Title
An NMR metabolomic study on the effect of alendronate in ovariectomized mice.

Permalink
https://escholarship.org/uc/item/13q6p47n

Journal
PloS one, 9(9)

ISSN
1932-6203

Authors
Chen, Shin-Yu
Yu, Hui-Tzu
Kao, Ju-Po
et al.

Publication Date
2014

DOI
10.1371/journal.pone.0106559

Peer reviewed
An NMR Metabolomic Study on the Effect of Alendronate in Ovariectomized Mice

Shin-Yu Chen¹²³, Hui-Tzu Yu¹, Ju-Po Kao⁴, Chung-Chun Yang⁴, Shen-Shih Chiang¹², Darya O. Mishchuk⁵, Jeng-Leun Mau¹²*, Carolyn M. Slupsky⁵⁶*

¹ Department of Food Science and Biotechnology, National Chung Hsing University (NCHU), Taiwan, R.O.C., ² NCHU-UCD Plant and Food Biotechnology Center, NCHU, Taiwan, R.O.C., ³ Agricultural Biotechnology Center, NCHU, Taiwan, R.O.C., ⁴ Veterinary Medical Teaching Hospital, College of Veterinary Medicine, NCHU, Taiwan, R.O.C., ⁵ Department of Food Science and Technology, University of California Davis, Davis, California, United States of America, ⁶ Department of Nutrition, University of California Davis, Davis, California, United States of America

Abstract

Alendronate sodium (Fosamax) is most widely used for the prevention and treatment of osteoporosis. It is a type of anti-resorptive agent that reduces the risk of fractures by changing bone turnover and bone mineral density. We investigated the effect of Fosamax on a mouse model of osteoporosis. Twenty-seven female C57BL/6J/Narl mice were divided into three groups: sham, ovariectomized (OVX) and OVX + Fosamax (Fosamax). After 23 weeks, bone density of femurs was analyzed using microcomputed tomography (micro-CT), and serum was analyzed for osteoblast and osteoclast activity, as well as metabolites using nuclear magnetic resonance (NMR) spectroscopy. Fosamax increased bone mineral density and cortical bone thickness, and decreased osteoblast activity slightly. Fosamax did not significantly change osteoclast activity. Serum metabolomics revealed that Fosamax had profound effects on overall metabolism, as significantly higher concentrations of metabolites associated with energy metabolism (including TCA-cycle intermediates and glucose), 3-hydroxybutyrate, taurine, allantoin, acetate, and ethanol, as well as lower concentrations of aspartate were observed in the Fosamax-treated mice compared with the OVX mice. These results suggest that alendronate may work by increasing bone density through altered metabolic activity.

Citation: Chen S-Y, Yu H-T, Kao J-P, Yang C-C, Chiang S-S, et al. (2014) An NMR Metabolomic Study on the Effect of Alendronate in Ovariectomized Mice. PLoS ONE 9(9): e106559. doi:10.1371/journal.pone.0106559

Introduction

Osteoporosis is a major public health issue. It is a skeletal disease that is defined by decreased bone mass combined with microarchitectural deterioration of bone tissue resulting in a consequent increase in bone fragility and susceptibility to fracture. Osteoporosis typically presents later in life, particularly in postmenopausal women, and its prevalence is expected to increase dramatically in the coming decades due to an ageing population [1,2,3]. The reason postmenopausal women are more susceptible to osteoporosis is due to reduced ovarian function resulting in decreased estrogen. Estrogen directly affects bone turnover by stimulating osteoblast activity (which forms bone) through increasing osteoblast formation, differentiation, proliferation, and function, and inhibiting osteoclast activity (which resorbs bone) through inducing osteoclast apoptosis, and inhibiting osteoclast formation [4,5]. Thus, estrogen deficiency is directly related with bone loss, and postmenopausal estrogen deficiency causes accelerated bone loss. Postmenopausal osteoporosis affects 20% of women aged 60–69 years, and in the UK it was found that out of the 60,000 people who suffer osteoporotic hip fractures each year, 15–20% die from complications within a year [6].

Alendronate sodium (Fosamax), a nitrogen-containing bisphosphonate, is most widely used for the prevention and treatment of osteoporosis. Bisphosphonates accumulate in the mineral phase of bone and inhibit bone resorption through inhibition of osteoclast activity. The degree to which bone turnover and bone mineral density change upon treatment with anti-resorptive agents is directly correlated with a reduction in the risk of fractures [4,7–10]. However, many side effects of bisphosphonate medications, including severe suppression of bone turnover that may develop during long-term therapy, actually increase the risk of fracture [11,12]. Bisphosphonates can also cause osteonecrosis of the jaw (ONJ), with higher risk in oncology patients treated with high dose bisphosphonate therapy [13–15]. Other relevant possible side effects include gastrointestinal (GI) upset, musculoskeletal pain, atrial fibrillation, and esophageal cancer [16,17].

