ESEM Analysis of Mice Femurs with Varying SOST Levels

By Patrick McClay

Advisor: Scott Hazelwood

Department: Biomedical Engineering

Table of Contents

ABSTRACT

This project's goal was to analyze the properties of the cortical femur on 100 mice bones from Lawrence Livermore National Laboratory. Analysis was limited to imaging which determined the ratio of bone volume to total volume (BV/TV) and osteocyte lacunae density. Mice were altered to knock out their SOST gene: a negative regulator of bone formation. Twelve groups were created to differentiate their treatment, duration, and phenotype. Transgenic (TG) mice had an overexpression of the SOST gene: they carried a bacterial artificial chromosome. Mice with limb defects (DEF) were the offspring of two TG mice and carried twice the amount of SOST. Mice with SOST knockout (KO) treatment, knockout phenotype, and 12 months duration had a significantly lower BV/TV than all other groups except for WT SOST KO, 12 and 6 months: the p-value was 0.00. No mouse group had a significantly different osteocyte lacunae density: the p-value was .071. The results of a significant decrease in BV/TV was observed in the KO, 12 months because the mice had the longest time to fully develop the phenotype. The lack of a difference in the osteocyte lacunae density could be the result of an insufficient sample size.

INTRODUCTION

Osteoporosis is a common bone disease affecting 1 in 5 American women over the age of 50 [**1**]. In patients with osteoporosis, the bones can become so weak that anything from a fall to bending over can cause fracture [**2**]. Like most living tissue, bone is constantly being replaced and reformed. When the rate of formation falls below the rate of removal, also called resorption, osteoporosis occurs. A much less common bone disease, Van Buchem disease, has the opposite effect of osteoporosis and is caused when the bone forms more than it resorbs. Despite the rarity of this disease, it is used as an important research tool as scientists attempt to identify the mechanisms that affect bone resorption and formation. The main purpose of identifying and learning about theses mechanisms is to improve osteoporosis research because they are a key factor of the disease's cause.

In Van Buchem disease, the medullary cavity of long bone narrows (Figure 1) [**3**] as a result of excessive bone formation.

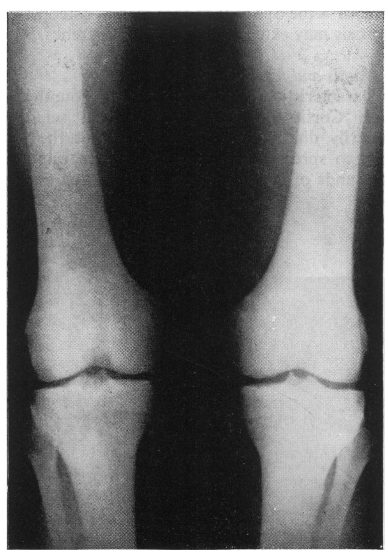


Figure 1: Long bones showing reduction in medullary canals [**3**].

Research Background

SOST is a gene that codes for the protein sclerostin, which is believed to be a negative regulator of bone formation. Overexpression of SOST has been shown to cause osteopenia, a mild form of osteoporosis; and lack of SOST results in too much bone formation and abnormally high bone mass and density [**4**]. Lawrence Livermore National Laboratory (LLNL) altered the SOST levels of 50 mice, sacrificed them, and removed their femur bones for testing. Although the tibia bone is easier to access, the femur was chosen because it is straighter and therefore improves mechanical testing. One hundred femur bones were separated into 10 different groups depending on their SOST treatment, phenotype, and time until sacrifice. The two treatment types were SOST knockout (KO) and SOST (TG), along with their respective wild type (WT) control littermates, and were analyzed for 6, 8, or 12 months. KO mice had a deficiency of SOST and TG mice had elevated expression of the gene. Phenotype classification was dependent on limb defects. Animals with limb defects (DEF) had twice the SOST: the result of mating two TG mice. Wild type (WT) mice, on the other hand, contained normal levels of SOST.

Bone Testing

Before this project began, a three point bending test was performed on the bones to test the basic mechanical properties of the bones. Afterward, this project used image analysis to find bone volume fraction, which is the bone's ratio of bone volume to total volume (BV/TV); total volume excludes the bone's medullary cavity. The volume of bone can be estimated from the area when only a two dimensional imaging analysis is available.

BV/TV is a useful parameter because it can be used to indicate signs of osteoporosis in bones [**5**].

Afterward, image analysis was used to find the osteocyte lacunae density of bone, which also indicates osteoporosis [**6**] [**7**]. The difference between live and dead osteocyte lacunae cannot be easily differentiated so the osteocyte lacunae density combines the two. In bones with osteoporosis, the osteocyte lacunae density decreases due to its decreased density.

Study Goals

The goal of this study is to find BV/TV and osteocyte lacunae density of the mice bones from LLNL and determine any differences between the 10 groups. This data can be used to explore the specific effects caused by manipulation of the SOST gene. Increased knowledge of the SOST gene aids in current research at laboratories, like LLNL, in their search for improved osteoporosis treatments.

METHODS

Bone Preparation

The 100 mice femur bones had already been broken in half due to previous mechanical testing. The proximal half of each bone was wrapped in an aluminum cylinder and embedded into acrylic pucks; each puck contained 4-6 bones. The side of the puck with the broken end of the bones was polished down using incrementally finer sandpaper and polishing pads: from 120 grit sandpaper down to polishing pads with 3µm polishing solution. Using a microscope, the pucks were analyzed at every step to ensure all scratches and imperfections were eliminated from the surface. The result was 20 pucks with smooth surfaces (Figure 2), which clearly displayed the cortical cross sections of each bone.

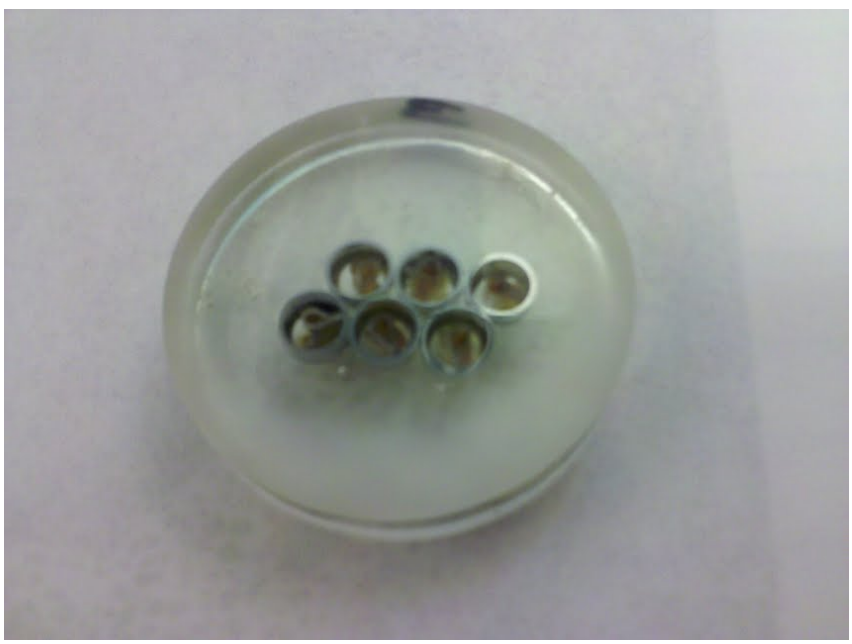


Figure 2: Bone samples embedded in a polished acrylic puck.

Imaging

The pucks were then imaged using an environmental scanning electron microscope (ESEM), specifically a Hitachi TM-1000 ESEM Tabletop Microscope (Figure 3).

Figure 3: Hitachi TM-1000 ESEM Tabletop Microscope located in Engineering IV was used to take every image.

Each puck was taped onto a loading tray, which was then placed in a device that could be used to adjust the height of the tray (Figure 4). The bar represents the clearance height of the machine and the ideal distance between the top of the puck and the bottom of the bar is 1mm, however, not every puck achieve this due to the curvature of the puck. The loading tray could be screwed up and down to adjust the height and resolution was improved when the 1mm distance was closely met.

