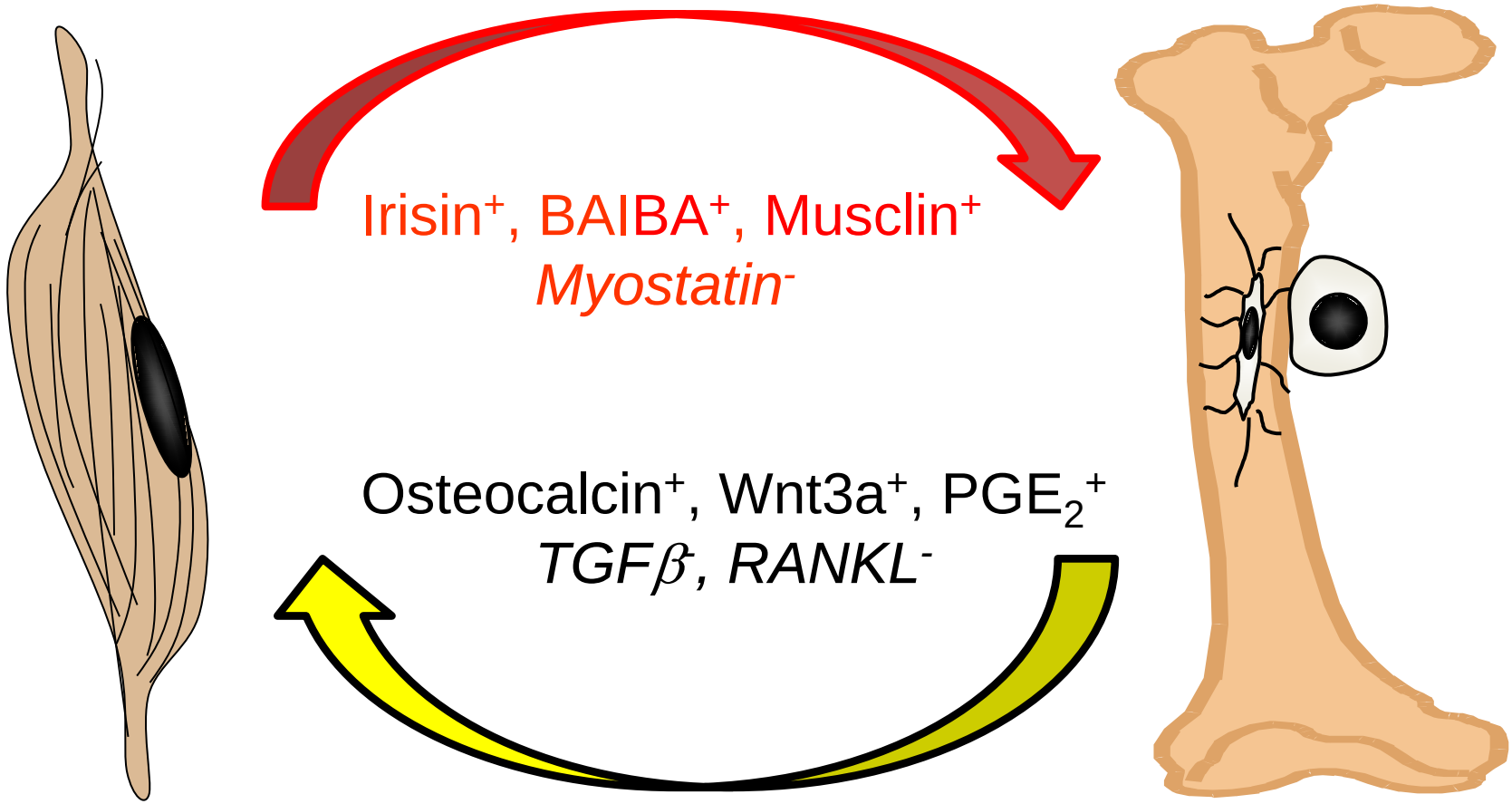
Room 522


Bone
Fat
Muscle
Young and active individual
Old and inactive individual


Resistance
'Pulley'
'Lever'
Force
Axis


Mechanical
Interaction
Biochemical
Communication

Factors Involved in Muscle-Bone Crosstalk



? Effects of Exercise, Age, Stress, Circadian Rhythm?

Mineral Balance and Tracer Methodologies in Clinical Research on Nutrition and Bone Health

Kathleen Hill Gallant, Ph.D.

Sunday, September 30

11:00 am – 12:00 pm

Room 518 C

Meet-the-Professor Session: Mineral Balance and Tracer Methodologies in Clinical Research on Nutrition in Bone Health

Speaker: Kathleen M. Hill Gallant, PhD, RD, Purdue University, West Lafayette, IN, USA
hillgallant@purdue.edu

Date/Time: Sunday, September 30th, 2018, 11:00AM, Montréal, Québec

Significance of the Topic

Good nutrition is undoubtedly important to bone health, particularly in critical stages of the life course, such as adolescent growth. However, clinical nutrition research studies often struggle to show efficacy or large effect sizes in randomized controlled trials of nutrients or dietary interventions on BMD or fractures. The potential reasons for this are many, but include 1) the reductionist nature of most RCTs to investigate single nutrients rather than whole diets, 2) the lifetime of exposures to nutritional factors that likely contribute to bone health that can't easily be captured in a well-controlled RCT, 3) the influence of background dietary intake causing noise that decreases the ability to detect effects of interventions during the RCT period, and 4) the limited ability to accurately assess nutrient intakes of study participants. The latter two issues can be overcome by controlled feeding studies. Controlled feeding studies allow for a known nutrient exposure and controlled environment and confounders, but immediately restrict the study duration that is realistically feasible. This precludes the use of clinical endpoints like fractures or even change in bone mineral density that would require longer duration studies to see effects resultant of the interventions. Whole-body mineral balance and isotopic tracer modeling (particularly of calcium) provide alternative outcomes related to bone and mineral metabolism that can be employed in these shorter-term controlled feeding studies. Additionally, kinetic modeling of tracer data can give unique information on pathways of calcium or phosphorus movement between body pools (e.g. intestinal absorption rate and transfer rates to and from bone).

