國立臺灣大學公共衛生學院流行病學與預防醫學研究所

碩士論文

Institute of Epidemiology and Preventive Medicine
College of Public Health
National Taiwan University

Master Thesis

應用核磁共振氫譜技術探討台灣女性代謝體特徵與低骨質密度之關聯研究

Association of Metabolome with Low Bone Mineral Density in Taiwanese Women Revealed by ^{1}H NMR Spectroscopy

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中華民國 101 年 7 月
July, 2012

國立臺灣大學碩士學位論文
口試委員會審定書

論文中文題目

應用核磁共振譜技術探討台灣女性代謝體特徵與低骨質密度之關聯研究

論文英文題目

Association of Metabolic Profiles with Low Bone Mineral Density in Taiwanese Women Revealed by ^{1}H NMR spectroscopy

本論文係 尤瀅淑君（學號 R99849016）在國立臺灣大學流行病學與預防醫學研究所完成之碩士學位論文，於民國 101 年 07 月 23 日承下列考試委員審查通過及口試及格，特此證明。

口試委員：程蘊菁 （簽名）

（指導教授）

林靖愉

孝先高

丘政民

誌謝

本篇碩士論文能順利完成，背後最重要的推手莫過於指導教授程蘊菁博士。在程老師不辭辛苦與悉心指導之下，才得已讓學生能完成此艱鉅的任務，在此相當感謝老師辛勤的付出，很榮幸能擔任程老師的指導學生。另外亦感謝共同指導教授林靖愉博士給予許多研究上的協助和建議，以及林老師的研究助理梁皓然學長耐心地教導我操作代謝體學的分析方法，使學生獲益良多。同時也感謝台大北護分院蔡克嵩院長、中研院統計所副所長丘政民教授，在百忙之中仍能撥冗前來指教學生的論文研究，並給予中肯的建議。

在進行代謝體學實驗的期間裡，多虧了中研院生醫所核磁共振實驗室裡的研究員和同仁們，總是熱心又親切地幫助我解決許多難題，才能使我順利收集實驗資料供研究分析。再者，整個碩士班修業期間，也很感謝程老師研究團隊和同屆的醫師同學們，大家能一起互助共進。

最後，感謝我親愛的家人和朋友們，不斷給予許多支持和鼓勵！感謝流預所各位老師們諄諄教導！能建立此基石，皆歸功於所有在我身旁無限供給能量予我的每一個人，謝謝您們。

尤瀅淑 謹誌 2012.8

摘要

背景：近期已有研究指出骨質疏鬆症與體內不斷循環的特定代謝物具有相關；然而，若單靠過去研究所探討的少數幾項代謝物，很難對此複雜的致病機轉做詳確的解釋。如今，代謝體學方法可全面性的檢視人體中代謝情形和潛在的臨床意義。截至目前為止，未有研究加以驗證人體中代謝體特徵和骨質密度之間的相關。

方法：本研究橫斷式研究設計，自 2009 到 2010 年間招募 610 位年齡介於 40 至 55 歲之間在台北美兆健康檢查中心參加健康檢查的台灣女性。使用雙能量放射線儀測量隻腰椎骨質密度，以骨質密度值的高低排序後研究族群依人數均分為三等份，定義骨質密度最高的第二和第三分群為「高骨質密度」組別，而最低的第一群為「低骨質密度」組別。而血漿代謝體特徵資料是透過核磁共振氫譜實驗分析所收集而成。運用無監督的主成分分析、監督的偏最小二乘法判別分析法及邏輯斯迴歸模式，來分析代謝體特徵與骨質密度的相關。

結果：研究結果顯示，無監督的主成分分析的分數散佈圖無法完善地分離高低骨質密度的組別；監督的偏最小二乘法判別分析法亦沒有能力區別高低骨質密度的組別。根據停經狀態加以分層之後，可透過偏最小二乘法判別分析法於停經婦女群體觀察到其代謝體特徵與高低骨質密度顯著的差異(R^2= 0.12；Q^2=0.04；$P_{permutation}$=0.03)。此外，在停經婦女身上，會隨著 glutamine 的濃度升高，使得低骨質密度的風險增加；同時，低骨質密度也與 lactate、acetone、lipid 和 very low density lipoprotein 濃度降低具有相關。

結論： 這是第一篇研究透過核磁共振氫譜實驗所建立的代謝體學與骨質密度之研究，我們成功地於停經婦女群體辨識出一群代謝物和低骨質密度具有關聯。其中，特別是 glutamine 的濃度升高會增加低骨質密度發生的風險，可能會因此導致骨骼流失。

關鍵詞：代謝體特徵、代謝體學、核磁共振氫譜、骨質疏鬆症、停經、女性

Abstract

Background. Osteoporosis has been related to the alteration of specific circulating metabolites previously. However, studies with few metabolites may have difficulty to explain the pathogenesis of this complex syndrome. Metabolome provide an overview of the metabolism status in human body and potential clinical implication. Up to date, no study has investigated the association between metabolome and bone mineral density (BMD).