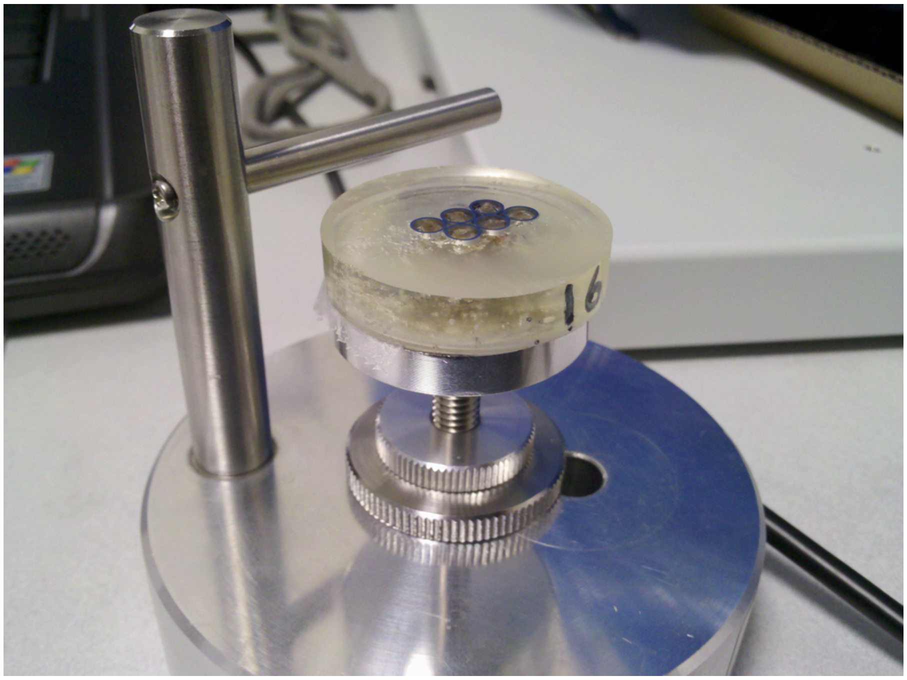


Figure 4: Loading tray inside height adjuster.

The loading tray and puck were then placed into the machine and the platform was adjusted to re-center the sample (Figure 5). Adjustments were made with two knobs on the front of the machine (Figure 3). These knobs used finely threaded screws to adjust the position of the platform in the x and y directions.

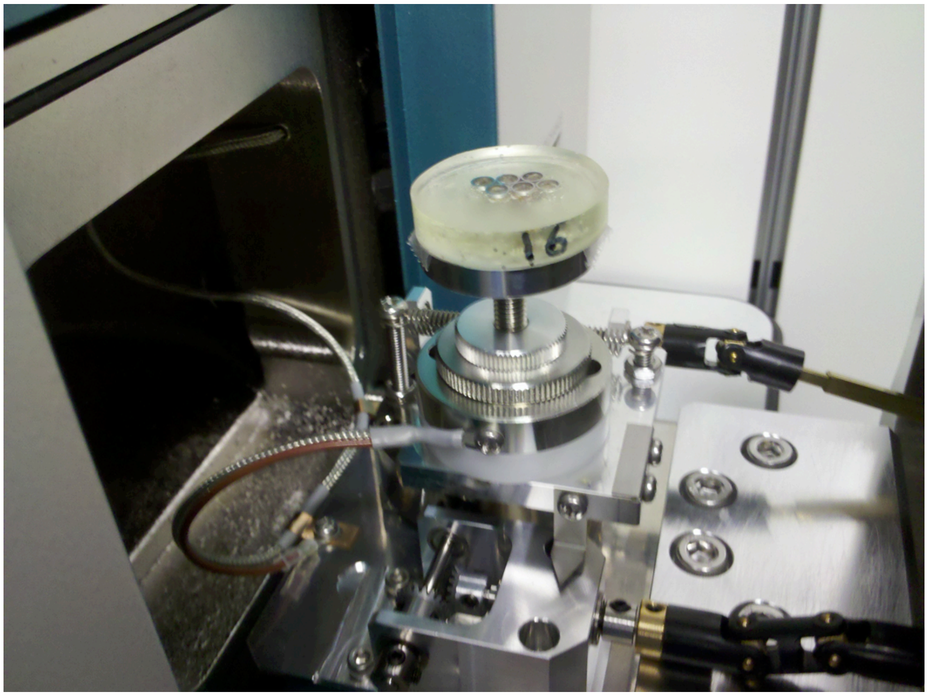


Figure 5: Loading area of ESEM.

The platform was slid back into the machine and pushed shut until the sides are completely sealed. A green button was pushed on the front of the machine, which activated a compressor to evacuate the chamber (Figure 3). The microscope's computer program was then accessed on a laptop located next to the machine. The start button was pushed and images would appear on the screen (Figure 6). Magnification, focus, brightness and contrast were adjusted using buttons on the computer program. To adjust the location of the image, knobs on the front of the machine were used (Figure 3). Four images were taken of each bone: one of the entire bone, and three at different positions around the bone (Figure 6). The full image was used to calculate the bone volume fraction. The images of the three positions were used to calculate the osteocyte lacunae density. Three images were consistently taken in the same relative locations in every

bone (Figure 5), which increased the accuracy of the osteocyte lacunae density calculations. It was necessary to take multiple images for the osteocyte lacunae density because it required a higher resolution of images to be able to identify individual osteocyte lacunae.

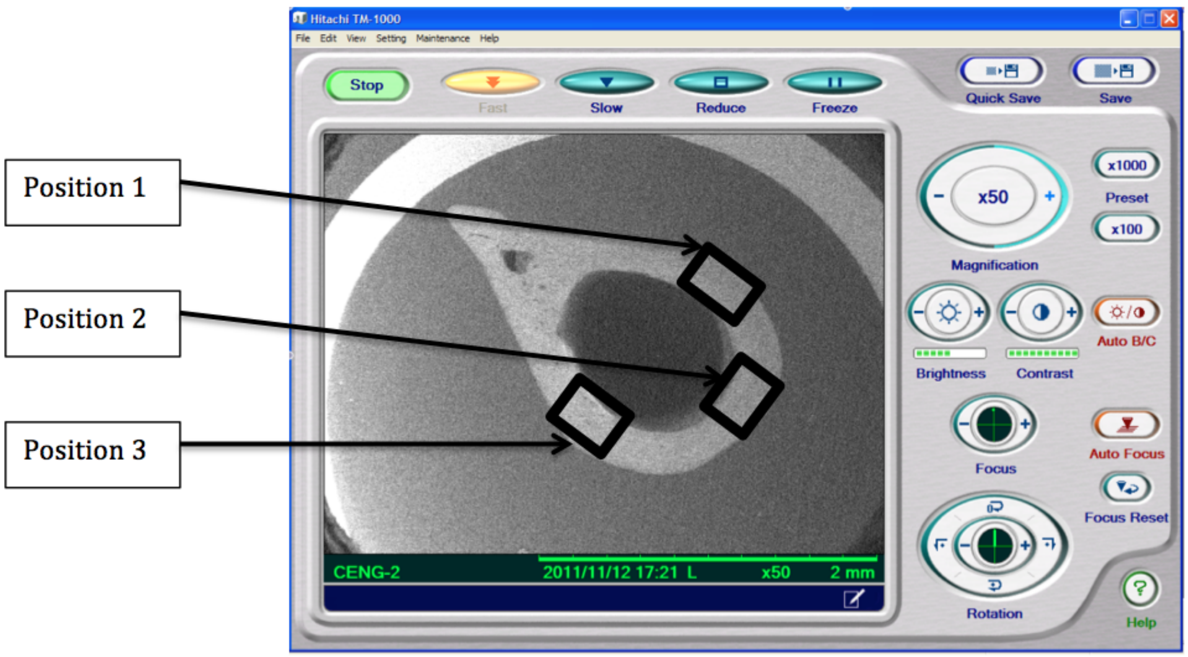


Figure 6: Screen capture of ESEM program screen. The three positions indicate the three locations individual images were taken on every bone.