Learning Objectives

After participating in this session, attendees should be able to:

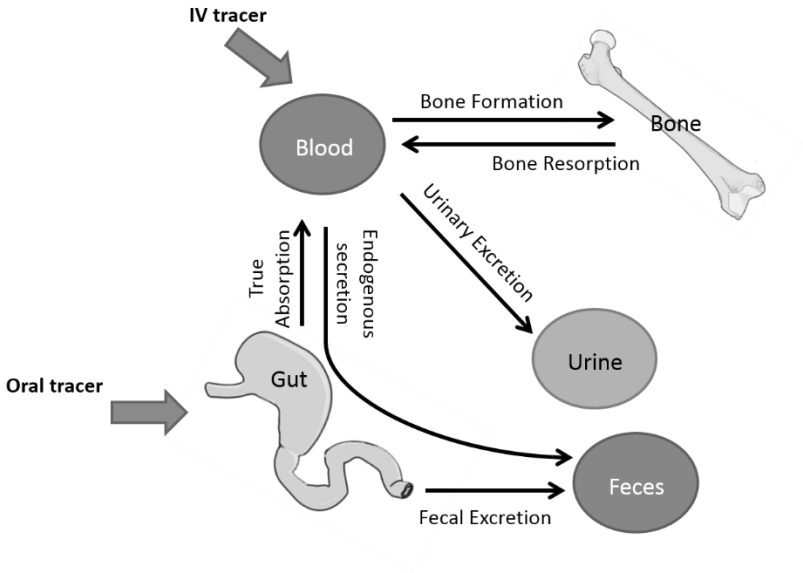
- Identify key characteristics of well-designed calcium and phosphorus balance studies.
- Identify strengths and limitations to the balance study approach in bone and mineral research.
- Describe how isotopic tracers can be used to enhance balance studies for more sophisticated data analyses and outcomes.
- Describe examples of clinical research applications for classic balance studies and kinetics in bone and mineral research, including some specific examples of balance and kinetics studies that have produced significant knowledge related to mineral nutrition.

Outline/Points of Interest

- **Overview of Balance Study Methodology**
    - Classic metabolic balance studies measure total inputs minus total outputs to give a whole-body picture of retention or deficit, typically expressed as a rate of mass retained or loss per day.
    - Well-designed balance studies include a controlled diet that has been 1) analyzed for accurate nutrient content (that is consistent from day-to-day during the study),

2) is prepared with precision (i.e. individual ingredients weighed during preparation), and 3) consumed completely by participants.
    - An adequately long run-in period on the study diet is needed prior to the formal balance study. This equilibration period is to ensure that subjects are in "steady-state" prior to the balance measurements. Regarding balance studies, steady-state means that daily inputs and outputs there are constant in the body system.
    - To ensure complete consumption of the study diet, complete collection of excreta, and to minimize chance of consumption of non-study foods and beverages, and even to control level of physical activity, an inpatient environment is necessary. Outpatient studies are possible with the use of meal pack-outs and home urine and fecal collections, but control over these factors is reduced, resulting in greater errors.
    - Urine and fecal collections should be complete and accurately recorded for time and volume (or weight) of the collection. Accurate timing and pooling of 24-hour urine collections is essential and is enhanced in an inpatient setting where study staff can oversee this process.
    - Compliance indicators are used in well-designed balance studies and include:
        - Weigh-back and chemical analysis of leftovers (if any)
        - Pill counts of any supplements given as part of the study (e.g. calcium supplements)
        - Fecal markers such as the minimally-absorbed polyethylene glycol m.w. 3350 (PEG 3350) can be used for several purposes: % fecal recover of PEG gives information on fecal collection compliance, fecal Ca:PEG or P:PEG ratios can be used to demonstrate steady state (i.e. steady ratios day-to-day), or PEG can be used to adjust fecal mineral measurements (e.g. if 3 g/d PEG are given, then fecal daily fecal Ca output could be adjusted for 3g of fecal PEG output once steady state is achieved). *(1)*
        - Urinary creatinine excretion should be relatively constant day-to-day based on the muscle turnover and renal function remaining constant day-to-day during the balance period. Thus, it can be used to show urine collection compliance and to adjust daily urine values to an average daily creatinine excretion determined over the course of the balance period.
- **Calcium and Phosphorus Balance and Kinetics** *(1)*
    - Calcium balance and kinetics presents a particularly useful tool in the bone research field due to the distribution of whole-body calcium with approximately 99% of the body's calcium residing in bone.
        - Whole-body calcium balance (rate of retention or loss per day) can be translated into estimates of predicted bone gains or losses based on assuming a relatively constant percentage of bone mineral content as calcium (32.2%) *(2)*
            - e.g. Calcium balance data from studies conducted in adolescent boys *(3)* and girls *(4)* closely aligns with the rate of bone accrual observed from longitudinal DXA measurements in adolescents*(5)*.
                - These comparisons support the ability of short-term balance studies to predict long-term skeletal calcium gains, at least during adolescent growth *(6)*.

- Calcium balance studies can be augmented with the use of isotopic tracers for modeling calcium kinetics.
    - Calcium kinetic modeling gives rates of transfer between pools, including fractional absorption, endogenous fecal calcium excretion, bone formation, resorption, and bone balance.