Methods. This is a cross-sectional study. A total of 610 healthy Taiwanese women aged 40 to 55 were recruited from MJ Health Screening Center between 2009 and 2010. High and low bone mineral density (BMD) was defined as the 2^{nd} plus 3^{rd} tertiles and the 1^{st} tertile of BMD, respectively. The plasma metabolome were evaluated by ^{1}H-nuclear magnetic resonance spectroscopy. Principal components analysis (PCA), partial least-squares discriminant analysis (PLS-DA), and logistic regression model were used to assess the association between metabolome and BMD.

Results. The unsupervised PCA showed no visual separation between low and high BMD levels; the supervised PLS-DA model was also unable to distinguish high and low BMD groups. After stratification by menopausal status, high and low BMD groups can be differentiated in post-menopausal women using PLS-DA (R^2= 0.12;

Q^2=0.04; $P_{permutation}$=0.03). In addition, elevated level of glutamine was associated with the risk of low BMD among postmenopausal women; while low BMD is characterized by decreased levels of lactate, acetone, lipid, and very low density lipoprotein in the same sub-population.

Conclusion. This is the first study using ^{1}H NMR-based metabolomic approach and successfully identified a group of metabolites representative for postmenopausal women with low BMD. Especially, elevated level of glutamine may lead to bone loss via increased risk of low BMD.

Keywords. *Metabolome, metabolic profiles, metabolomics, metabonomics, nuclear magnetic resonance, NMR, bone mineral density, osteoporosis, menopause, women.*

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Chapter 1. Introduction

1.1 Importance of Osteoporosis

Osteoporotic fractures are a major cause of morbidity, disability, and the subsequent premature death in the elderly.[1] According to World Health Organization criteria, osteoporosis is defined as bone mineral density (BMD) is 2.5 standard deviations (SD) below the mean. In the United States, spinal osteoporosis was more prevalent among Mexican American (24.4% for women and 4.6% for men) than that among either non-Hispanic blacks (5.3% for women) or non-Hispanic whites (10.9% for women and 2.2% for men) aged 50 years or older in National Health and Nutrition Examination Survey (NHANES) from 2005 to 2008.[2] In the 2005 to 2008 Nutrition and Health Survey in Taiwan (NAHSIT 2005-2008), the prevalence of osteoporosis at lumbar spine is 12.6 % in women and 4.3% in men aged 50 years or older.[3] Furthermore, BMD testing, impaired vision, and neuromuscular deficits before age 65 are recommended for predicting the risk of future fracture[4], which has known to associated with high mortality. As the population aging fast worldwide, osteoporosis has become an important public health issue.

1.2 Bone-Related Proteins and Metabolites

Decreased bone mass occurs when bone resorption is excessive or bone formation is decreased. Bone turnover markers (e.g., alkaline phosphatase, osteocalcin, type I collagen cross-linked N-telopeptide, hydroxyproline and the pyridinium crosslinks, etc.) have been used to monitor bone homeostasis and predict fracture risk.[5,6] However, 20% to 30% of women with high bone turnover rate may be misclassified as osteoporosis patients in the future.[7] On the other hand, some studies found that elevated homocysteine, which interferes with the cross-linking of collagen in bone, may predict osteoporotic fractures.[8-10] However, the association between homocysteine and BMD has been inconsistent.[8,9,11,12] Moreover, *in vitro* and *in vivo* studies showed that nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) essential modulator -binding domain (NBD) peptide[13] and polyunsaturated fatty acids[14] inhibit osteoclastogenesis for bone loss. In summary, past studies only explored few metabolites and thus may have difficulty on predicting the complex syndrome, osteoporosis.

1.3 NMR-based Metabolomics

Metabolomics based on Nuclear Magnetic Resonance spectroscopy (NMR)

spectrometry allow the detection of a wide range of structurally diverse metabolites simultaneously as well as metabolome for the discovery of novel biomarkers.[15] The NMR spectrum is made up of peaks from each chemically distinct hydrogen atom in the molecule, and the peak position of a given hydrogen on the frequency axis is known as a chemical shift [unit: parts per million (ppm), often termed a δ value] from that of a reference substance.[15-17] In addition, NMR is a robust and reliable technique for metabolomic applications with high reproducibility.[15,18,19] The NMR-based spectroscopic method usually uses urine or blood plasma/serum as the primary sample sources of metabolic fingerprint data.[20,21] Generally, as compared with urine, blood sample has less pH variations, dietary or drug effects.[15,22] Therefore, NMR-based metabolomics gradually become an important tool in predicting metabolites for different research purpose, e.g., drugs, diet, lifestyle, environment, stimuli and genetic modulations.[15]