Bone Volume Fraction

To calculate the bone volume fraction, all the images were taken directly from the ESEM machine and opened in ImageJ. The scale on each image had to be set for ImageJ to know the distance each pixel of the image represented. Every time the ESEM machine saved an image, the scale was included on the bottom of the image in the form of a bar with a labeled distance. To insert the scale on ImageJ a line was drawn across the bar and the *Set Scale* function was used (Figure 7).

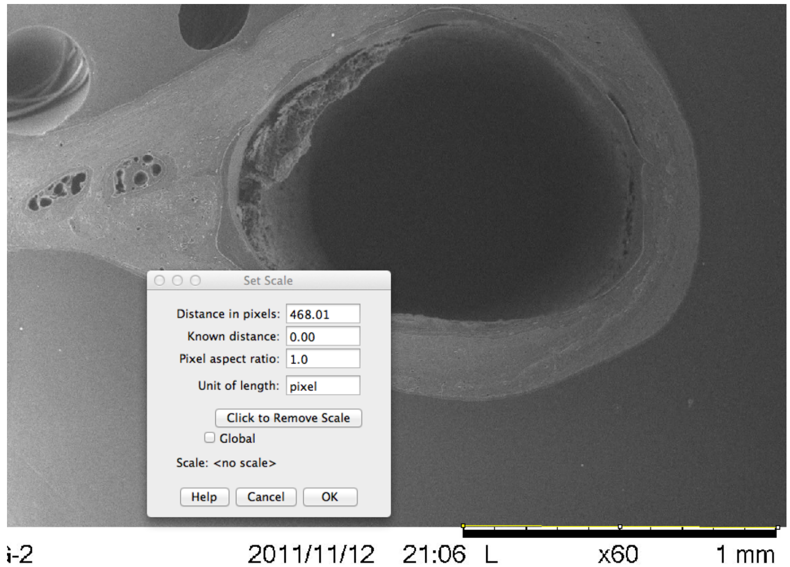


Figure 7: Set scale function on ImageJ.

Next, the area of the entire bone was found. Using the drawing tool, the bone was completely outlined (Figure 8) and the area was captured and stored.

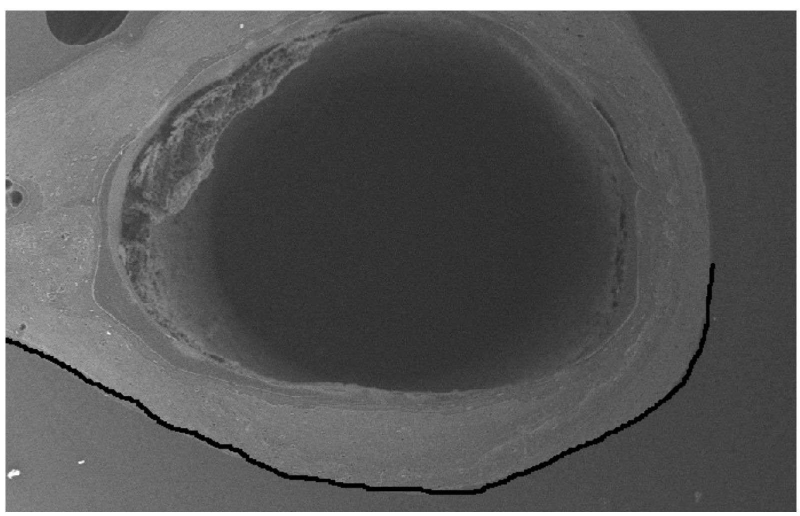


Figure 8: Outlining of a bone.

To determine the areas of the inner cavities, a threshold was used based on the shade of the gray scale image (Figure 9). This allowed every inner cavity of the bone to be easily selected and each area individual area stored (Figure 10). A special note of the area of the

medullary cavity was made because it would be used in later analysis. This function could not be performed earlier to outline the entire bone because the range in gradient was too great across the entire bone for the outline to be individually selected.

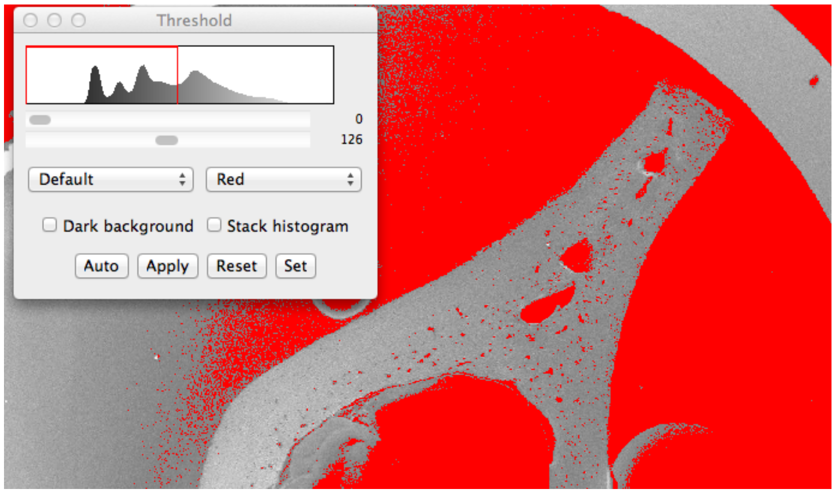


Figure 9: Threshold of the bone.

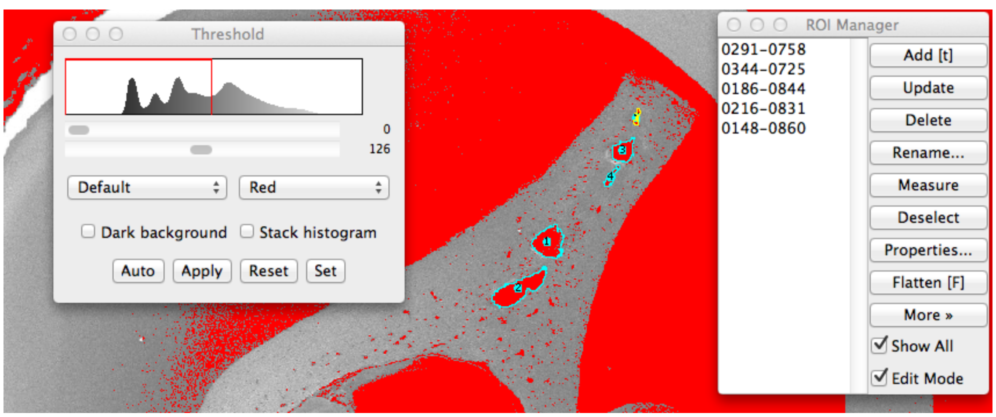
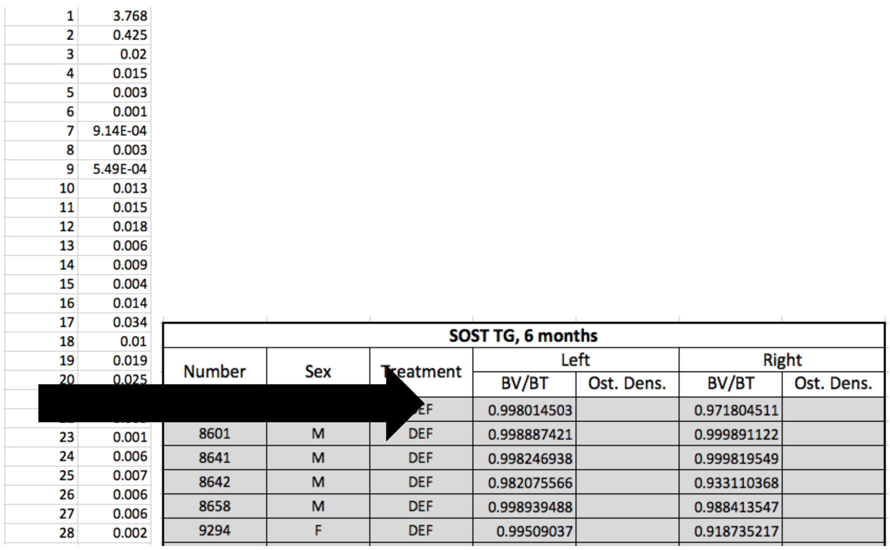


Figure 10: Areas of the individual cavities selected and stored.