K.M. Hill Gallant, 2018

- Foods, beverages, and supplements can be labeled with an isotopic tracer by intrinsic or extrinsic methods. Intrinsic labeling refers to the isotope being incorporated into the plant or animal source as it is growing or when a supplement is being synthesized; extrinsic labeling refers to adding the isotope to the food/beverage/supplement in a form that is thoroughly mixed. It relies on the assumption that the tracer exchanges with the endogenous calcium (or whatever the substance being traced). Several applications of extrinsic calcium isotope labeling have been validated against intrinsic labeling techniques, including for milk *(7)* and wheat flour used to make bread *(8)*.
- Bone turnover by Ca-45 kinetic modeling has been cross-validated against dynamic histomorphometry *(9)*. This supports the validity of using calcium kinetic studies for assessing bone turnover at the whole-skeleton.
- There are many useful calcium isotope options, both stable and radioactive. This allows for more versatile applications:

Ca Isotopes	**^{42}Ca, ^{44}Ca, ^{46}Ca**	**^{45}Ca**	**^{47}Ca**	**^{41}Ca**
Type of energy emitter	Stable	Low energy β-	High energy β-, γ	Electron Capture
Half-Lives	N/A	163 d	4.5 d	100,000 y
Tracer lifetime for measurements	Weeks	Months	Months	Many years→ a lifetime
Health risk	None	Radioactivity exposure	Radioactivity exposure	Negligible
Cost of dose	$$$	$	$	$
Cost of analysis	$$	$	$	$$$
Applications	Full kinetic modeling; calcium absorption studies; particularly useful for studies in children*(10)*	Full kinetic modeling; calcium absorption studies;	Whole-body calcium retention	Deep labeling of bone; whole-bone calcium retention, net bone turnover

- Calcium-41 is unique in that it is a rare isotope that can be used to "deep label" bone. After an equilibration period of 150 days, urine ^{41}Ca:^{40}Ca ratio can be measured to indicate response in net bone turnover to a treatment. Because an individual's bone is then labeled for life with the isotope, multiple treatments can be studied on the same subjects. A recent review of this methodology has been published (11).

- Unlike calcium balance and kinetics, phosphorus balance and kinetics are not proxies for bone balance or turnover. This is because the distribution of body phosphorus in bone, while high at 85%, is not like the near complete (~99%) distribution of body calcium in bone. Still, strong relationships are expected between bone metabolism and phosphorus retention and kinetics.

- Also, unlike calcium, full phosphorus kinetic modeling has not been done, so rates of transfer between body pools, at present, are only estimated and many knowledge gaps exist.

- Phosphorus does not enjoy the variety of isotopes that calcium provides. There are essentially two useful phosphorus radioisotopes, and no stable isotopes beyond the near 100% naturally abundant ^{31}P.
  - ^{32}P is a high-energy β- emitter with a half-life of 14.3 days. Due to its high energy, its use in humans has been very limited.
  - ^{33}P is a low-energy β- emitter with a half-life of 25.3 days. Useful for phosphorus tracers studies in humans due to its low energy and longer half-life compared with ^{32}P.

- **Clinical Research Application Examples**
  - The following examples of how balance and kinetics studies have been used in clinical research to advance understanding of mineral nutrition and bone health.
  - **Adolescent Dietary Calcium Requirements**
    - Calcium balance studies in adolescents provided calcium intakes for maximal calcium retention that became the basis of the calcium RDA for this age group *(4, 6).*
  - **Effects of Dietary Protein on Bone Calcium**
    - Studies have used calcium kinetic modeling *(12)* and whole-body ^{47}Ca gamma-counting to demonstrate that high protein diets in the presence of adequate calcium do not risk bone calcium loss, but instead promote greater intestinal calcium absorption *(13, 14)*
  - **Ca-41 Technology for Rapid Screening of Bone Turnover Effects** *(11)*
    - Calcium-41 was used in post-menopausal women to compare the antiresportive effects of various phytoestrogen sources with those of risendronate and estrogen – in total, 6 interventions were tested in each *subject (15).*
  - **CKD Calcium and Phosphorus Balance Studies**
    - Patients with chronic kidney disease (CKD) have disordered bone and mineral metabolism which leads to high risk of vascular calcification as

well as bone fragility fractures. Calcium and phosphorus balance and isotopic tracer studies can give valuable information on whole-body calcium and phosphorus physiology the is unattainable by other methods *(16).*

- Calcium and phosphorus balance studies including full calcium kinetic modeling (17) have demonstrated that patients with moderate-stage CKD have, on average, neutral calcium balance at a calcium intake of around 1000 mg/d, but go into high calcium retention when calcium intake is increased to 2500 mg/d, but with no change in phosphorus retention. Additionally, kinetic data show that these patients still have relatively normal intestinal calcium absorption at this stage of disease. However, these studies also show a high degree of variability in calcium and phosphorus retention in patients within a relatively narrow range of kidney function and on the same controlled diets.
- A secondary analysis of the phosphorus balance data uncovered that 24-hour urine phosphorus, which has long been considered a proxy for phosphorus absorption, is highly variable in these patients even on a controlled intake, and that it is not related to net phosphorus absorption, but instead inversely related to whole-body phosphorus retention *(18).* This demonstrates the need for phosphorus balance and kinetic studies for better measurement of phosphorus absorption in this disease state.

- **Strengths and Limitations of Use of Balance and Kinetic Studies in Mineral Nutrition Research**
  - Strengths
    - Well-controlled/defined exposure (nutrient/diet) → very useful for nutrition research
    - Proxy outcomes related to bone → e.g. calcium retention related to bone mass/accrual, bone turnover from calcium kinetics related to histomorphometry measures
    - Whole-body balance of calcium or phosphorus gives information that plasma Ca or P can't
    - Can detect changes in balance and kinetics in response to an intervention in shorter duration studies, in contrast to the longer studies needed for changes in BMD.
    - Modeling of isotopic tracers can give specific components of calcium or phosphorus metabolism unattainable by other methods (e.g. rate of endogenous calcium excretion into the intestine)

  - Limitations
    - Expensive and labor intensive
      - Typically limits sample size feasible
      - Study duration is limited. Ca-41 can be use in longer-term studies, but not feasible to achieve controlled diet for long-term.
        - Thus, "lifetime exposure" effects of nutrients elude this methodology

- Balance and kinetic studies *tend* to also be reductionist for the sake of controlled design. But, it is still possible to test effects of whole diets/patterns.
- Radioactivity risk, associated environmental controls
- Expertise in kinetic modeling needed (but simplified methods have been published, e.g. *(19))*

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Risk Prediction Models

Lisa Langsetmo, Ph.D.