1.4 Aims

Previous studies evaluated limited number of candidate metabolites, which is hard to address the overview of the metabolism status in human body. Up to date, the association between metabolome and BMD has only been explored in ovariectomized

rats.[23,24] Therefore, this study adopted the high-throughput ^{1}H NMR-based 'metabonomic/metabolomic' approach[20,21] to assess the association between plasma metabolome and BMD in healthy middle-aged women. In addition, because estrogen deficiency-induced bone loss is observed in postmenopausal women, who are more susceptible to fragility fractures than premenopausal women,[25] this study further accessed the effect modification by menopausal status.

Chapter 2. Materials and Methods

2.1 Study Population

This was a cross-sectional study. A total of 773 Taiwanese women aged 40 to 55 were recruited from MJ Health Screening Center, Taipei, Taiwan, between October 2009 and August 2010. Each participant filled out a self-reported questionnaire and provided a blood sample. The outcome of this study was BMD (g/cm^2) at lumbar spine. Participants with the following conditions or diseases were excluded (n=163): (1) received hormone replacement therapy (n=34) or other medications (e.g., steroid, n=3) that may affect BMD, (2) lack of BMD at lumbar spine (n = 51), (3) lack of blood samples (n=45), (6) or lack of NMR data (n=30). A total of 610 women were included for data analyses (Figure 1). Informed consent was obtained from each participant. The study protocol has been approved by the institutional review boards of MJ Health Screening Center and College of Public Health, National Taiwan University.

A self-report questionnaire was administered to collect information on demography, life style (e.g., smoking, alcohol consumption, calcium supplement, and regular exercise), menopausal status, disease history (e.g., hypertension and diabetes), and medication history (e.g., steroid, anti-inflammatory drugs, Chinese herb, and gastrointestinal drugs).

2.2 Measurement of Bone Mineral Density

BMD (g/cm^2) was measured at the lumbar spine by using dual-energy X-ray absorptiometry (DXA, GE Lunar Health Care, DPX-L, USA), which was calibrated by a standard automated test program provided by the manufacturer. BMD was tertiled (T1, T2, and T3) based the whole population. High BMD was defined as T2 plus T3 (reference group) and low BMD was defined as T1 (comparison group, Figure 2).

2.2 Sample Collection and Preparation

Fasting blood samples were collected in tubes containing sodium ethylene diamine tetraacetic acid (EDTA) from each participant. After centrifugation, plasma samples were stored in a −80°C freezer. Before NMR procedures, 200 µl of plasma was diluted with a ratio of 1:2 in physiological saline [0.9% (w/v) NaCl solution] with 10% D_2O. Solution in Eppendorf tubes were then centrifuged at 15,000*g* for 5 min at 4℃, and 550 µl of supernatant was transferred into 5 mm NMR tubes.

2.3 1D ^{1}H NMR Spectroscopy

All NMR spectra were acquired at 500.13 MHz using Avance-500 spectrometers (Bruker, Fremont, CA) at a ^{1}H frequency with 300 K internal probe temperature at

High Field Nuclear Magnetic Resonance Center in Academia Sinica, Taiwan.

For the plasma samples, Carr-Purcell-Meiboom-Gill (CPMG)-presat pulse sequence (relaxation delay-90°-(t -180°-t)n -90°-acquired-free induction decay) was acquired from 1D ^{1}H NMR analysis. For each sample, 128 scans were collected into 64K computer data points using a spectral width of 10,000 Hz (20 ppm), with a total echo time of 64 ms (*t*= 400 μs) during the relaxation delay (RD, 2.0 s) and an acquisition time of 1.63 s.[15] Additionally, all 1D spectra were applied for analysis before Fourier transformation with zero-filled to exponential line-broadenings of 0.5 Hz. The acquired NMR spectra were phased, baseline-corrected, and then calibrated by the methyl lactate doublet (δ 4.12 ppm) using TopSpin 2.0 (Bruker Biospin Ltd.).

2.4 NMR Spectral Pre-Processing

After acquisition of the NMR data, each spectrum was then segmented into 1,880 chemical shift bins between δ 0.2 and δ 10.0, corresponding to a bin width of 0.005 ppm (2.5 Hz), using custom-written *ProMetab* software Version 3.3 in MATLAB (Version 7.0.1, The MathWorks, Natick, MA).[26] Following the removal of the residual water (δ 4.50-6.00 ppm) and EDTA (δ 3.58-3.65 ppm and δ 3.17-3.23 ppm),[27] the area within each spectral bin was integrated to yield a 1×1572 vectors

containing intensity-based descriptors of the original spectrum. The total spectral area of the remaining bins was normalized to unity to facilitate comparison between the spectra. The binned data was subject to the generalized log transformation ($\lambda = 8 \times 10^{-9}$),[28] and the columns were mean-centered by Pareto scaling[29,30] before multivariate analysis.