All of the area data from ImageJ was then transferred to Excel (Figure 11) with the area of the entire bone area and the medullary cavity labeled separately. To calculate the area of the total bone, the area of the medullary cavity was subtracted from the area of the entire bone perimeter. The bone volume area was calculated by subtracting the area of

every non-bone cavity from the bone volume. The area of bone volume was then divided by the total volume and multiplied by 100 to obtain BV/TV.



1	3.768
2	0.425
3	0.02
4	0.015
5	0.003
6	0.001
7	9.14E-04
8	0.003
9	5.49E-04
10	0.013
11	0.015
12	0.018
13	0.006
14	0.009
15	0.004
16	0.014
17	0.034
18	0.01
19	0.019
20	0.025
[illegible]	[illegible]
[illegible]	[illegible]
23	0.001
24	0.006
25	0.007
26	0.006
27	0.006
28	0.002

SOST TG, 6 months						
Number	Sex	Treatment	Left		Right	
			BV/BT	Ost. Dens.	BV/BT	Ost. Dens.
[illegible]	[illegible]	[illegible]	0.998014503		0.971804511	
8601	M	DEF	0.998887421		0.999891122	
8641	M	DEF	0.998246938		0.999819549	
8642	M	DEF	0.982075566		0.933110368	
8658	M	DEF	0.998939488		0.988413547	
9294	F	DEF	0.99509037		0.918735217	

Figure 11: ImageJ data transferred to excel.

Osteocyte Lacunae Density

To calculate osteocyte lacunae density, each of the three positions for each bone were analyzed separately in ImageJ. First, for each position, the scale was set using the same procedure as previously described for BV/TV. Next, an area of bone of was outlined; the area was made as big as possible to increase accuracy (Figure 12).

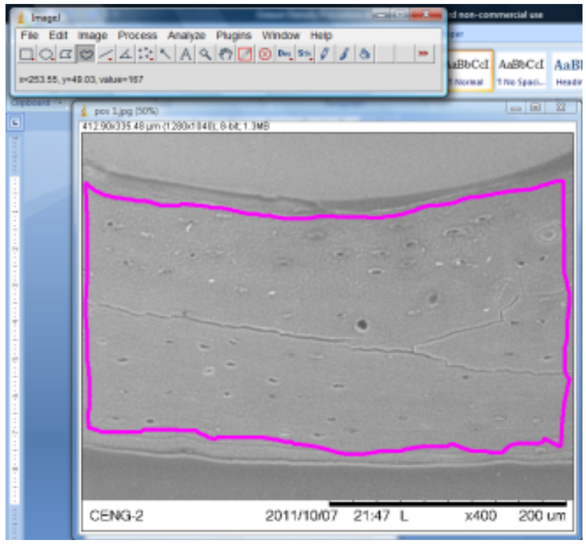


Figure 12: A selection of bone is being taken on one of the three positions taken of each bone.

In order to improve later analysis, everything outside of the selection was cleared away (Figure 13). The area of the selection was recorded and saved into Excel.

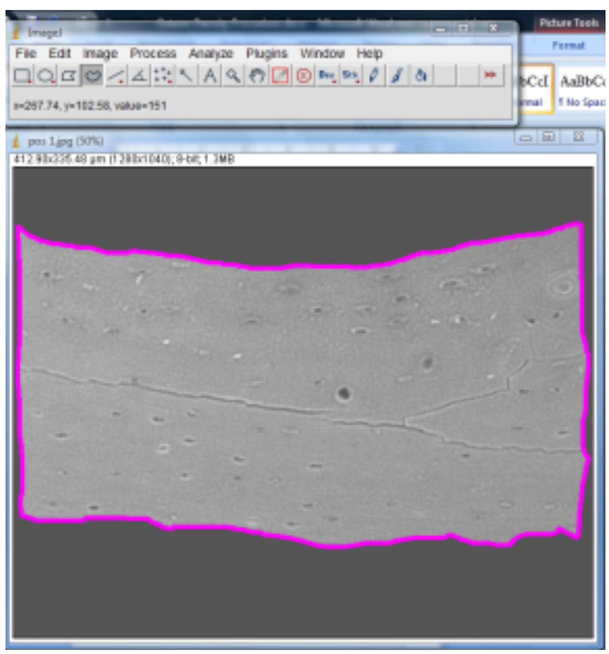


Figure 13: The outside of the selection area was cleared away to improve threshold analysis.

The threshold function was used to differentiate between different shades of gray. Osteocyte lacunae are significantly darker than the rest of the bone so they become selected as red (Figure 14).

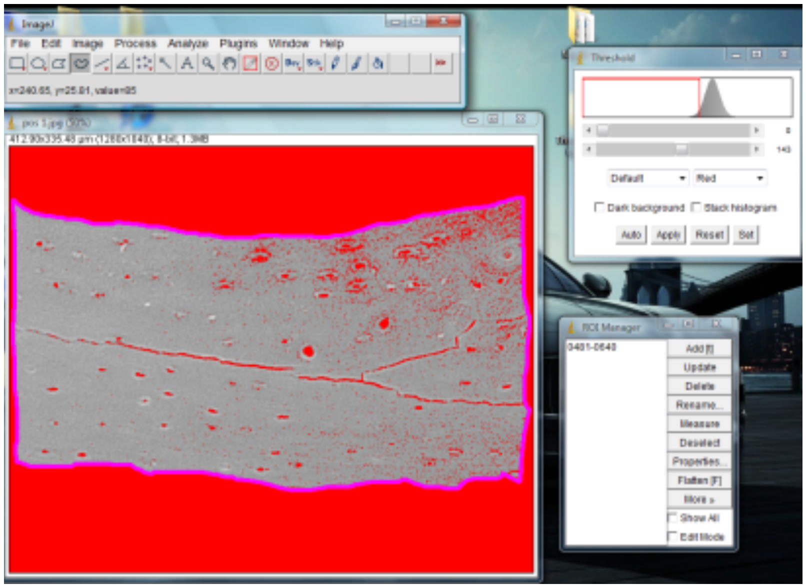


Figure 14: The threshold was set to differentiate between osteocyte lacunae and bone.

To determine the number of osteocyte lacunae inside the selected area, the analyze particles tool was used (Figure 15). It was set to count areas between 10μm^2 and 200μm^2 to capture osteocyte lacunae and avoid counting other imperfections [**8**] [**9**]. To avoid counting overly long objects that are not osteocyte lacunae, the circularity was set to capture between .2 and 1 circular features. In some bones, the image resolution was too poor to use this technique, so the osteocyte lacunae were counted manually using the multipoint selection tool.

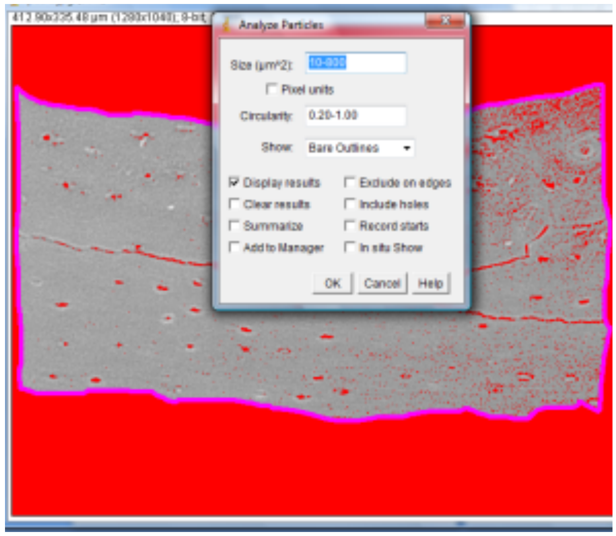


Figure 15: The analyze particles tool was used to automatically count the number of osteocyte lacunae in a given area.

ImageJ returned an outline of every feature it counted (Figure 16), and a list of everything counted with its corresponding area (Figure 17). The outlines were checked to ensure nothing other than osteocyte lacunae were counted. The list was used to obtain the number of osteocyte lacunae in the area, which was then entered into Excel.