Sunday, September 30

11:00 am – 12:00 pm

Room 525

Assessing Performance, Validity, and Accuracy of Fracture Prediction Tools

Significance of the Topic

Management of our patients' fracture risk boils down to predicting and preventing these events. Hence, fracture prediction models have become essential research and clinical management tools in our field. Clinicians use them for prediction of fracture risk for individual patients, and researchers use them for prediction in populations. For example, the value of new diagnostic tests of bone mass, microarchitecture, or quality will depend in large part on how well they improve prediction of fractures in clinically relevant populations. However, the value of fracture prediction models depends on their accuracy and validity.

The primary goal of this session is to provide and discuss a practical checklist by which clinicians, journal article reviewers, and clinical researchers who are not statisticians can evaluate the performance characteristics of a prediction model. This is NOT intended to be a state of art exposition on advances in the statistics of prediction models.

Learning Objectives

- Understand the basic criteria of good prediction model performance
- Understand prediction model calibration and discrimination and the difference between them
- Understand the bias that may occur from the competing risk of mortality
- Be able to apply a checklist to judge whether a fracture prediction model may be useful clinically, or whether a journal article's claims about a fracture prediction model are likely to be true.

Checklist for Evaluation of Prediction Models

1. What is the sample size and demographic characteristics of the population in which the prediction model was developed?

2. How many outcome events (fractures) occurred in this population?

3. Initial selection of candidate predictor variables
    a. By what criteria were predictor variables considered as candidates for the model?
    b. How many predictor variables were considered (e.g., tested) in the prediction model?

4. What statistical model techniques (e.g., logistic, proportional hazards, Poisson) were chosen, and what was the rationale for the choice?
    a. Were appropriate post-regression diagnostic tests done to be sure that the models were well specified?

5. How were missing values handled?
    a. Were study participants dropped if they had missing values?

b. If missing values were imputed, was the method of imputation described and referenced?

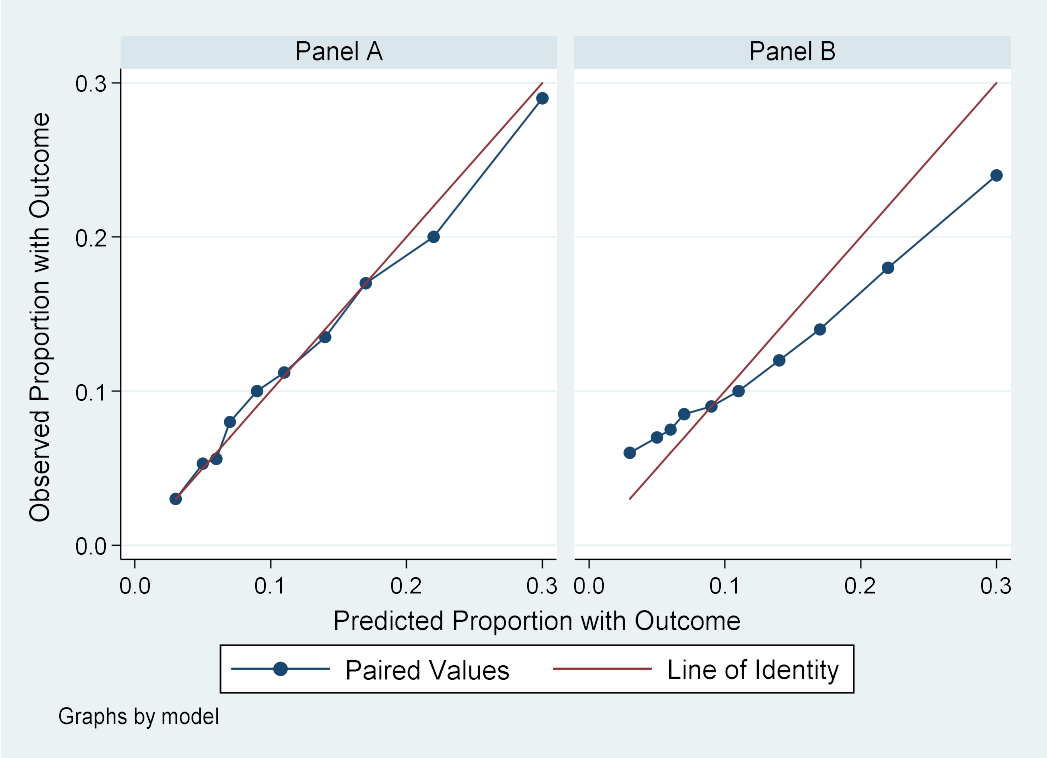
6. Model calibration; How well do observed actual fracture probabilities agree with predicted fracture probabilities from the model and has calibration been evaluated across the spectrum of fracture risk?

7. Model discrimination; how well does the model discriminate those who will from those who will not have a fracture?
    a. What statistic was used to assess model discrimination?
    b. If the discrimination of two models are being compared, was this performed using appropriate statistical methods?

8. Model validation
    a. How was internal validation done?
    b. Has the model's performance been tested in different populations than the one in which it was developed by independent investigators?