There are few reports on the relationship between postmenopausal osteoporosis treatment with Fosamax and overall metabolism. To understand the global changes associated with Fosamax use, we investigated ovariectomized mice treated with Fosamax to determine its effects on serum metabolites using NMR spectroscopy. These results are compared with bone density using...
Effect of Alendronate in Ovariectomized Mice

Materials and Methods

Ethics statement

This study was performed in strict accordance with animal use protocols approved by National Chung Hsing University Institutional Animal Care and Use Committee (IACUC, approval number: 99–62). All surgery was performed under anesthesia, and all efforts were made to minimize suffering.

Animals and design

Twenty-seven female C57BL/6JNarl mice aged 7 weeks were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and acclimated to conditions for 5 weeks before the start of the experiment. Animals were housed in an air-conditioned room in autoclavable cages (4-5 mice per cage) with ventilating tops and stainless steel lips (BioLASCO, Taipei, Taiwan) with 12 h light/dark illumination cycles at a constant temperature of 25±2°C and humidity of 65±5%. Tapvei aspen bedding was purchased from Young Li (New Taipei, Taiwan) and changed every week. Drinking water and food (basal diet Lab 5001 (Purina Mills, St. Louis, MO, USA)) were supplied every week. Drinking water and food (basal diet Lab 5001 (Purina Mills, St. Louis, MO, USA)) were supplied every week. Drinking water and food (basal diet Lab 5001 (Purina Mills, St. Louis, MO, USA)) were supplied every week. Drinking water and food (basal diet Lab 5001 (Purina Mills, St. Louis, MO, USA)) were supplied every week. Drinking water and food (basal diet Lab 5001 (Purina Mills, St. Louis, MO, USA)) were supplied every week. Drinking water and food (basal diet Lab 5001 (Purina Mills, St. Louis, MO, USA)) were supplied every week. Drinking water and food (basal diet Lab 5001 (Purina Mills, St. Louis, MO, USA)) were supplied every week. Drinking water and food (basal diet Lab 5001 (Purina Mills, St. Louis, MO, USA)) were supplied every week.

Bilateral ovariectomy (OVX) was performed on 19 twelve weeks old mice after intramuscular injection of an anesthetic cocktail composed of 0.03 mL Zoletil 50 and 0.01 mL 0.2% Xylazine, as described by Lasota [18]. Another eight mice were given sham surgery. At 2 weeks post-surgery (week 0), 9 of the 19 ovariectomized mice were randomly assigned to the OVX (no treatment) group, and 10 to the Fosamax (treatment) group. The Fosamax group was then fed Fosamax plus (Merck, Whitehouse Station, NJ, USA) once a week based on the human equivalent dose [19], and contained 70 mg alendronic acid and 2800 IU of cholecalciferol. Blood was collected from the cheeks of mice after 12 weeks of treatment to measure osteocalcin and alkaline phosphatase (ALP). After 23 weeks, mice were sacrificed by CO2 asphyxiation, and blood was collected by cardiac puncture. Tissue samples for histomorphometry were removed from each femur, and the bone was placed in ethanol until analysis. For serum, blood was allowed to clot for 15 minutes on ice, centrifuged at 5,000 rpm for 15 min, and the top layer was collected and stored at −80°C until ready for analysis.

Histomorphometric analysis using microcomputed tomography

To perform qualitative and quantitative analysis of bone, the right femurs were analyzed using a high performance in vivo micro-CT Skyscan 1076 (Skyscan, Kontich, Belgium), with image field at pixel size 9 μm. Three-dimensional images were reconstructed using CTvol (Skyscan). The distal femoral metaphysis was analyzed from a region that was 1.0 mm below the growth plate and 1.5 mm in length. For quantitative analysis, the software CTAn (Skyscan) was used to obtain the following parameters within the region of interest (ROI): bone volume/tissue volume (BV/TV), bone surface/bone volume (BS/BV), bone surface/tissue volume (BS/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), trabecular pattern factor (Tb.Pf), structure model index (SMI), connectivity density (Conn.Dn) and trabecular bone mineral density (Tb.BMD). The SMI quantifies the plate-rod characteristics of the 3D trabecular structure [20]. The Conn.Dn was calculated using the Euler method [21]. Cortical bone parameters (including cortical bone shell thickness, cortical BMD, endosteal circumference (EC), periosteal circumference (PC) and cross-sectional area) were analyzed in the middle of the diaphysis of the femur.

Measurement of serum indicators of osteoblast and osteoclast activity, and serum concentrations of calcium and phosphate

We determined the activity of osteoblast and osteoclast cells using serum indicators rather than histomorphometry. Serum concentrations of alkaline phosphatase (ALP), osteocalcin, procollagen I C-terminal propeptide (PICP), pyridinoline (PYD), cross-linked N-telopeptide of type I collagen (NTX) were all measured using commercial kits according to manufacturer’s instructions. (ALP: Alkaline phosphatase activity fluorometric assay kit (Biovision, Mountain View, CA, USA); osteocalcin: Mouse osteocalcin ELISA kit (Biomedical Technologies, Stoughton, MA, USA); PICP: ELISA kit for mouse PICP (USCN Life Science, Wuhan, Hubei, China); PYD: Mouse pyridinoline ELISA kit (CUSABIO, Wuhan, Hubei, China); NTX: ELISA kit for mouse NTX (USCN Life Science, Wuhan, Hubei, China)). Calcium was determined with a calcium colorimetric assay kit (Biovision, Mountain View, CA, USA), and phosphate was measured with a phosphate colorimetric assay kit (Biovision, Mountain View, CA, USA).