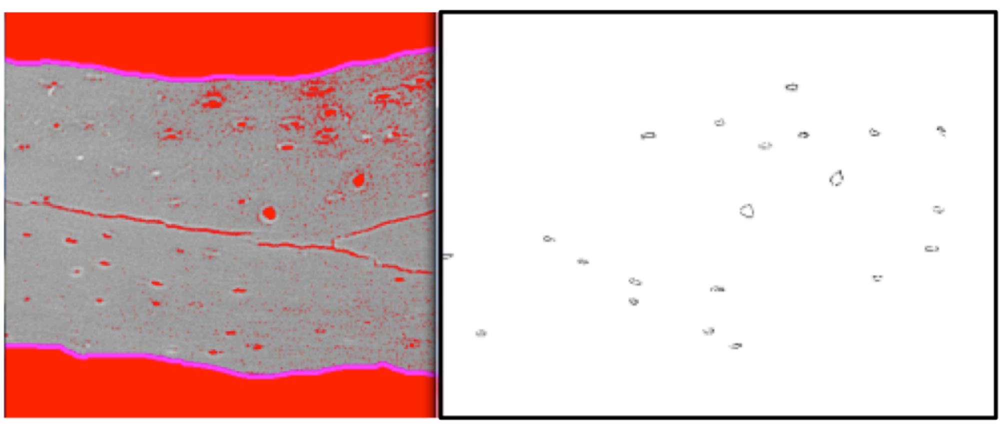


Figure 16: The outlines of every object counted by ImageJ were used to ensure only osteocyte lacunae were counted.

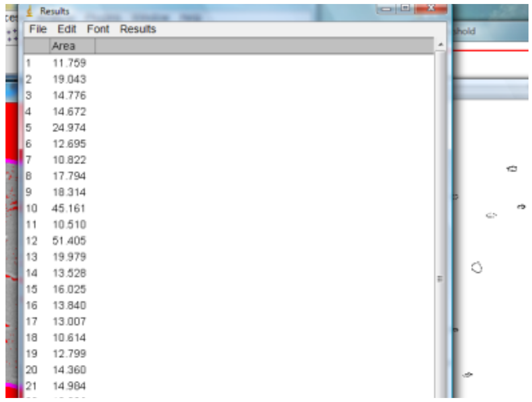


Figure 17: A list of every feature counted and its corresponding area was returned by the analyze particles function.

This process was then repeated on each bone for the second and third positions. In Excel, the osteocyte lacunae density was found for each of the three individual positions by dividing the number of osteocytes by the area of the selection. Finally, the three numbers were averaged together to obtain the osteocyte lacunae density for the entire bone.

RESULTS

Bone Volume Fraction

Data from excel was transferred from excel into Minitab to obtain the results below. In Table 1, only the 12 month KO group had an average bone volume fraction below 90%. The 12 month KO group's entire 95% confidence interval was below 90% (

```
                       ---+---------+---------+---------+------
DEF SOST TG, 6 months                             (--*--)
DEF SOST TG, 8 months                    (----*----)
KO SOST KO, 12 months  (-----*----)
KO SOST KO, 6 months                      (---*---)
TG SOST TG, 6 months                     (-----*-----)
TG SOST TG, 8 months                     (-----*-----)
WT SOST KO, 12 months              (------*-------)
WT SOST KO, 6 months                  (--*---)
WT SOST TG, 6 months                                   (-*--)
WT SOST TG, 8 months                         (----*----)
                       ---+---------+---------+---------+------
                       85.0      90.0      95.0     100.0
                                       BV/TV [%]
```

Figure 18).

Table 1: Basic statistics of bone volume fraction data.

Phenotype with Treatments	Mean [%]	Standard Deviation [%]	Number of Samples
DEF SOST TG, 6 months	98.114	2.617	17
DEF SOST TG, 8 months	94.5	3.3	6
KO SOST KO, 12 months	86.27	7.13	5
KO SOST KO, 6 months	95.079	1.72	9
TG SOST TG, 6 months	95.44	6.8	4
TG SOST TG, 8 months	95.52	2.88	4
WT SOST KO, 12 months	93.21	5.59	3
WT SOST KO, 6 months	92.661	2.399	11
WT SOST TG, 6 months	98.674	1.328	22
WT SOST TG, 8 months	97.05	3.4	7

```
                        ---+---------+---------+---------+------
DEF SOST TG, 6 months                               (--*--)
DEF SOST TG, 8 months                      (----*----)
KO SOST KO, 12 months   (-----*----)
KO SOST KO, 6 months                         (---*---)
TG SOST TG, 6 months                        (-----*-----)
TG SOST TG, 8 months                        (-----*-----)
WT SOST KO, 12 months                 (------*-------)
WT SOST KO, 6 months                     (--*---)
WT SOST TG, 6 months                                  (-*--)
WT SOST TG, 8 months                            (----*----)
                        ---+---------+---------+---------+------
                        85.0      90.0      95.0     100.0
                                        BV/TV [%]
```

Figure 18: Individual 95% CIs for bone volume fraction mean based on pooled standard deviation.

There was a significant statistical between the groups as demonstrated by the p-value of 0.00 (Figure 19). The statistical power of this data was 100% at a significance level of 5%. A Tukey test (Table 2) shows the mice in 12 month KO group had a statistically lower BV/TV than all groups besides the 6 and 12 month WT groups of the KO mice. A boxplot of the BV/TV data visually shows the 12 month KO group is less than the others (Figure 20).

```
Factor                    Type   Levels  Values
Phenotype with Treatment  fixed      10  DEF SOST TG, 6 months, DEF SOST TG, 8
                                         months, KO SOST KO, 12 months, KO SOST
                                         KO, 6 months, TG SOST TG, 6 months, TG
                                         SOST TG, 8 months, WT SOST KO, 12
                                         months, WT SOST KO, 6 months, WT SOST
                                         TG, 6 months, WT SOST TG, 8 months


Analysis of Variance for BV/BT %, using Adjusted SS for Tests

Source                    DF   Seq SS  Adj SS  Adj MS     F      P
Phenotype with Treatment   9   881.47  881.47   97.94  9.78  0.000
Error                     78   780.78  780.78   10.01
Total                     87  1662.25


S = 3.16386   R-Sq = 53.03%   R-Sq(adj) = 47.61%


Unusual Observations for BV/BT %

Obs  BV/BT %      Fit  SE Fit  Residual  St Resid
 10  91.8735  98.1139  0.7673   -6.2404     -2.03 R
 33  96.1416  86.2730  1.4149    9.8686      3.49 R
 34  79.2944  86.2730  1.4149   -6.9787     -2.47 R
 35  79.3185  86.2730  1.4149   -6.9545     -2.46 R
 50  85.5090  95.4429  1.5819   -9.9339     -3.63 R
 55  87.8185  93.2088  1.8267   -5.3903     -2.09 R
 57  98.9733  93.2088  1.8267    5.7645      2.23 R
 97  90.4917  97.0507  1.1958   -6.5591     -2.24 R

R denotes an observation with a large standardized residual.
```

```
Factor                      Type   Levels  Values
Phenotype with Treatment    fixed      10  DEF SOST TG, 6 months, DEF SOST TG, 8
                                           months, KO SOST KO, 12 months, KO SOST
                                           KO, 6 months, TG SOST TG, 6 months, TG
                                           SOST TG, 8 months, WT SOST KO, 12
                                           months, WT SOST KO, 6 months, WT SOST
                                           TG, 6 months, WT SOST TG, 8 months


Analysis of Variance for BV/BT %, using Adjusted SS for Tests

Source                      DF   Seq SS  Adj SS  Adj MS     F      P
Phenotype with Treatment     9   881.47  881.47   97.94  9.78  0.000
Error                       78   780.78  780.78   10.01
Total                       87  1662.25


S = 3.16386   R-Sq = 53.03%   R-Sq(adj) = 47.61%


Unusual Observations for BV/BT %

Obs  BV/BT %      Fit  SE Fit  Residual  St Resid
 10  91.8735  98.1139  0.7673   -6.2404     -2.03 R
 33  96.1416  86.2730  1.4149    9.8686      3.49 R
 34  79.2944  86.2730  1.4149   -6.9787     -2.47 R
 35  79.3185  86.2730  1.4149   -6.9545     -2.46 R
 50  85.5090  95.4429  1.5819   -9.9339     -3.63 R
 55  87.8185  93.2088  1.8267   -5.3903     -2.09 R
 57  98.9733  93.2088  1.8267    5.7645      2.23 R
 97  90.4917  97.0507  1.1958   -6.5591     -2.24 R

R denotes an observation with a large standardized residual.
```

Figure 19: ANOVA general linear model of bone volume fraction (BV/BT) versus phenotype with treatment.