Outline of Presentation

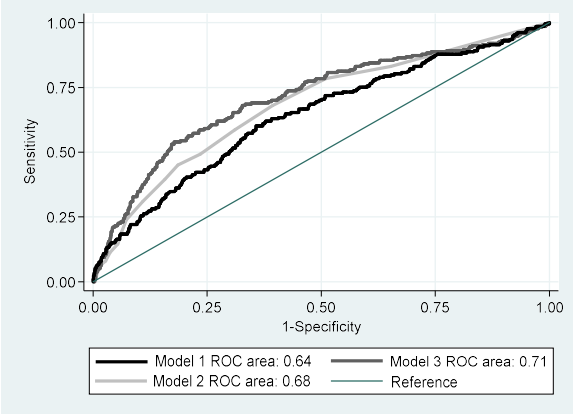
A. Potential sources of bias leading to overestimation of how well a model predicts fractures.
B. Calibration Example



C. Fracture prediction model discrimination

1. Discrimination slope and Integrated Discrimination Index

2. Receiver Operating Characteristics (ROC) Curves



3. Example of Categorical Net Reclassification Index (Hypothetical)

Fracture Cases				
		Model B Prediction		
		No Fracture	**Fracture**	**Totals**
Model A Prediction	**No Fracture**	30	7	38
	Fracture	3	110	112
	Totals	32	118	150
Fracture Non-Cases				
		Model B Prediction		
Model A Prediction	**No Fracture**	672	6	678
	Fracture	92	80	172
	Totals	764	86	850

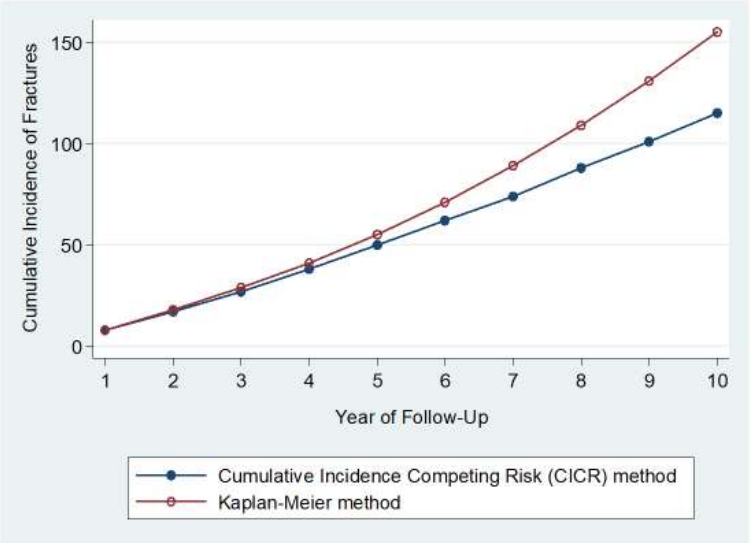
*NRI for Cases: (7-3)/150 = 0.027;

p-value calculation: z=0.027 /sqrt[(7/150)+(3/150)/150]; p-value=0.09*(27)*

^NRI for Non-Cases: (92-6)/850 = 0.101;

p-value calculation: z=0.10 /sqrt[(92/850)+(6/850)/850]; p-value<0.001*(27)*

D. Competing risk of mortality (and other outcomes)



Year	1	2	3	4	5	6	7	8	9	10
Original Total	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
Survivors	1000	956	910	862	813	764	714	665	616	568
No. Fractures	8	9	10	11	11	12	13	13	13	14
CICR Rate*	8	9	10	11	11	12	13	13	13	14
K-M Rate^	8	10	11	12	14	16	18	20	22	24

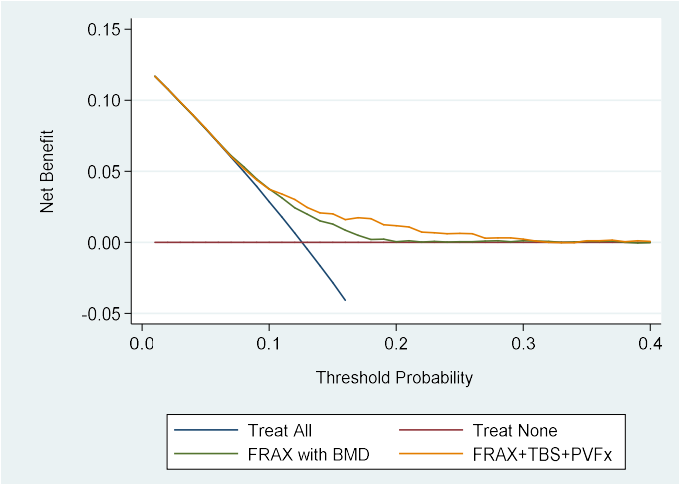
E. Decision Curve Analysis

Net Benefit: Trade-off of true positives and false positives

Formula: Net Benefit =[#True Positives – #False Positives*(tp/(1-tp)] / N,

Where tp is threshold probability, N is total number of sample

Decision Curve is plot of Net Benefit over range of threshold probabilities



Example from MrOS: prediction of major osteoporotic fracture for men with Femoral Neck T-Score < -1

- FRAX with BMD vs
- FRAX with BMD plus TBS plus prevalent radiographic vertebral fracture

(Schousboe JT, et al. 2016 JBMR 2016; 31(3): 690-697)

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Skeletal Regeneration: Stem Cell Therapy

Pamela Robey, Ph.D.

Sunday, September 30

11:00 am – 12:00 pm

Room 519 A

Skeletal Regeneration: Stem Cell Therapy

Pamela Gehron Robey, Ph.D., NIDCR/NIH/DHHS, Bethesda, MD, USA

Significance of the topic [excerpted from (1)]:

"Cell-based therapies are a new frontier in skeletal medicine, and are often heralded as holding much promise for modifying disease progression and repairing or replacing damaged or degenerating tissues. Cell-based therapy encompasses the fields of engineered tissues, direct cell application, and cell-derived products (e.g., platelet rich plasma and extracellular vesicles). Within the bone and cartilage fields, cell-based therapies are mainly permanent cell replacement therapies, whole tissue engineering, transient cell therapies, and conventional tissue grafts, particularly for the treatment of injury or degeneration of the skeletal system (2).