Serum Metabolomics

For serum metabolomics measurement, serum samples were prepared by thawing and centrifuging at maximum speed for 20 min and 4°C to remove particulate matter. The serum supernatant was subsequently filtered through Amicon 3,000 molecular weight (MW) cutoff filters to remove protein and lipid particles. If needed, the serum filtrates were diluted with deionized water to a total volume of 207 μL and 23 μL of an internal standard (Chenomx Inc., Edmonton, Alberta, Canada) containing 4.8366 mM 3-(trimethylsilyl)-1-propanesulfonic acid-d6 (DSS-d6) and 0.2% Na3 in 98% D2O was added. The pH value was adjusted to 6.8 for each sample by adding small amounts of 1 N HCl or NaOH. 180 μL of each sample was transferred into a 3 mm NMR tube, and samples were stored at 4°C until NMR data acquisition (within 24 h of sample preparation).

NMR spectra were acquired using the Bruker noesyp1drl experiment on a Bruker Avance 600 MHz NMR spectrometer equipped with a SampleJet as previously described [22]. Identification and quantification of metabolites were accomplished using Chenomx NMRSuite 7.6 (Chenomx Inc., Edmonton, Canada) [23]. For each mouse, 55 metabolites were measured and quantified from the serum which included 2-hydroxybutyrate, 2-oxoglutarate, 2-oxoisocaproate, acetate, acetocacetate, acetone, alanine, allantoin, arginine, ascorbate, asparagine, aspartate, betaine, carnitine, choline, citrate, creatine, creatinine, dimethylamine (DMA), ethanol, formate, fumarate, glucose, glutamate, glutamine, glycerol, glycine, histidine, isoleucine, lactate, leucine, lysine, malate, mannose, methanol, methionine, N,N-dimethylglycine (DMG), acetylcarnitine, ornithine, phenylalanine, proline, pyruvate, serine, serotonin, succinate, taurine, threonine, trimethylamine (TMA), tryptophan, tyrosine, uracil, urea, uridine and valine. Samples were corrected for dilution by multiplying by a correction factor calculated by the ratio of the final sample volume divided by the initial volume of serum. Those samples for which there was limited volume were excluded from NMR analysis.
Statistics

Unless otherwise indicated, all comparisons between the Fosamax group and either the sham or OVX group regarding bone measurements were made using Student’s t-test, and comparison between multiple groups were made using ANOVA followed by Bonferroni’s multiple t test. For metabolite data, all comparisons were made using Kruskal-Wallis non-parametric analysis followed by Dunn’s multiple comparison test. For time-course data (body weight), analysis was accomplished using repeated measures two-way ANOVA. Statistical significance was defined as \( p < 0.05 \). All data are expressed as mean ± standard error (SEM) or mean ± standard deviation (SD). Data sets were analyzed for statistical significance using Prism GraphPad 6 software (GraphPad Software Inc., San Diego, CA). Multivariate data analysis (Principal Component Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA)) was performed on log10 - transformed metabolite concentrations using SIMCA-P (version 13.0, Umetrics, Umeå, Sweden) as described in Slupsky [24]. The number of components was chosen using a 7-fold cross validation rule. The quality of the models were judged by the goodness-of-fit parameter (R2X), and the predictive ability (Q2) defined as equation 2.

Results

Total body weight

Average body weight for each group of mice over 23 weeks after surgery is shown in Figure 1. Analysis by repeated measures 2-way ANOVA revealed a significant effect of time \( (p < 0.0001) \) and treatment \( (p = 0.0011) \) on each group, and a significant interaction effect \( (p < 0.0001) \). Calculation of the % increase in body weights of each of the groups at week 22 revealed an increase of 23% (sham), 36% (OVX) and 47% (Fosamax), compared with the starting weights. The corresponding R2 and Q2 calculated and compared with the original model.

Micro-computed tomography analysis

After 23 weeks of treatment, the trabecular bone parameters in the distal femur were analyzed by micro-CT and are shown in Figure 3. No significant difference was observed between sham and OVX mice for any of the parameters; however, Tb.BMD, BV/TV, BS/TV, Tb.Th, Tb.N and Conn.Dn were significantly different between the Fosamax-treated mice and the sham or OVX mice as shown by separation in the first component, whereas a smaller difference between sham and OVX mice was observed in the second component. Twelve metabolites were primarily responsible for the difference between OVX and Fosamax-treated mice after 23 weeks of treatment, and are summarized in Table 1. TCA-cycle intermediates (citrate, 2-oxoglutarate, malate, fumarate, and succinate), amino acids (taurine), glucose, 3-hydroxybutyrate (a ketone), and others (acetate, allantoin, and ethanol) were significantly higher in Fosamax-treated mice compared with OVX, while aspartate was significantly lower.