Table 2: Grouping information using Tukey Method and 95.0% confidence[1].

Phenotype with Treatments	Group Letters		
DEF SOST TG, 6 months	A		
DEF SOST TG, 8 months	A	B	
KO SOST KO, 12 months			C
KO SOST KO, 6 months	A	B	
TG SOST TG, 6 months	A	B	
TG SOST TG, 8 months	A	B	
WT SOST KO, 12 months	A	B	C
WT SOST KO, 6 months		B	C
WT SOST TG, 6 months	A		
WT SOST TG, 8 months	A	B	

[1] Means that do not share a letter are significantly different.

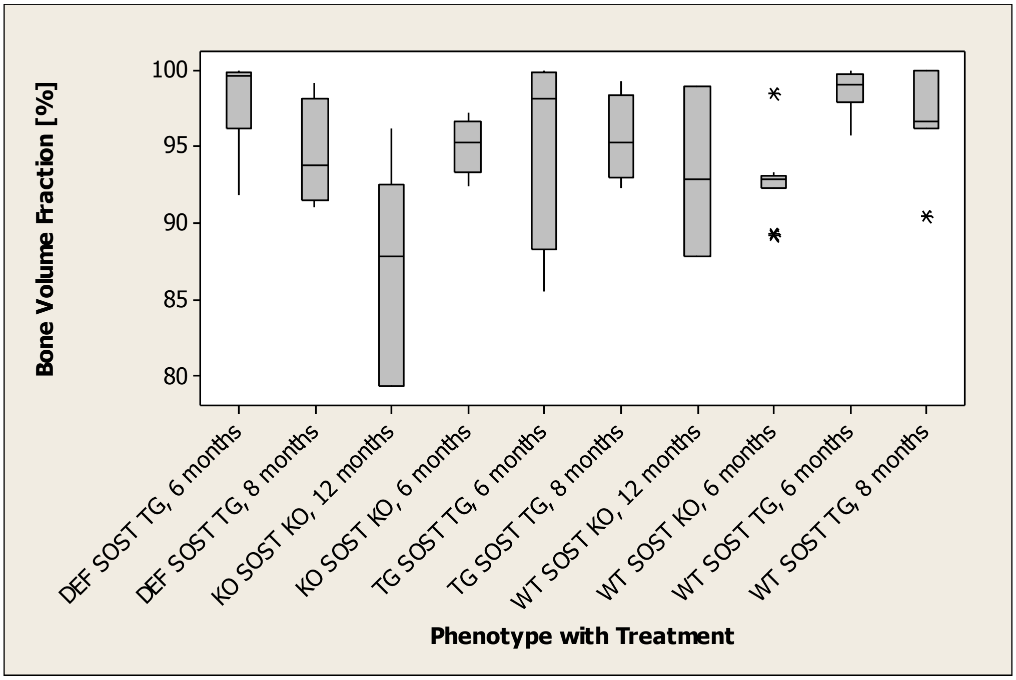


Figure 20: Boxplot of BV/TV results.

Osteocyte Lacunae Density

The standard deviation of all the groups was below 90 for every mouse group (Table 3).

Figure 21 shows the overlap between each group's 95% confidence intervals.

Table 3: Basic statistics of osteocyte lacunae data.

Phenotype with Treatments	Mean [O.L./mm^2]	Standard Deviation [O.L./mm^2]	Number of Samples
DEF SOST TG, 6 months	658.1	154.1	18
DEF SOST TG, 8 months	843.1	99.7	8
KO SOST KO, 12 months	740	171.6	10
KO SOST KO, 6 months	638.3	132.5	10
TG SOST TG, 6 months	642.6	268.1	4
TG SOST TG, 8 months	769.4	240.2	4
WT SOST KO, 12 months	804.2	221	4
WT SOST KO, 6 months	678.7	205.6	12
WT SOST TG, 6 months	773.6	120.1	22
WT SOST TG, 8 months	784.9	151	8

```
                        --------+---------+---------+---------+-
DEF SOST TG, 6 months          (----*----)
DEF SOST TG, 8 months                    (------*-------)
KO SOST KO, 12 months              (-----*------)
KO SOST KO, 6 months        (------*-----)
TG SOST TG, 6 months    (----------*----------)
TG SOST TG, 8 months             (---------*----------)
WT SOST KO, 12 months              (----------*---------)
WT SOST KO, 6 months           (-----*-----)
WT SOST TG, 6 months                   (----*---)
WT SOST TG, 8 months                 (------*-------)
                        --------+---------+---------+---------+-
                              600       750       900      1050
                          Osteocyte Lacunae Density [O.L./mm²]
```

Figure 21: Individual 95% CIs for osteocyte lacunae mean based on pooled standard deviation.

There was not a statistically significant difference between the groups as demonstrated by the p-value of 0.71 (Figure 22). The statistical power of this data was 23.1% with a confidence interval of 5%. A Tukey test (Table 4) shows that no group is different form the others because they are all in the same column. A boxplot of the osteocyte lacunae density data shows the relative proximity of each group's results (Figure 23).

```
Factor                      Type   Levels  Values
Phenotype with Treatment    fixed      10  DEF SOST TG, 6 months, DEF SOST TG, 8
                                           months, KO SOST KO, 12 months, KO SOST
                                           KO, 6 months, TG SOST TG, 6 months, TG
                                           SOST TG, 8 months, WT SOST KO, 12
                                           months, WT SOST KO, 6 months, WT SOST
                                           TG, 6 months, WT SOST TG, 8 months


Analysis of Variance for Ost/mm., using Adjusted SS for Tests

Source                      DF   Seq SS   Adj SS  Adj MS     F      P
Phenotype with Treatment     9   435582   435582   48398  1.85  0.071
Error                       90  2358975  2358975   26211
Total                       99  2794557


S = 161.898   R-Sq = 15.59%   R-Sq(adj) = 7.15%


Unusual Observations for Ost/mm.

Obs  Ost/mm.     Fit  SE Fit  Residual  St Resid
 48   351.02  642.59   80.95   -291.56     -2.08 R
 50   970.74  642.59   80.95    328.15      2.34 R

R denotes an observation with a large standardized residual.
```

Figure 22: ANOVA general linear model of osteocyte lacunae density (Ost/mm.) versus phenotype with treatment.

Table 4: Grouping information using Tukey Method and 95.0% confidence[2].

Phenotype with Treatments	Group Letters		
DEF SOST TG, 6 months	A		
DEF SOST TG, 8 months	A		
KO SOST KO, 12 months	A		
KO SOST KO, 6 months	A		
TG SOST TG, 6 months	A		
TG SOST TG, 8 months	A		
WT SOST KO, 12 months	A		
WT SOST KO, 6 months	A		
WT SOST TG, 6 months	A		
WT SOST TG, 8 months	A		