The scientific, public, and biomedical healthcare industry excitement for cell-based therapies has grown exponentially over the past decade. Over 18 billion U.S. dollars have been invested in publicly traded cell therapy companies between 2011 and 2016 (3). As of 2016, there were over 500 clinics in the United States alone marketing "stem cell" therapies (4). Between 2008 and 2012, the growth rate of stem cell scientific publications grew at greater than twice the rate of all publications worldwide, with nearly 30,000 manuscripts published in 2012 (5). This flourishing field not only presents growth and potential therapeutic promise, but increasingly presents the scientific and medical communities with new challenges (6,7).

The clinical problems associated with cell-based therapies are becoming increasingly acute. In one report sampling 1,052 publications regarding stem cell clinical trials, of the 393 completed cell based trials, only 45% had reported their results, with some trials disclosing results directly through press releases, bypassing peer review contrary to the recommendations of the International Society for Stem Cell Research (8,9). Further, many stem cell tourism clinics register trials to provide the appearance of legitimacy without the intention of trial completion or disclosure of data, making the actual disclosure rates of stem cell clinical trial data significantly lower."

The issues indicated above for "stem" cell therapies also pertain to the field bone repair. Yet,despite the challenges and drawbacks, progress is being made through the conduct of concerted studies on the cell sources, scaffolds and uses thereof, along with a recognition of how one characterizes the outcomes of pre-clinical studies of bone regeneration.

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

1. the scope of the problem in treating skeletal diseases, injuries and defects
2. the basic components of tissue engineering/regenerative medicine
3. the differences between stem cell-based therapies (tissue engineering) and cell-based therapies (regenerative medicine)
4. the methods to characterize the nature of bone regeneration

Outline:

Scope of the problem
Components of tissue engineering therapies
Functional characterization of BMSCs/SSCs
Applications of BMSCs/SSCs in tissue engineering and regenerative medicine (TE/RM)
Characterization of tissue repair by SSCs/BMSCs

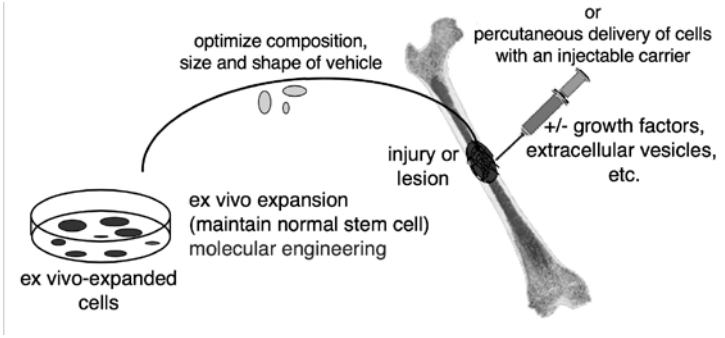
Scope of the problem [excerpted from The Burden of Musculoskeletal Diseases at http://www.boneandjointburden.org/]

"Musculoskeletal diseases affect more than one out of every two persons in the United States age 18 and over, and nearly three out of four age 65 and over. Trauma, back pain, and arthritis are the three most common musculoskeletal conditions reported, and for which health care visits to physicians' offices, emergency departments, and hospitals occur each year. The rate of musculoskeletal diseases far outstrips that of circulatory diseases and respiratory diseases, which affect about one in three persons, with the majority reporting relatively easily treatable conditions such as chronic hypertension or hay fever and bronchitis.

The cost of treating major musculoskeletal diseases, which often includes long-term pain and disability, is also greater than for treatment of many other common health conditions. Yet research dollars to identify causes, create new treatments, and reduce pain and disability remain much lower than that of other health conditions."

"With the aging of the US population, musculoskeletal diseases are becoming a greater burden every year. The pages of this site (Burden of Musculoskeletal Diseases) illustrate the magnitude of musculoskeletal diseases on the US population, and provide a small slice of the cost and impact on the US economy. The aggregate economic impact of musculoskeletal conditions is increasing rapidly. This reflects both the increase in prevalence and increase in per person costs described above. In constant dollars, persons with musculoskeletal conditions accounted for an aggregate economic impact of $367.1billion in 1996–1998 and $796.3 billion in 2009–2011, an increase of 117 percent in real terms. Using the more conservative estimates of the incremental impact of musculoskeletal conditions beyond what one would expect of persons with the same demographic characteristics as those with musculoskeletal conditions, such conditions still accounted for an increment of $212.7 billion in 2009–2011, an increase of 119 percent compared to the $97.3 billion figure for 1996–1998." [excerpted from 10].

Components of tissue engineering



Tissue engineering is generally composed of three components, used either singly or in combination with one another: cells, scaffolds and growth factors (or other exogenous factors such as extracellular vesicles). It is necessary to optimize each of the components that are used for specific animal species (murine and human cells often differ from one another in their requirements), the site that is under construction [embryonic origin, type of bone (cortical versus cancellous)], and the function that the new bone is expected to perform (e.g., weight-bearing versus non-weight-bearing). Particular attention must be paid to the choice of appropriate cell sources (described below) and scaffolds. It is often not appreciated that commercially available scaffolds are sold as "bone fillers," and many are not osteoconductive. Careful testing is needed to show that the scaffold can support the formation of bone and its marrow in vivo. The importance of marrow relates to the fact that the SSC is found in marrow as a pericyte, located on the adluminal surface of marrow sinusoids. Consequently, the present of marrow in BMSC/SSC-generated transplants is a surrogate marker for the presence of the skeletal stem cells (11).