2.5 Statistical Analyses

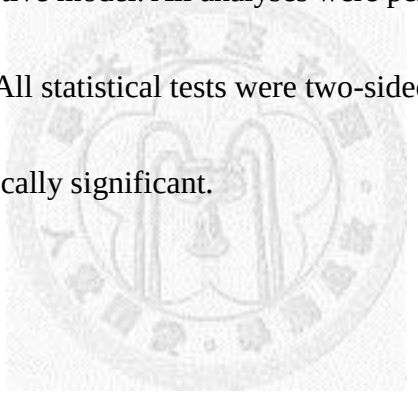
The reduced and normalized NMR spectral binned data were uploaded to Metaboanalyst 2.0 (http://www.metaboanalyst.ca)[31] for principal components analysis (PCA), an unsupervised approach, and partial least-squares discriminant analysis (PLS-DA), a supervised method, to identify which metabolites contributed to specific clusters. PCA is used to reduce high-throughput metabolomic data into principal components without referring to the BMD level, and then the low-dimensional PCA score plots were visually inspected for natural separation of the two BMD levels (low vs. high, Figure 2). In contrast, PLS-DA uses previous knowledge about the BMD level during the classification process by multiple linear regression technique to find the direction of maximum covariance between metabolome and the BMD level. The PLS-DA score plots were used to assess if the metabolome can be separated based on BMD levels. Furthermore, PLS-DA was determined by the number of latent variables

below to build the model. A default 10-fold internal cross validation was employed, from which Q^2 (expressing the cross-validated predictive capability), R^2 (explained variance) and variable importance in projection (VIP) score (coefficient reflects the relative importance of each variable) were used to extract important plasma metabolites (threshold>1.5) in the PLS-DA model.

Student's t test (for normally-distributed continuous variables) and Pearson's χ^2 test (for categorical variables) were performed to compared the baseline distribution of potential confounders by BMD level (T1 vs. T2 plus T3). Logistic regression model was used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) in women with low BMD (T1) versus high BMD (T2 plus T3) for selective metabolites (VIP>1.5). All models were adjusted for age, menopausal status (yes vs. no), body weight (continuous), height (continuous), waist circumference (continuous), creatinine (>79 mg/dL, yes vs. no), regular exercise (≥2 times/week for 30 minutes or more, yes vs. no), and serum alkaline phosphatase (ALP≥ 60 IU, yes vs. no). These variables were selected by either stepwise model selection (Slentry=0.15, Slstay=0.15) or because of its biological importance to BMD level. Serum creatinine and ALP were dichotomized by its median because quite few participants had abnormal level in this healthy population.

Because menopausal status significantly affect BMD level, effect modification

by menopausal status (yes/no) was explored by comparing a model with terms for main effects and interaction terms to the models for main effect only using the likelihood ratio test. Stratified analysis was performed to assess the relationship between metabolome and BMD by menopausal status. Areas under the receiver operating characteristic (ROC) curve (AUC) were calculated to evaluate the performance of the candidate metabolites. An AUC value of 1.0 represents a prediction model with 100% sensitivity and 100% specificity, while an AUC value of 0.5 correspond to a poor predictive model. All analyses were performed by using SAS 9.2 (SAS Institute, Cary, NC). All statistical tests were two-sided and a *P* value less than 0.05 was considered statistically significant.



Chapter 3. Results

3.1 Characteristics of the Study Population

This cross-sectional study included 610 healthy women aged 40 to 55. As compared to women with high BMD, women with low BMD were older (47.6 vs. 45.4 years old), had lower body weight (53.1 vs. 57.0 kg), body mass index (21.5 vs. 22.7 kg/m^2), waist circumference (71.6 vs.73.6 cm), fasting glucose level (97.2 vs. 101.4 mg/dL), and creatinine level (0.78 vs. 0.80 mg/dL), and had higher alkaline phosphatase level (67.3 vs. 60.2 IU), and more postmenopausal women (36.5% vs. 11.4%, Table 1). The distributions of diastolic blood pressure, triglyceride, high-density lipoprotein cholesterol, total cholesterol, hypertension status, smoking history, alcohol consumption, calcium supplement, and regular exercise were similar between high and low BMD.

3.2 Metabolome of the BMD Levels

Metabolome obtained from unsupervised PCA showed no visual separation between low and high BMD levels as well as after stratified by menopausal status (Figure 3).