Discussion

Type 1 (postmenopausal) osteoporosis is characterized by hormonal deprivation resulting in accelerated bone turnover and bone loss [25]. The OVX rat and mouse are classic models used to study postmenopausal bone loss since removal of the ovaries mimics postmenopausal hormonal changes that occur in humans, including decreases in estrogen and progesterone [26]. In this study, OVX mice were compared with OVX mice treated with Fosamax, as well as mice that received sham surgery. Interestingly, weight gain of OVX mice over 23 weeks was higher than sham mice, even though their food intake was similar. It has previously been shown that estrogen deficiency can cause higher body weight and body fat deposition [27,28], which may partially explain our results.

Metabolomics can measure changes in the levels of metabolites found in biological fluids and tissues, which can produce a
quantitative and comprehensive indication of metabolic pathway activity. It can offer information to understand the effect of diet, drugs and disease, and assessment of a disease state [29,30]. Few metabolic differences between OVX and sham mice were observed. One interesting difference was the significantly lower glycine concentration in OVX mice compared with sham. Glycine has been shown to be inversely associated with cholesterol, phospholipid, free fatty acid, and triglyceride accumulation [31], which may indicate changes in fat metabolism. Lower dimethylamine, and ethanol may be related to changes in gut microbial metabolism, and lower allantoin may indicate lower oxidative stress [32] in mice with ovariectomy compared with sham.

Despite the minor metabolic changes that occurred in the OVX mice, they were similar to sham mice with respect to bone-related measurements. We speculate that the age of the mice may have been a factor. Indeed, a report evaluating age-related changes in trabecular architecture in C57BL/6J mice showed that the trabecular bone volume (BV/TV) is greatest at 6–8 weeks of age, and declines steadily thereafter. Moreover, trabecular bone loss, as measured by trabecular number (Tb.N), is most rapid between 2–6 months of age, with a more gradual decline thereafter [33]. Our study commenced when the mice were 3 months of age (12 weeks), and continued until the mice were ~9 months of age (35 weeks).

Fosamax-treated ovariectomized mice revealed increased Tb.BMD, BV/TV, Tb.Th, Tb.N, and cortical bone thickness as well as decreased Tb.Sp, and SMI compared with both OVX and sham, which demonstrates that Fosamax appears to increase bone mass and strength as has been previously reported [34–36]. Furthermore, some reports indicate that bisphosphonates suppress the resorption and formation of bones, thereby decreasing bone turnover and lowering bone loss [37,38]. Several serum indicators of osteoblast and osteoclast activity, indicators of bone turnover, were examined. Indicators of osteoblast activity included ALP, osteocalcin, and PICP. Fosamax-treated mice exhibited lower ALP, but no significant difference in osteocalcin or PICP at week 23 compared with the OVX mice. Various studies have shown that alendronate treatment either decreases serum osteocalcin significantly, or brings about no significant change in osteoblast activity [34,37,39], which supports the observations presented here. Interestingly, osteocalcin levels in Fosamax-treated mice at week 12 were higher than sham or OVX mice, and higher than all three groups of mice at 23 weeks. We speculate that the cholecalciferol found in Fosamax may be responsible for the initial increase of osteocalcin observed at 12 weeks [40]. No significant difference in either of the osteoclast activity indicators (PYD and NTXI) after Fosamax treatment for 12 or 23 weeks was observed.

Administration of Fosamax for 23 weeks to OVX mice resulted in a significantly higher rate of weight gain over the course of the experiment compared with both sham and OVX mice. While the OVX mice receiving Fosamax treatment had a slightly elevated intake of chow compared with sham, intake was not significantly different between OVX and Fosamax-treated mice, suggesting that other factors may be responsible for the increased rate of weight gain of Fosamax mice. Interestingly, other studies in rats have shown that alendronate treatment results in higher body weight compared with OVX [28]. One possibility of the increased

Figure 1. Body weight of mice over 23 weeks. Body weight of sham (circle), OVX (square), and OVX + Fosamax (triangle) C57BL/6 mice over 23 weeks. Time 0 represents the time of surgery. Analyzed by 2-way analysis of variance (ANOVA). Each value is expressed as mean ± SEM. doi:10.1371/journal.pone.0106559.g001

Figure 2. Weekly food intake. Average weekly food intake of sham C57BL/6 female mice compared with ovariectomized mice with and without Fosamax treatment during weeks 8–23. Analyzed by analysis of variance (ANOVA) followed by Bonferroni’s multiple t test. Statistical significance is defined as p < 0.05. Each value is expressed as mean ± SD. doi:10.1371/journal.pone.0106559.g002
Figure 3. Micro-CT femur parameters. Bone characteristics of sham C57BL/6 female mice compared with ovariectomized mice with and without Fosamax treatment for 23 weeks. (A–J) Bone parameters of the distal femur analyzed by micro-CT. (A) Tb.BMD, trabecular bone mineral density; (B) BV/TV, bone volume/tissue volume; (C) BS/TV, bone surface/tissue volume; (D) Tb.Th, trabecular thickness; (E) Tb.N, trabecular number; (F) Conn.Dn, connectivity density; (G) BS/BV, bone surface/bone volume; (H) Tb.Sp, trabecular separation; (I) Tb.Pf, trabecular pattern factor; (J) SMI, structure model index. (K–O) Cortical bone parameters of the middle diaphysis analyzed by micro-CT. (K) Thickness, cortical bone shell thickness; (L) cortical BMD, cortical bone mineral density; (M) EC, endosteal circumference; (N) PC, periosteal circumference; (O) cross-sectional, cross-sectional area. Analyzed by analysis of variance (ANOVA) followed by Bonferroni’s multiple t test. Statistical significance is defined as p<0.05. Each value is expressed as mean ± SD.
doi:10.1371/journal.pone.0106559.g003