Cell sources [see (11)]

The good	The bad*	Currently, the ugly
Bone marrow stromal cells	dental pulp cells	hESCs
trabecular bone cells	adipose derived cells	(non-autologous)
periosteal cells	muscle derived cells	iPSCs
circulating skeletal cells (endogenous cells)	placenta, amniotic fluid, etc	trans-differentiated cells
adherent cells from cord blood	cells from virtually any connective tissue	

"mesenchymal stem cells"

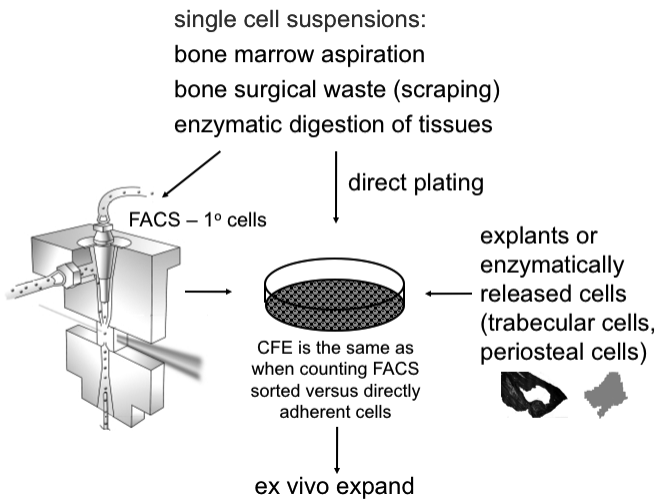
*may be pericytes in some tissues

A read of the current literature would suggest that virtually any population of "mesenchymal stem cells" would fill the order of being able to regenerate bone. However, based on rigorous analyses, "MSCs" from non-skeletal sources do NOT make bone in vivo, unless they are treated with BMPs, which will temporarily induce any fibroblastic cell to form bone. However, this induced bone is often not enduring. To date, the most efficacious cells for regenerating bone are BMSCs/SSCs. Periosteal cells are also able to reform bone, but do not appear to support blood formation. Circulating skeletal cells and cells in umbilial cord blood have also been identified. However, these cells have not yet been proved to be stem cells, by rigorous criteria (i.e., the ability of the progeny of a single cell to differentiate into functional parenchyma of a tissue, and are able to self-renew). Many of the cells identified as "MSCs" have not been shown to fulfill these essential criteria. Furthermore, the regeneration and MAINTENANCE of bone relies on the presence of a SSC within the BMSC population. Without the SSC, injured bone would not be regenerated; bone resorbed by osteoclasts during tissue turnover would not be replaced. Currently, the most reliable source of cells for skeletal regeneration are periosteal cells, trabecular cells isolated from bone, and bone marrow stromal

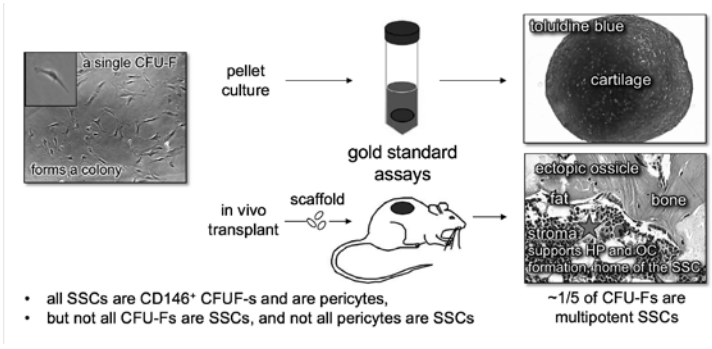
cells. While there are reports of human embryonic cells and induced pluripotent stem cells forming bone, few have performed in vivo transplantation assays to verify their osteogenic differentiation.

Cell Isolation

Single cell suspensions are created by mechanical disruption of bone marrow aspirates, and by scraping surgical waste with sterile scalpels to release bone marrow from trabecular bone. These cells are immature osteogenic cells (BMSCs), a subset of which SSCs. Bone marrow aspirates provide the opportunity to isolate cells by cell surface markers prior to culture. There are many different sorting strategies. For human samples, red blood cells are first eliminated, followed by elimination of $CD45^+/CD34^+$ blood cells and endothelial cells, followed by positive selection with CD146. Using freshly isolated cells provides the opportunity to determine the colony forming efficiency of the cell population; i.e., the ability of a single cell to grow in a density-independent fashion to form a colony. More mature osteogenic cells can be obtained by treating fragments of trabecular bone that that have been ground to a consistency of sand with collagenase. Collagenase released cells are heterogeneous with respect to their maturity BMSCs, and cells lying on the surface of bone (osteoblasts at various stages of maturity and bone surface lining cells). More homogeneous populations can be obtained by culturing the collagenase-treated bone fragments in low calcium medium. After several weeks in culture, cells emerge from the chips of bone and proliferate (11). These trabecular bone cells have been shown to form bone in vivo, but do not support hematopoiesis. Cells can also be derived from periosteum by either using explant cultures, or by digesting with collagenase to generate single cells. It is also important to assess the number of SSCs in the BMSC population by colony forming efficiency (CFE) assays, which are, to date, the closest approximation of the number of SSCs within the freshly isolated BMSC population. While it is unlikely that "purified" stem cells would be used directly for tissue regeneration due to their rarity, it is important to document the presence of a stem cell subset, which is required for appropriate tissue turnover [11].