For supervised approach, PLS-DA score plots revealed little evidence of separation between high and low BMD clusters (Figure 4a). Some parameters of PLS-DA model were applied to compare high and low BMD and results using one component again showed little evidence of classification (variation: R^2=0.03, predictive capability: Q^2 of 10-fold cross validation=-0.002, 1000 random permutation test: p=0.07, Table 2). After stratification by menopausal status, metabolome showed better separation by high and low BMD levels in menopausal women (Figure 4b and 4c; R^2=0.12, Q^2=0.04, permutation test p=0.03) but not in premenopausal women (R^2=0.01, Q^2=-0.02, permutation test p=0.76).

3.3 Candidate Metabolites and the Risk of Low BMD

Given the success of the PLS-DA model in classifying high and low BMD among postmenopausal women (Table 2), a total of 7 significantly altered metabolites (VIP > 1.5) were identified based on published data[16,17] and Chenomx NMR suite (Chenomx Inc., Alberta, Canada). As compared with high BMD, low BMD was related to decreased levels of plasma lactate, acetone, lipid, very low lipoprotein (VLDL), and glucose, and elevated acetate and glutamine (Table 3).

For multivariate logistic regression, women who had an elevated level of glutamine was significantly associated with 1.6-fold increased risk of low BMD (AOR=1.55, 95% CI=1.03-2.33, Table 4). In addition, no significant difference between high and low BMD level was found for lactate, acetone, acetate, lipid, VLDL, and glucose (Table 4). However, menopausal status significantly modified the association between lactate (P interaction=0.004), acetone (P interaction=0.01), acetatate (P interaction=0.02), lipid (P interaction=0.04), VLDL (P interaction=0.02), glutamine (P interaction=0.04) and the risk of low BMD (Table 5). After stratification, postmenopausal women with elevated level of lactate (AOR=0.55, 95% CI= 0.33-0.92), acetone (AOR=0.51, 95% CI= 0.31-0.85), lipid (AOR=0.04, 95% CI=0.001-0.91), and VLDL (AOR=0.49, 95% CI=0.27-0.90) had a significantly decreased risk of low BMD (Table 5). No significant association was observed for premenopausal women. In contrast, postmenopausal women with elevated level of glutamine had an increased risk of low BMD (AOR=6.04, 95% CI=1.57-23.21, Table 5). No significant association was observed for acetate and glucose in either pre- or postmenopausal women.

Comparing the ROC curves of model 1 (lactate, acetone, lipid, VLDL, and glutamine), model 2 (variables in model 1 plus acetate and glucose), and glutamine alone, the AUC was 0.59 for model 1 (95% CI=0.55-0.64, Figure 5a), 0.60 for model 2

(95% CI=0.55-0.65), and 0.54 for glutamine alone (95% CI=0.50-0.59) with limited ability to classify high and low BMD. After stratification by menopausal status, the AUC became 0.57 for model 1 (95% CI=0.52-0.63, Figure 5b), 0.60 for model 2 (95% CI=0.55-0.66), and 0.53 (95% CI=0.48-0.58) for glutamine alone for premenopausal women; AUC was 0.69 for model 1 (95% CI= 0.67-0.72, Figure 5c), 0.70 for model 2 (95% CI= 0.67-0.73), and 0.65 (95% CI= 0.63-0.68) for glutamine alone in postmenopausal women. For pairwise comparisons between models using ROC contrast tests, ROC curves were different between model 2 and glutamine alone in all participants (p=0.03), premenopausal women (p=0.02), and postmenopausal women (p=0.01, Table 6), respectively. In addition, ROC curve of glutamine alone was significant different to ROC curve of model 1 in postmenopausal women (p=0.03, Table 6).

Chapter 4. Discussion

4.1 Main findings

This is the first epidemiologic study used ^{1}H NMR-based metabolomic approach to investigate the association between plasma metabolome and the risk of low BMD. Before stratification by menopausal status, metabolome were not associated with the risk of low BMD; significant association was observed in postmenopausal women only. Reactive oxygen species has been related to age-related and estrogen-dependent bone loss.[32] Therefore, estrogen plays an important role in maintaining bone strength by diminish oxidative stress in bone and bone marrow in women. As estrogen level decreases significantly after menopause, this may explain menopausal status affect the association of metabolome with BMD level.

4.2 Evidences of Metabolites and Bone Metabolism

Among the top 7 metabolites identified by PLS-DA model (VIP>1.5), elevated levels of lactate, acetone, lipid, and VLDL were significantly associated with decreased risk of low BMD; instead, glutamine showed significant increased risk of low BMD and acetate and glucose showed no association. Below details the postulated

mechanism of each metabolite with BMD (Figure 7).