Figure 4. Micro-CT three-dimensional images. Femur images of sham, OVX and OVX + Fosamax C57BL/6 female mice treatment for 23 weeks. The distal femur region of: (A) sham mice, (B) OVX mice, and (C) Fosamax-treated mice. Femoral mid-diaphysis of: (D) sham mice (E) OVX mice, and (F) Fosamax-treated mice.
doi:10.1371/journal.pone.0106559.g004
weight gain could be the neo-formation of bone tissue [41]. However, other factors may also affect body weight, including changes in global metabolism through changes in adiponectin, leptin and energy metabolism (glucose, 3-hydroxybutyrate and taurine), as well as activity of osteoblast cells. Interestingly, adiponectin and leptin, the main circulating peptides secreted by adipose tissue, have been shown to be involved with regulation of bone metabolism [42,43], and treatment with alendronate for 12 months has been shown to slightly decrease serum leptin [44].

One interesting observation was a decrease in osteocalcin at week 23 compared with week 12 in Fosamax-treated mice. Osteoblasts are known to secrete hormones that affect energy metabolism. Indeed, mice deficient in osteocalcin are obese, having decreased β-cell proliferation and secretion, greater insulin resistance, and decreased energy expenditure [45–47]. Insulin is an important molecule associated with bone remodeling and energy metabolism [48]. While insulin was not directly measured in this study, serum metabolites of Fosamax-treated mice were compared with OVX mice, revealing higher serum glucose and higher 3-hydroxybutyrate in Fosamax-treated mice compared with OVX mice. Hyperglycemia has been correlated with diabetic ketoacidosis, which can result in stimulation of lipolytic pathways, resulting in free fatty acids and ketone bodies in the blood [49]. However, it is puzzling that there is an almost 2-fold higher concentration of osteocalcin in Fosamax-treated mice at week 12, which then appears to return to levels similar to OVX and sham mice at week 23. It is tempting to speculate that this change in osteocalcin levels may lead to insulin resistance and lipolysis [48], followed by increased weight gain and risk of diabetes [39,45,47]. Indeed, most of the increased weight gain of the Fosamax treated mice was in the later part of the experiment, and these results could suggest that prolonged use of the drug may have negative consequences. However, this will need to be elucidated in a further study. Indeed, there is much controversy in the literature on the correlation of bisphosphonate therapy with glycemia, diabetes and body weight [50–52], and whether these observations are restricted to mice or may be extended to humans remains to be clarified.
Table 1. Comparison of serum metabolite concentrations after 23 weeks.

| Metabolite* (μM) | Sham      | OVX       | Fosamax   | p-value | Adjusted p-value OVX vs Fosamax | Adjusted p-value Sham vs OVX |
|------------------|-----------|-----------|-----------|---------|---------------------------------|-------------------------------|
| TCA cycle        |           |           |           |         |                                 |                               |
| Citrate          | 293.1±24.6| 263.5±38.2| 365.2±37.6| <0.0001 | 0.0020                          | >0.99                         |
| 2-Oxoglutarate   | 32.3±12.0 | 35.3±27   | 49.7±98   | 0.0010  | 0.0202                          | >0.99                         |
| Malate           | 106.4±26.6| 93.0±38.4 | 176.4±47.4| 0.0010  | 0.0055                          | >0.99                         |
| Fumarate         | 10.9±5.0  | 13.0±50   | 19.6±5.1  | 0.0067  | 0.0693                          | >0.99                         |
| Succinate        | 436.8±139.9| 389.7±138.3| 679.8±187.3| 0.0165  | 0.0341                          | >0.99                         |
| Amino acids      |           |           |           |         |                                 |                               |
| Aspartate        | 39.1±10.3 | 38.2±9.0  | 23.8±7.2  | 0.0020  | 0.0325                          | >0.99                         |
| Taurine          | 460.8±95.1| 467.6±116.0| 669.2±127.6| 0.0040  | 0.0341                          | >0.99                         |
| Glycine          | 313.8±41.1| 249.8±42.5| 289.5±38.5| 0.0482  | 0.5338                          | 0.0468                        |
| Energy metabolism|           |           |           |         |                                 |                               |
| Glucose          | 13188.6±2517.5| 14309.8±1897.3| 18547.8±2747.4| 0.0007  | 0.0468                          | >0.99                         |
| Ketones          |           |           |           |         |                                 |                               |
| 3-Hydroxybutyrate| 110.8±44.8| 76.2±17.9 | 168.5±63.8| 0.0052  | 0.0081                          | 0.6103                        |
| Others           |           |           |           |         |                                 |                               |
| Acetate          | 65.1±19.2 | 44.6±16.1 | 78.7±14.9 | 0.0095  | 0.0110                          | 0.2787                        |
| Allantoin        | 165.6±50.3| 100.4±12.2| 138.7±32.8| 0.0047  | 0.0609                          | 0.0089                        |
| Ethanol          | 188.4±81.4| 85.1±35.5 | 300.1±233.9| 0.0025  | 0.0062                          | 0.0397                        |
| Dimethylamine    | 4.1±1.5   | 1.9±0.7   | 2.7±0.6   | 0.0015  | 0.4062                          | 0.0012                        |