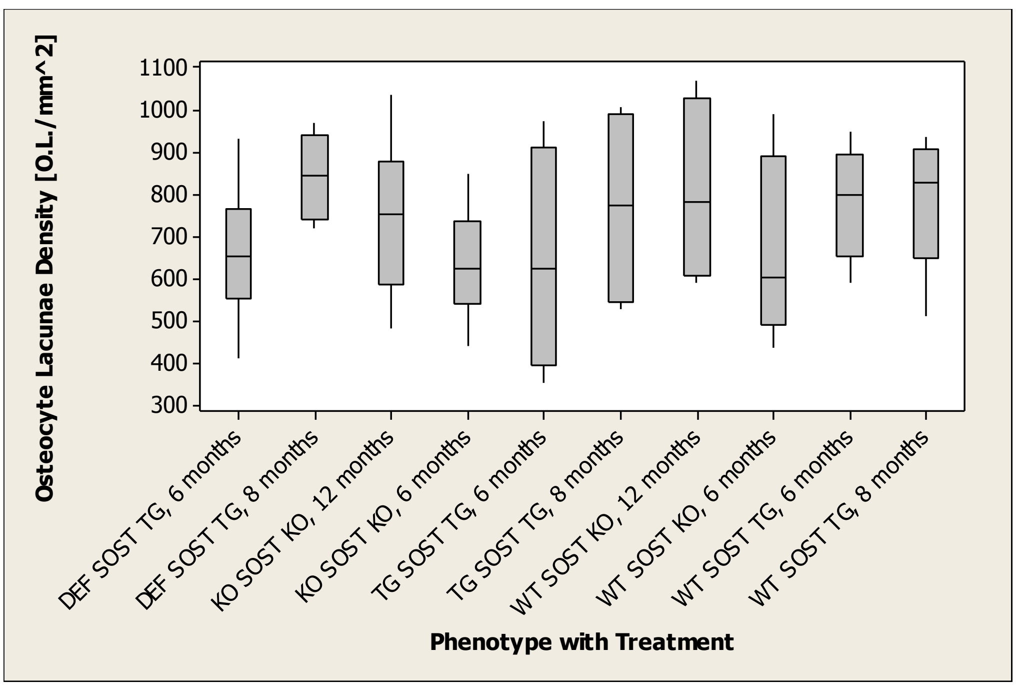


Figure 23: Boxplot osteocyte lacunae density results.

---

[2] Means that do not share a letter are significantly different.

DISCUSSION

Bone Volume Fraction

The group with a significantly lower bone volume fraction was the 12 month KO group. This group had the longest time to fully develop the phenotype, indicating the lack of the SOST gene eventually created the intended effect on the bones. The lack of significance in other groups could have been the result of an insufficient amount of time for the phenotype to develop.

Unanalyzed Bones

The number of bones does not add up to 100. For certain bones, labeled N/A in Appendix A, a bone volume fraction could not be taken because the resolution was not high enough. This resulted from pucks where the height could not be adjusted to 1mm below the adjustment bar due to the curvature of the puck. The curvature of the pucks also caused problems for another type of testing that was to be done after imaging, nano-indentation testing, so the pucks were re-done using the other half of the bones and a different method of sanding. In future research, the image analysis could be repeated with these flatter pucks to obtain results from every bone.

Other bones, labeled broken in Appendix A, could not be analyzed because were still broken at the cross section being analyzed. In future research this could be avoided if these bones were grouped together into pucks that could be sanded down to their unbroken state.

Osteocyte Lacunae Density

Although there was not a significant difference in the data, the p-value .071 was close to .05 and could have been the result of the low statistical power of 23.1%. Anything under 60% is considered to be low for this type of testing; therefore 23.1% is much too low to develop significant results. To raise the statistical power, the experiment could be repeated on a larger scale with more samples.

Future Image Analysis

Bone mineralization analysis is a technique that would yield useful results for osteoporosis research by revealing the specific composition of each bone [**10**]. This technique is done using an SEM machine and samples with known mineralization properties. The shades of gray from the bone samples are compared to the known samples to determine bones mineralization. Figure 24 demonstrates an example of what would result.

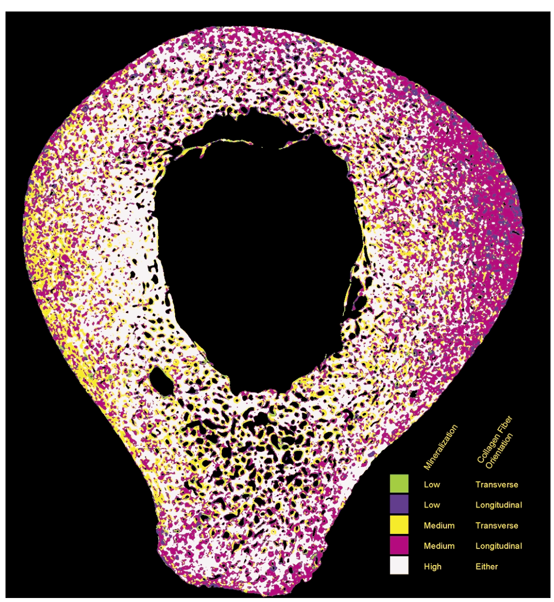


Figure 24: Bone mineralization analysis [**11**].

REFERENCES

[1] A.D.A.M. Medical Encyclopedia. (2010, Novemeber) US National Library of Medicine. [Online]. http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0001400/

[2] Mayo Foundation for Medical Education and Research. (2011, December) Mayo Clinic. [Online]. http://www.mayoclinic.com/health/osteoporosis/DS00128

[3] Philip Jacobs, "Van Buchem Disease," *Postgraduate Medical Journal*, pp. 497-505, August 1977.

[4] Cristal Yee, Deepa Murugesh, Richard Harland, Gabriela Loots Nicole M. Collette, "Preaxial polydactyly caused by hyperactive WNT signaling in Sclerostin/Sostdc1 double knockouts," *Developmental Biology*, pp. 264-274, 2011.

[5] Dietrich von Stechow, David Zurakowski, Ralph Mu¨ller,Brian D. Snyder Ara Nazarian, "Bone Volume Fraction Explains the Variation in Strength and Stiffness of Cancellous Bone Affected by Metastatic Cancer and Osteoporosis ," *Calcified Tissue International*, pp. 368-379, 2008.

[6] Geoffrey M. Henebry Geoffrey Dougherty, "Lacunarity analysis of spatial pattern in CT images of vertebral trabecular bone for assessing osteoporosis ," *Medical Engineering and Physics*, pp. 192-138, 2002.

[7] D.D. van der Meer, R. Huiskes, P. Lips M.G. Mullender, "Osteocyte density changes in aging and osteoporosis," *Bone*, pp. 109 - 113, 1996.

[8] C.D.L. Thomas, J.G. Clement, F. De Carlo, A.G. Peele K.M. Hannah, "Bimodal distribution of osteocyte lacunar size in the human femoral cortex as revealed by micro-CT ," *Bone*, pp. 866-871, August 2010.

[9] Gastone Marotti, Giorgia Volpi, Davide Zaffe, Silvana Palazzini, Francesca Remaggi, and Maria A. Muglia Valerio Can, "Size and Density of Osteocyte Lacunae in Different Regions of Long Bones," *Calcified Tissue International*, pp.

558-563, 1982.

[10] J. G. Skedros, E. G. Vajda, K. N. Bachus, and B. R. Constantz R. D. Bloebaum, "Determining Mineral Content Variations in Bone Using Backscattered Electron Imaging ," *Bone*, pp. 485-490, 1997.

[11] Aron Blayvas, Alan Boyde, Peter G.T. Howell, John G. Clement, Timothy G. Bromage Haviva M. Goldman, "Correlative Light and Backscattered Electron Microscopy of Bone — Part II: Automated Image Analysis," *Scanning*, pp. 337-344, 2000.