Functional characterization of the differentiation of BMSCs/SSCs

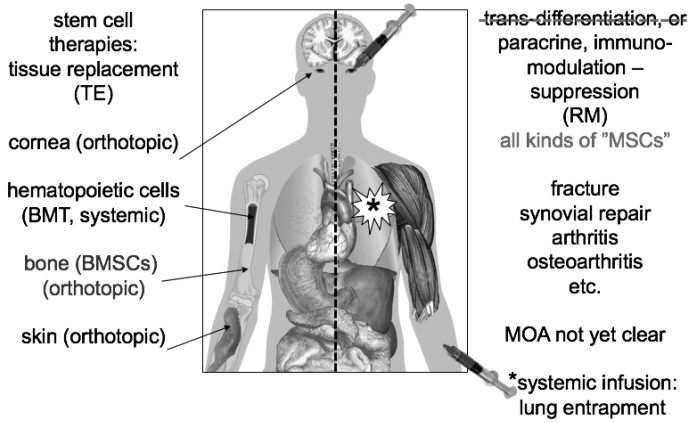


Characterization of the differentiation capacity of skeletal stem cells relies on a series of rigorous assays. For cartilage formation, the chondrogenic pellet culture is the gold standard, in which one must see bona fide chondrocytes lying in lacunae, surrounded by extracellular matrix that stains purple with toluidine blue (metachromasia). For the osteogenesis assay, alizarin red S cannot distinguish between dystrophic calcification induced by dead and dying cells versus matrix mineralization. In addition, if the cells make the enzyme alkaline phosphatase, the enzyme cleaves β-glycerophosphate that is in the osteogenic differentiation medium, and when the phosphate concentration in the medium becomes high enough, calcium phosphate precipitates, and it too stains with alizarin red S, but it is not hydroxyapatite. In the adipogenic assay, many cells take up lipid from the serum in the medium and do not synthesize lipids de novo. In vivo transplantation with an appropriate scaffold is the gold standard by which to assess osteogenic and adipogenic differentiation (12).

Applications of BMSCs/SSCs in tissue engineering and regenerative medicine (TE/RM)

To date, there are only a few examples of successful bona fide stem cell therapies: blood reconstitution with populations containing hematopoietic stem cells, corneal regeneration by populations of limbal cells

containing limbal stem cells, skin regeneration with epidermal stem cells that contain stem cells, and a number of small studies regenerating bone with SSCs/BMSCs [reviewed in (13, 14)]. On the other hand, the notion emerged that SSCs/BMSCs (and other types of "MSCs") could be infused systemically or locally injected to treat generalized diseases and disorders, or injuries. Initially, a long list of studies suggested that these infused cells could "trans-differentiate" into cells outside of their lineage (e.g., SSCs/BMSCs could form neurons, cardiomyocytes, etc.) based on the expression of a few markers. Subsequently, more rigorous studies that followed indicated that trans-differentiation is a rare event, if it occurs at all, and proof of functionality of these trans-differentiated cells was lacking. Yet some studies reported beneficial effects of "MSCs" in treating a long list of diseases and disorders in animal models and in humans (12).



It was hypothesized that infused or directly injected cells exert paracrine effects that encourage local stem/progenitor cells to begin the repair process, or that they were exerting immunomodulatory and immunosuppressive effects that would bring about improvement. However, it is well known that upon systemic infusion, "MSCs" of all types are rapidly cleared by the lungs and rarely escape from the circulation. They rapidly disappear, even upon direct injection without a scaffold or carrier. Consequently, the mechanism(s) of action have not been well elucidated, and are very unclear. Furthermore, these putative effects have not been pinpointed to the rare subset of stem cells that are present within any "MSC" population, and cannot be correctly called a "stem" cell therapy. The putative effects are brought about by the entire cell population. In addition, it is also not clear that "MSCs" are unique in this regard, as it has been demonstrated that skin fibroblasts exert similar effect**.** Many studies have not used a negative control cell type to show the specificity of "MSCs" in these treatments (12).

Characterization of tissue repair by SSCs/BMSCs [excerpted in part from (1)]

"An optimal experimental approach to evaluating cell-based therapies for enhancing skeletal tissue repair/regeneration would be to initiate studies in small animals, focusing on cellular, molecular, functional, and mechanical outcome measures. Once these models provide proof of principle in multiple laboratories for the utility of a specific cell preparation in augmentation of repair, additional investigation would be completed in larger animal models. Subsequent successful outcomes in the large animal models, with inclusion of appropriate safety and efficacy profiles, would identify prime methods for human clinical trials. "

There is no single method to evaluate cell-based experiments; however, there are a number of techniques that can be used to rigorously establish the efficacy and the mechanism by which bone regeneration occurs after application of a cell-based therapy. First, it must be determined if the newly made bone is made by the donor cells or recipient cells. If donor cells are not present, it is indicative of the fact that the donor cells themselves did not participate in bone regeneration, but rather that they induced local cells to repair the bone. In pre-clinical studies, identification of the donor or the recipient origin of the bone is determined through the use of markers human cells, in the case of xeno-transplants, or presence of a reporter in the donor cells or in the recipient. Localization of a marker or a reporter should be coupled with histological analysis to determine the cell type that is expressing the marker or reporter, and with localization of a maker of mature osteogenic cells (e.g., bone sialoprotein or osteocalcin). Functional outcomes should be determined by mechanical testing, which can be augmented by determination of the material and structural properties (e.g., cortical versus trabecular bone, bone mineral density, etc., as determined by microCT analyses). Lastly, the time course of repair should be evaluated at short, mid and long-term time points in order to determine the fate of transplanted cells.

"Cell-based therapies are an area of public confusion and are subject to increasing regulatory, scientific, and public safety scrutiny. To ensure that the promise and scientific potential of this field are met, and that public and regulatory trust in the field is upheld, basic and translational scientists can implement currently available technologies to increase the scientific rigor supporting cell-based therapies. Further, increasing focus on mechanism and cell fate determination can improve the utility and accuracy of the scientific conclusions drawn from these experiments. Such advancements will inform intelligent clinical trial design, strengthen the scientific foundation for clinical translation, and drive the discovery of cell derived products that may be used for treatment

of musculoskeletal conditions. Such an approach, using currently available techniques, will greatly enhance the societal value of the scientific efforts put forth in these fields, and will more rapidly lead to safe, proven, efficacious therapies for musculoskeletal disease."

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