*Significantly different metabolites between OVX and Fosamax-treated mice or Sham and OVX mice. Each value is expressed as mean ± SD. P-values were calculated using the Kruskal-Wallis test, and adjusted p-values were calculated using Dunn's multiple comparison test. Alpha was set to 0.05.

doi:10.1371/journal.pone.0106559.t001
Other metabolites that may be connected with osteoporosis include 3-hydroxybutyrate and taurine [53-56]. 3-hydroxybutyrate has been shown to reduce bone absorption, maintain normal bone function and femur bone mineral density, thus reducing osteoporosis [53]. We observed higher 3-hydroxybutyrate in Fosamax-treated treated mice. Our histomorphometric results also showed taurine was higher in Fosamax-treated mice. Interestingly, taurine supplementation has been shown to increase bone mineral density in rats [34,55]. It can bind divalent cations, including Mg²⁺, and has an important role in bone remodeling and skeletal development [56].

Our results showed that while Fosamax increased bone mineral density and lowered bone loss, it only decreased serum osteoblast activity slightly, and did not affect osteoclast activity. Serum metabolomics revealed that Fosamax primarily affected energy metabolism. Specifically, Fosamax increased the concentrations of serum glucose, 3-hydroxybutyrate and taurine, which have been shown to be positivity related to bone health, but also significantly increased the concentrations of TCA cycle intermediates, suggesting that mitochondrial function may also be affected by Fosamax treatment. Significantly increased weight gain of Fosamax-treated mice, particularly in the later weeks of treatment, suggest that long-term Fosamax use appears to impact metabolism, but it is unclear at this stage how these metabolic changes may lead to complications observed with long-term use of bisphosphonates. Future studies will explore changes in serum insulin, adiponectin, and leptin with treatment, as well as determine global metabolic changes at 12 weeks to compare with 24 weeks to determine the length of time of maximum benefit.

Acknowledgments
We would like to thank the National Laboratory Animal Center of Taiwan for technical support in Micro-CT experiment.

Author Contributions
Conceived and designed the experiments: SYC JPK CCY SSC JLM CMS. Performed the experiments: SYC HTY DOM. Analyzed the data: SYC CMS. Contributed reagents/materials/analysis tools: JPK CCY SSC JLM CMS. Contributed to the writing of the manuscript: SYC CMS JLM.