Lactate. Lactate is the end product of glycolysis derived from glucose under low oxygen conditions.[33] Lactate is also a crucial intermediate for regulating collagen biosynthesis during osteogenesis.[34] This may explain that increased level of lactate was associated with decreased risk of low BMD via increased bone formation.

Acetone. Acetone is spontaneous produced in the body by the decarboxylation of acetoacetate, which refers to ketone bodies.[35] In human plasma, concentration of acetone was (generally higher than acetoacetate) correlated with fasting and diabetic ketosis.[36] However, recent studies suggested that BMD was increased in patient with Type 2 diabetes[37-39] via increased mechanical loading and release of hormonal factors (oestrogen, leptin and adiponectin).[40,41] This is also observed in our study, that is, women with the symptom of diabetes mellitus (i.e., fasting glucose > 100 mg/dL) had higher acetone (Figure 6) and lower risk of low BMD after menopause than those without elevated level of fasting glucose.

Lipid & VLDL. Some evidence showed that the amount of body lipid across a broad range of body fat can be reflected by leptin levels,[42] which related to differentiation of stromal cells to osteoblasts and thus prevent further loss of bone.[43,44] This may explain our finding that lipid and VLDL were associated with decreased risk of low BMD, which was consistent with other studies.[45,46] However, our findings do not support the

previous lipid hypothesis of osteoporosis, which suggests that LDL oxidation and atherogenic lipid profiles promote bone loss,[47-49] probably because the characteristics of this study population was differ from previous studies and adjusted different confounding factors in models.

Glutamine. Glutamine may regulate bone metabolism via osteoclast and can interconvert to glutamate (Glu), a major neuromediator of the central and peripheral nervous systems.[50,51] In addition, Glu may lead to bone resorption via bone cells express Glu receptors, especially on osteoclast,[52] this supports our finding that elevated glutamine was associated with elevated risk of low BMD. Moreover, some studies have reported that estrogen facilitates the neuroprotection against oxidative stress and reduces glutamatergic excitotoxicity.[53,54] This further explains our finding that elevated glutamine was associated with increased risk of low BMD among postmenopausal women. However, this finding showed different metabolome as compared with those from ovariectomized rats.[23,24,55,56] It is possible that epidemiologic and experimental studies differ in many ways, e.g., controlling for confounding effect, sample size, and biological mechanism, etc.

4.3 Strengths and limitations

This study had several strengths. First, this study had relatively large sample

size, especially for metabolomic study on human participants. Second, previous studies mainly focused on the association of limited metabolites with BMD in postmenopausal women.[57,58] This study used high-throughput and unbiased approach to evaluate the metabolome and includes both pre- and postmenopausal women, which opens a broad view on research in similar topics. In addition, this study additionally adjusted for numerous important confounders (age, body weight, menopausal status, creatinine, waist circumference, serum alkaline phosphatase), which provides more reliable results.

This study had some limitations. First, the cross-sectional design does not allow us to assess causal inference. Second, this population is relatively healthy and thus it is not easy to differentiate the metabolome between high and low BMD groups. This is also why this study did not use clinical cutoff points for BMD level (Figure 2) and some biochemical values, e.g., serum alkaline phosphatase and creatinine. Third, this study only assessed metabolome from plasma but not other biomarkers, e.g., urine. This is because plasma can provide data on lipid-soluble compounds,[22] which has known with extensive association with osteoporosis.[45-49] However, lipid profile is not available in urine sample as it is hydrophilic. Last, the use of sodium EDTA tubes for collecting blood samples prevented us from obtaining the information on some metabolites, e.g., glycerol, choline, and valine, etc.[16,27] However, a recent study

showed that even though some endogenous metabolites might be obscured by the anticoagulant peaks, the information of these anticoagulants binding metabolites can be identified by other signals [e.g., glycerol (δ 3.87), choline (δ 3.50), valine (δ 2.28)], which are available in our NMR spectra.[27] After removal of anticoagulants, the inter-sample variation in these lipoprotein signals would allow us to perform the statistical analyses.

4.4 Conclusions

This study, for the first time, identified metabolome for predicting the risk of low BMD in postmenopausal women. Metabolomics can quantify a large-scale of metabolites to characterize response to drugs, diet, lifestyle, environmental stimuli and genetic modulations.[15] Future studies exploring gene-metabolite interactions using liquid chromatograph/mass spectrometry for low abundant metabolites and animal studies are warranted to shed light on the association between metabolome and BMD in human.

References

1. Prentice A. Diet, nutrition and the prevention of osteoporosis. Public Health Nutr 2004;7:227-43.

2. Dawson-Hughes B, Looker AC, Tosteson AN, Johansson H, Kanis JA, Melton LJ, 3rd. The potential impact of the National Osteoporosis Foundation guidance on treatment eligibility in the USA: an update in NHANES 2005-2008. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA 2012;23:811-20.