APPENDIX A

Phenotype with Treatment	Puck	Number	Sex	Side	BV/BT %	Ost/mm.
DEF SOST TG, 6 months	Puck 10	8587	M	R	97.18045113	929.321252
DEF SOST TG, 6 months	Puck 10	8601	M	R	99.98911223	844.985965
DEF SOST TG, 6 months	Puck 10	8641	M	R	99.98195489	563.129473
DEF SOST TG, 6 months	Puck 10	8642	M	R	93.31103679	844.081721
DEF SOST TG, 6 months	Puck 10	8658	M	R	98.84135472	738.623354
DEF SOST TG, 6 months	Puck 13	9530	F	L	99.879727	663.480486
DEF SOST TG, 6 months	Puck 13	9294	F	L	99.50903704	582.621042
DEF SOST TG, 6 months	Puck 13	9528	F	L	Broken	593.953058
DEF SOST TG, 6 months	Puck 13	9295	F	L	99.60762268	513.065813
DEF SOST TG, 6 months	Puck 14	9294	F	R	91.87352166	409.404429
DEF SOST TG, 6 months	Puck 14	9528	F	R	95.14175287	622.432466
DEF SOST TG, 6 months	Puck 14	9295	F	R	99.75925166	727.278852
DEF SOST TG, 6 months	Puck 14	9530	F	R	95.24538388	720.041497
DEF SOST TG, 6 months	Puck 9	8587	M	L	99.80145026	420.493011
DEF SOST TG, 6 months	Puck 9	8601	M	L	99.88874209	452.769316
DEF SOST TG, 6 months	Puck 9	8641	M	L	99.82469381	722.349732
DEF SOST TG, 6 months	Puck 9	8642	M	L	98.2075566	641.335895
DEF SOST TG, 6 months	Puck 9	8658	M	L	99.89394883	857.202937
DEF SOST TG, 8 months	Puck 17	5588	F	L	90.96450815	757.641258
DEF SOST TG, 8 months	Puck 17	5589	F	L	91.60155125	734.021873
DEF SOST TG, 8 months	Puck 17	8007	F	L	99.12653201	939.836080
DEF SOST TG, 8 months	Puck 17	8008	F	L	93.93939394	717.189692
DEF SOST TG, 8 months	Puck 18	5588	F	R	93.57715966	969.498734
DEF SOST TG, 8 months	Puck 18	5589	F	R	97.80028455	855.692354
DEF SOST TG, 8 months	Puck 18	8007	F	R	Broken	937.932691
DEF SOST TG, 8 months	Puck 18	8008	F	R	N/A	832.837202
KO SOST KO, 12 months	Puck 5	7592	M	L	N/A	737.943931
KO SOST KO, 12 months	Puck 5	7544	M	L	N/A	480.530761
KO SOST KO, 12 months	Puck 5	7593	M	L	N/A	876.885895
KO SOST KO, 12 months	Puck 5	7565	M	L	N/A	529.481689
KO SOST KO, 12 months	Puck 5	7594	M	L	N/A	603.457860
KO SOST KO, 12 months	Puck 6	7544	M	R	87.75437344	682.893748
KO SOST KO, 12 months	Puck 6	7565	M	R	96.1416311	764.943771
KO SOST KO, 12 months	Puck 6	7592	M	R	79.29436003	808.716137
KO SOST KO, 12 months	Puck 6	7593	M	R	79.31851852	878.416036

KO SOST KO, 12 months	Puck 6	7594	M	R	88.85620915	1036.835289
KO SOST KO, 6 months	Puck 1	9198	M	L	95.86474245	438.734603
KO SOST KO, 6 months	Puck 1	9200	M	L	93.38061466	846.114795
KO SOST KO, 6 months	Puck 1	9201	M	L	95.31151668	698.393695
KO SOST KO, 6 months	Puck 1	9202	M	L	96.08345534	846.152544
KO SOST KO, 6 months	Puck 1	9203	M	L	Broken	622.110427
KO SOST KO, 6 months	Puck 2	9198	M	R	92.41810051	553.185260
KO SOST KO, 6 months	Puck 2	9200	M	R	95.16433103	627.617937
KO SOST KO, 6 months	Puck 2	9201	M	R	97.18608169	619.427260
KO SOST KO, 6 months	Puck 2	9203	M	R	97.09459459	496.299769
KO SOST KO, 6 months	Puck 2	9202	M	R	93.20695103	635.231396
TG SOST TG, 6 months	Puck 7	9293	F	L	96.56518345	729.810577
TG SOST TG, 6 months	Puck 7	9296	F	L	99.92768371	351.024916
TG SOST TG, 6 months	Puck 8	9296	F	R	99.76963351	518.776598
TG SOST TG, 6 months	Puck 8	9293	F	R	85.50898204	970.735730
TG SOST TG, 8 months	Puck 17	5592	F	L	95.70575111	943.968468
TG SOST TG, 8 months	Puck 17	5599	F	L	99.23639581	600.620321
TG SOST TG, 8 months	Puck 18	5592	F	R	92.27326067	527.646934
TG SOST TG, 8 months	Puck 18	5599	F	R	94.86130931	1005.500330
WT SOST KO, 12 months	Puck 7	7545	M	L	87.81852082	1067.911845
WT SOST KO, 12 months	Puck 7	7564	M	L	Broken	589.236773
WT SOST KO, 12 months	Puck 8	7545	M	R	98.97330595	658.355884
WT SOST KO, 12 months	Puck 8	7564	M	R	92.83464567	901.394030
WT SOST KO, 6 months	Puck 3	9207	M	L	92.81624154	895.255084
WT SOST KO, 6 months	Puck 3	9208	M	L	92.31863442	868.367331
WT SOST KO, 6 months	Puck 3	9209	M	L	92.83507224	988.661587
WT SOST KO, 6 months	Puck 3	9240	M	L	93.33333333	971.444387
WT SOST KO, 6 months	Puck 3	9239	M	L	93.03266434	746.384480
WT SOST KO, 6 months	Puck 3	9238	M	L	92.5014997	474.068018
WT SOST KO, 6 months	Puck 4	9207	M	R	89.3183201	561.569549
WT SOST KO, 6 months	Puck 4	9208	M	R	93.07100662	628.825432
WT SOST KO, 6 months	Puck 4	9209	M	R	92.35119451	436.455181
WT SOST KO, 6 months	Puck 4	9238	M	R	89.2372767	456.986489
WT SOST KO, 6 months	Puck 4	9239	M	R	98.4572076	538.835926
WT SOST KO, 6 months	Puck 4	9240	M	R	N/A	577.342983
WT SOST TG, 6 months	Puck 11	8599	M	L	99.88566265	946.010718
WT SOST TG, 6 months	Puck 11	8600	M	L	99.98843931	904.460346
WT SOST TG, 6 months	Puck 11	8640	M	L	98.6162574	929.993211

WT SOST TG, 6 months	Puck 11	8656	M	L	99.9864743	639.968872
WT SOST TG, 6 months	Puck 11	8657	M	L	99.79941899	612.400314
WT SOST TG, 6 months	Puck 12	8599	M	R	99.75478668	779.669646
WT SOST TG, 6 months	Puck 12	8600	M	R	99.87856777	602.734607
WT SOST TG, 6 months	Puck 12	8640	M	R	98.11316993	668.209190
WT SOST TG, 6 months	Puck 12	8656	M	R	99.50112237	653.711282
WT SOST TG, 6 months	Puck 12	8657	M	R	99.75325516	941.236941
WT SOST TG, 6 months	Puck 15	9526	F	L	98.65635268	673.321137
WT SOST TG, 6 months	Puck 15	9527	F	L	99.37274525	820.314599
WT SOST TG, 6 months	Puck 15	9529	F	L	99.78833197	798.960178
WT SOST TG, 6 months	Puck 15	8643	F	L	95.69006206	890.745365
WT SOST TG, 6 months	Puck 15	8603	F	L	98.00542991	815.975400
WT SOST TG, 6 months	Puck 15	8602	F	L	96.38268567	633.380192
WT SOST TG, 6 months	Puck 16	8602	F	R	96.66873874	903.560923
WT SOST TG, 6 months	Puck 16	9526	F	R	99.73579283	771.558634
WT SOST TG, 6 months	Puck 16	9527	F	R	97.84447162	589.563778
WT SOST TG, 6 months	Puck 16	9529	F	R	97.87401575	792.973448
WT SOST TG, 6 months	Puck 16	8643	F	R	96.86631818	814.995977
WT SOST TG, 6 months	Puck 16	8603	F	R	98.6765759	835.412647
WT SOST TG, 8 months	Puck 19	5598	F	L	96.20034542	836.214102
WT SOST TG, 8 months	Puck 19	5597	F	L	99.9605164	762.664376
WT SOST TG, 8 months	Puck 19	5590	F	L	99.99143505	907.050865
WT SOST TG, 8 months	Puck 19	5593	F	L	Broken	511.256621
WT SOST TG, 8 months	Puck 20	5590	F	R	90.49166229	896.473947
WT SOST TG, 8 months	Puck 20	5593	F	R	96.69959167	934.069588
WT SOST TG, 8 months	Puck 20	5597	F	R	96.21212121	821.386515
WT SOST TG, 8 months	Puck 20	5598	F	R	99.79956207	609.714916