References
1. Burge R, Dawson-Hughes B, Solomon DH, Wong JB, King A, et al. (2007) Incidence and economic burden of osteoporosis-related fractures in the United States, 2005–2025. J Bone Miner Res 22: 465–473.
2. Chang KL, Hu YC, Hsieh BS, Hsu HW, et al. (2013) Combined effect of soy isoflavones and vitamin D₃ on bone loss in ovariectomized rats. Nutrition 29: 251–257.
3. Kanis JA, the WHO Study Group (1994) Assessment of fracture risk and its application to screening for postmenopausal osteoporosis: a WHO report. Osteoporos Int 4: 306–381.
4. Farzana MF, Ventura A, Marzano F, Cavallo L (2013) Postmenopausal osteoporosis: the role of immune system cells. Clin Dev Immunol 2013: 1–6.
5. Riggs BL, Khosla S, Melton III LJ (2002) Sex steroids and the construction and conservation of the adult skeleton. Endocr Rev 23: 279–302.
6. Taladikar VS, Chambers T, Manyonda I (2012) Exploiting the antioxidant effect of soy isoflavones and vitamin D₃ on bone loss in ovariectomized rats. Nutrition 29: 251–257.
7. Sambrook PN, Geusens P, Ribot C, Soliman JA, Ferrer-Barriendos J, et al. (2004) Alendronate produces greater effects than raloxifene on bone density and bone turnover in postmenopausal women with low bone density: results of EFFECT (Efficacy of FOSAMAX versus EVISTA Comparison Trial) International. J Intern Med 255: 305–311.
8. Rusell RGG, Watts NB, Ehtene FF, Rogers MJ (2008) Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy. Osteoporos Int 19: 733–759.
9. Kendler DL, Rusc C, Benhamou CL, Brown JP, Lillested M, et al. (2010) Effects of denosumab on bone mineral density and bone turnover in postmenopausal women transitioning from alendroantrate to placebo. J Bone Miner Res 25: 72–81.
10. Delmas PD, Seeman E (2004) Changes in bone mineral density explain little of the reduction in vertebral or nonvertebral fracture risk with anti-resorptive therapy. Bone 34: 599–604.
11. Olvina CV, Zervath JF, Rao DS, Malaunf N, Grotzalch FA, et al. (2005) Severely suppressed bone turnover: a potential complication of alendronate therapy. J Clin Endocrinol Metab 90: 1294–1301.
12. Neustadt J, Pieczenik S (2012) Osteoporosis: beyond bone mineral density. A special report on the state of osteoporosis research. NBI Health website. Available: http://www.nbihhealth.com/supporting/osteoportunositeport.pdf. Accessed 2014 Jun 7.
13. Varun B, Srikumar T, Nair BJ, Joseph AP (2012) Bisphosphonate induced osteonecrosis of jaw in breast cancer patients: A systematic review. J Oral Maxillofac Pathol 16: 210–214.
14. Yamazaki T, Yamori M, Tanaka S, Yamamoto K, Suni E, et al. (2013) Risk factors and indices of osteoclasts in the jaw in osteoporosis patients: results from a hospital-based cohort study in Japan. PLoS One 8: e79376.
15. Khan AA, Sándor GK, Dore E, Morrison AD, Alsahl M, et al. (2009) Bisphosphonate associated osteonecrosis of the jaw. Rheumatol 36: 478–490.
16. Watts NB, Diab DL (2010) Long-term use of bisphosphonates in osteoporosis. J Clin Endocrinol Metab 95: 1555–1563.
17. Sener G, Kapucu C, Cetinl S, Cikler E, Ayangogo-Dilger G (2005) Gastroprotective effect of leuteosine recepter blocker montelukast in alendronait-induced lesions of the rat gastric mucosa. Prostaglandins Leukot Essent Fatty Acids 72: 11–18.
18. Lasaot A, Danovska-Klenovska D (2004) Experimental osteoporosis- different methods of ovariectomy in female rats. Rocz Akad Med Bialymst 49 Suppl 1: 129–131.
19. U.S. Department of Health and Human Services Food and Drug Administration (2002) Estimating the safe starting dose in clinical trials for therapeutics in adult healthy volunteers. FDA website. Available: http://www.fda.gov/ODRHB/DOCKETS/98fr/02d-0492-gd0001-vol1.pdf. Accessed 2014 Jun 7.
20. Härlebrand T, Rüegsegger P (1997) Quantification of bone microarchitecture with the structure model index. Comput Methods Biomech Biomed Engin 1: 15–23.
21. O’Sullivan A, Willoughby RE, Mihoshch D, Akarraz B, Cabezal-Sanchez C, et al. (2013) Metabolomics of cerebrospinal fluid from humans treated for rabies. J Proteome Res 12: 481–490.
22. Weljie AM, Newton J, Mercier P, Carlson E, Shinsky CM (2006) Targeted profiling quantitative analysis of H NMR metabolomics data. Anal Chem 78: 4430–4442.
23. Slipsky CM, Rankin KN, Wagner J, Fu H, Chang D, et al. (2007) Investigations of the effects of gender, diurnal variation, and age in human urinary metabolite profiles. Anal Chem 79: 6995–7004.
24. Vs K, K P, Ramesh M, Venkatesan V (2013) The association of serum osteocalcin with the bone mineral density in post menopausal women. J Clin Diagn Res 7: 814–816.
25. Rendina E, Henhure KD, Davis MR, Marlow D, Clarke SL, et al. (2013) Dried plums unique capacity to reverse bone loss and alter bone metabolism in postmenopausal osteoporosis model. PlOeB 8: e60609.
26. Zhao X, Wu ZK, Zhang Y, Yan YB, He Q, et al. (2011) Anti-osteoiposis activity of Cibotium barometz extract in ovariectomy-induced bone loss in rats. J Ethnopharmacol 137: 1083–1088.
27. Abdell-Satee KA, Mansour H (2012) Effects of leptin on metabolic bone turnover in ovariectomy rats. Acta Endocrinol 8: 189–198.
28. Nicholson JK, Lindon JC (2008) Systems biology: Metabonomics. Nature 455: 1054–1056.