3. Lin YC, Pan WH. Bone mineral density in adults in Taiwan: results of the Nutrition and Health Survey in Taiwan 2005-2008 (NAHSIT 2005-2008). Asia Pac J Clin Nutr 2011;20:283-91.

4. Raisz LG. Clinical practice. Screening for osteoporosis. N Engl J Med 2005;353:164-71.

5. Kanis JA. Diagnosis of osteoporosis and assessment of fracture risk. Lancet 2002;359:1929-36.

6. Garnero P, Hausherr E, Chapuy MC, Marcelli C, Grandjean H, Muller C, Cormier C, Breart G, Meunier PJ, Delmas PD. Markers of bone resorption predict hip fracture in elderly women: the EPIDOS Prospective Study. J Bone

Miner Res 1996;11:1531-8.

7. Garnero P, Mulleman D, Munoz F, Sornay-Rendu E, Delmas PD. Long-term variability of markers of bone turnover in postmenopausal women and implications for their clinical use: the OFELY study. J Bone Miner Res 2003;18:1789-94.

8. McLean RR, Jacques PF, Selhub J, Tucker KL, Samelson EJ, Broe KE, Hannan MT, Cupples LA, Kiel DP. Homocysteine as a predictive factor for hip fracture in older persons. N Engl J Med 2004;350:2042-9.

9. van Meurs JB, Dhonukshe-Rutten RA, Pluijm SM, van der Klift M, de Jonge R, Lindemans J, de Groot LC, Hofman A, Witteman JC, van Leeuwen JP, Breteler MM, Lips P, Pols HA, Uitterlinden AG. Homocysteine levels and the risk of osteoporotic fracture. N Engl J Med 2004;350:2033-41.

10. Leboff MS, Narweker R, LaCroix A, Wu L, Jackson R, Lee J, Bauer DC, Cauley J, Kooperberg C, Lewis C, Thomas AM, Cummings S. Homocysteine levels and risk of hip fracture in postmenopausal women. J Clin Endocrinol Metab 2009;94:1207-13.

11. Enneman AW, van der Velde N, de Jonge R, Heil SG, Stolk L, Hofman A, Rivadeneira F, Zillikens MC, Uitterlinden AG, van Meurs JB. The association between plasma homocysteine levels, methylation capacity and incident

osteoporotic fractures. Bone 2012.

12. Gjesdal CG, Vollset SE, Ueland PM, Refsum H, Drevon CA, Gjessing HK, Tell GS. Plasma total homocysteine level and bone mineral density: the Hordaland Homocysteine Study. Arch Intern Med 2006;166:88-94.

13. Jimi E, Aoki K, Saito H, D'Acquisto F, May MJ, Nakamura I, Sudo T, Kojima T, Okamoto F, Fukushima H, Okabe K, Ohya K, Ghosh S. Selective inhibition of NF-kappa B blocks osteoclastogenesis and prevents inflammatory bone destruction in vivo. Nat Med 2004;10:617-24.

14. Yuan J, Akiyama M, Nakahama K, Sato T, Uematsu H, Morita I. The effects of polyunsaturated fatty acids and their metabolites on osteoclastogenesis in vitro. Prostaglandins Other Lipid Mediat 2010;92:85-90.

15. Beckonert O, Keun HC, Ebbels TM, Bundy J, Holmes E, Lindon JC, Nicholson JK. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. Nat Protoc 2007;2:2692-703.

16. Nicholson JK, Foxall PJ, Spraul M, Farrant RD, Lindon JC. 750 MHz ^{1}H and ^{1}H-^{13}C NMR spectroscopy of human blood plasma. Anal Chem 1995;67:793-811.

17. Nicholson G, Rantalainen M, Maher AD, Li JV, Malmodin D, Ahmadi KR,

Faber JH, Hallgrimsdottir IB, Barrett A, Toft H, Krestyaninova M, Viksna J, Neogi SG, Dumas ME, Sarkans U, The Molpage C, Silverman BW, Donnelly P, Nicholson JK, Allen M, Zondervan KT, Lindon JC, Spector TD, McCarthy MI, Holmes E, Baunsgaard D, Holmes CC. Human metabolic profiles are stably controlled by genetic and environmental variation. Mol Syst Biol 2011;7:525.

18. Keun HC, Ebbels TM, Antti H, Bollard ME, Beckonert O, Schlotterbeck G, Senn H, Niederhauser U, Holmes E, Lindon JC, Nicholson JK. Analytical reproducibility in ^{1}H NMR-based metabonomic urinalysis. Chem Res Toxicol 2002;15:1380-6.