29. DelBerardinis RJ, Thompson CB (2012) Cellular metabolism and disease: what do metabolic outsiders teach us? Cell 148: 1332–1144.
30. Senthilkumar R, Viswanathan P, Nalini N (2003) Glycine modulates hepatic lipid accumulation in alcohol-induced liver injury. Pol J Pharmacol 55: 603–611.
31. Dryland PA, Love DR, Walker MF, Dommes Y, Butt C, et al. (2008) Allantoin as a biomarker of inflammation in an inflammatory bowel disease mouse model: NMR analysis of urine. Open Biost Compl J 1, 1–6.
32. Glant V, Canalis E, Statham D, Bouxsein ML (2007) Age-related changes in osteocalcin with the bone mineral density in post menopausal women. J Intern Med 261: 229–237.
33. Glant V, Canalis E, Statham D, Bouxsein ML (2007) Age-related changes in trabecular architecture differ in female and male C57BL/6J mice. J Bone Miner Res 22: 1197–1207.
34. Cho DC, Kim KT, Jeon Y, Sung JK (2012) A synergistic bone sparing effect of curcumin and alendronate in ovariectomized rat. Acta Neurochir 154: 2215–2223.
35. Yamane H, Sakai A, Mori T, Tanaka S, Moridera K, et al. (2009) The anabolic action of intermittent PTH in combination with cathepsin K inhibitor or alendronate differs depending on the remodeling status in bone in ovariectomized mice. Bone 44: 1053–1062.
36. Davison KS, Siniomski KJ, Ashadi JD, Hanley DA, Goltzman D, et al. (2006) Bone strength: the whole is greater than the sum of its parts. Semin Arthritis Rheum 36: 22–31.
37. Seo SK, Yang HI, Lim KJ, Jeon YE, Choi YS, et al. (2012) Changes in serum levels of cartilage oligomeric matrix protein after estrogen and alendronate therapy in postmenopausal women. Gynecol Obstet Invest 74: 143–150.
38. Iwamoto J, Seki A, Takeda T, Sato Y, Yamada H, et al. (2006) Comparative effects of alendronate and alfacalcidol on cancellous and cortical bone mass and bone mechanical properties in ovariectomized rats. Exp Anim 55: 357–367.
39. Lacombe J, Karsevent G, Ferron M (2013) In vivo analysis of the contribution of bone resorption to the control of glucose metabolism in mice. Mol Metab 2: 496–504.
40. Clemens TL, Tang H, Maca S, Kesterson RA, Demayo F, et al. (1997) Analysis of osteocalcin expression in transgenic mice reveals a species difference in vitamin D regulation of mouse and human osteocalcin genes. J Bone Miner Res 12: 1570–1576.
41. Notomi T, Okimoto N, Okazaki Y, Nakamura T, Suzuki M (2003) Tower climbing exercise started 3 months after ovariectomy recovers bone strength of the femur and lumbar vertebrae in aged osteopenic rats. J Bone Miner Res 18: 140–149.
42. Wu N, Wang QP, Li H, Wu XP, Sun ZQ, et al. (2010) Relationships between serum adiponectin, leptin concentrations and bone mineral density, and bone biochemical markers in Chinese women. Clin Chim Acta 411: 771–775.
43. Legirán S, Brandi ML (2012) Bone mass regulation of leptin and postmenopausal osteoporosis with obesity. Clin Cases Miner Bone Metab 9: 145–149.
44. Sebastián-Ochoa A, Fernández-García D, Reyes-García R, Mezquita-Raya P, Rejas-Moreno P, et al. (2012) Adiponectin and leptin serum levels in osteoporotic postmenopausal women treated with raloxifene or alendronate. Menopause 19: 172–177.
45. Clemens TL, Karsenty G (2011) The osteoblast: an insulin target cell controlling glucose homeostasis. J Bone Miner Res 26: 677–680.
46. Lee NK, Sowa H, Hinoi E, Ferron M, Ahn JD, et al. (2007) Endocrine regulation of energy metabolism by the skeleton. Cell 130: 456–469.
47. Semenikovich CF, Teitelbaum SL (2007) Bone weighs in on obesity. Cell 130: 414–417.
48. Ng KW (2011) Regulation of glucose metabolism and the skeleton. Clin Endocrinol 75: 147–155.
49. Van Ness-Onnuru R, Hack JB (2013) Hyperglycemic crisis. J Emerg Med 45: 797–805.
50. Kaji H, Hira I, Inoue Y, Naito J, Sugimoto T, et al. (2009) Analysis of factors affecting increase in bone mineral density at lumbar spine by bisphosphonate treatment in postmenopausal osteoporosis. J Bone Miner Metab 27: 76–82.
51. Schwartz AV, Schafer AL, Grey A, Vittinghoff E, Palermo L, et al. (2013) Effects of antiresorptive therapies on glucose metabolism: results from the FIT, HORIZON-PFT, and FREEDOM trials. J Bone Miner Res 28: 1348–1354.
52. Hong SH, Koo JW, Hwang JK, Hwang YC, Jeong IK, et al. (2013) Changes in serum osteocalcin are not associated with changes in glucose or insulin for osteoporotic patients treated with bisphosphonate. J Bone Metab 20: 37–41.
53. Zhao Y, Zou B, Shi Z, Wu Q, Chen GQ (2007) The effect of 3-hydroxybutyrate on the in vitro differentiation of murine osteoblast MC3T3-E1 and in vivo bone formation in ovariectomized rats. Biomaterials 28: 3063–3073.
54. Choi MJ, DaMarco NM (2009) The effects of dietary taurine supplementation on bone mineral density in ovariectomized rats. Adv Exp Med Biol 643: 341–349.
55. Choi MJ, Chang KJ (2013) Effect of dietary taurine and arginine supplementation on bone mineral density in growing female rats. Adv Exp Med Biol 776: 335–345.
56. Jeon SH, Lee MY, Kim SJ, Joe SG, Kim GB, et al. (2007) Taurine increases cell proliferation and generates an increase in 
\[\text{Mg}^{2+}\] accompanied by ERK 1/2 activation in human osteoblast cells. FEBS Lett 591: 5929–5934.