19. Dumas ME, Maibaum EC, Teague C, Ueshima H, Zhou B, Lindon JC, Nicholson JK, Stamler J, Elliott P, Chan Q, Holmes E. Assessment of analytical reproducibility of ^{1}H NMR spectroscopy based metabonomics for large-scale epidemiological research: the INTERMAP Study. Analytical chemistry 2006;78:2199-208.

20. Nicholson JK, Lindon JC, Holmes E. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica 1999;29:1181-9.

21. Fiehn O. Metabolomics--the link between genotypes and phenotypes. Plant Mol

Biol 2002;48:155-71.

22. Gibney MJ, Walsh M, Brennan L, Roche HM, German B, van Ommen B. Metabolomics in human nutrition: opportunities and challenges. Am J Clin Nutr 2005;82:497-503.

23. Long WF, Li L, Chen HQ, Tang Y, He XL, Jing RZ. ^{1}H-NMR-based metabonomics analysis of plasma from osteoporotic rats induced by ovariectomy. Sichuan Da Xue Xue Bao Yi Xue Ban 2009;40:843-7.

24. Xue L, Wang Y, Liu L, Zhao L, Han T, Zhang Q, Qin L. A ^{1}HNMR-Based Metabonomics Study of Postmenopausal Osteoporosis and Intervention Effects of Er-Xian Decoction in Ovariectomized Rats. Int J Mol Sci 2011;12:7635-51.

25. Lerner UH. Bone remodeling in post-menopausal osteoporosis. J Dent Res 2006;85:584-95.

26. Viant MR. Improved methods for the acquisition and interpretation of NMR metabolomic data. Biochem Biophys Res Commun 2003;310:943-8.

27. Barton RH, Waterman D, Bonner FW, Holmes E, Clarke R, Nicholson JK, Lindon JC. The influence of EDTA and citrate anticoagulant addition to human plasma on information recovery from NMR-based metabolic profiling studies. Mol Biosyst 2010;6:215-24.

28. Purohit PV, Rocke DM, Viant MR, Woodruff DL. Discrimination models using

variance-stabilizing transformation of metabolomic NMR data. OMICS 2004;8:118-30.

29. Eriksson L, Johansson E, Kettaneh-Wold N, Wold S. Introduction to multi- andmegavariate dta analysis using projection methods (PCA & PLS): Umetrics; 1999.

30. van den Berg RA, Hoefsloot HC, Westerhuis JA, Smilde AK, van der Werf MJ. Centering, scaling, and transformations: improving the biological information content of metabolomics data. BMC Genomics 2006;7:142.

31. Xia J, Psychogios N, Young N, Wishart DS. MetaboAnalyst: a web server for metabolomic data analysis and interpretation. Nucleic Acids Res 2009;37:W652-60.

32. Manolagas SC. From estrogen-centric to aging and oxidative stress: a revised perspective of the pathogenesis of osteoporosis. Endocr Rev 2010;31:266-300.

33. Cohen SA. Amino acid analysis using precolumn derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. Methods Mol Biol 2000;159:39-47.

34. Sengupta S, Park SH, Patel A, Carn J, Lee K, Kaplan DL. Hypoxia and amino acid supplementation synergistically promote the osteogenesis of human mesenchymal stem cells on silk protein scaffolds. Tissue Eng Part A

2010;16:3623-34.

35. Frayn KN. Calculation of substrate oxidation rates in vivo from gaseous exchange. J Appl Physiol 1983;55:628-34.

36. Owen OE, Trapp VE, Skutches CL, Mozzoli MA, Hoeldtke RD, Boden G, Reichard GA, Jr. Acetone metabolism during diabetic ketoacidosis. Diabetes 1982;31:242-8.

37. Vestergaard P. Discrepancies in bone mineral density and fracture risk in patients with type 1 and type 2 diabetes--a meta-analysis. Osteoporos Int 2007;18:427-44.

38. Shan PF, Wu XP, Zhang H, Cao XZ, Gu W, Deng XG, Gu C, Liao EY. Bone mineral density and its relationship with body mass index in postmenopausal women with type 2 diabetes mellitus in mainland China. J Bone Miner Metab 2009;27:190-7.

39. Ma L, Oei L, Jiang L, Estrada K, Chen H, Wang Z, Yu Q, Zillikens MC, Gao X, Rivadeneira F. Association between bone mineral density and type 2 diabetes mellitus: a meta-analysis of observational studies. Eur J Epidemiol 2012;27:319-32.

40. Adami S. Bone health in diabetes: considerations for clinical management. Curr Med Res Opin 2009;25:1057-72.

41. Felson DT, Zhang Y, Hannan MT, Anderson JJ. Effects of weight and body mass index on bone mineral density in men and women: the Framingham study. J Bone Miner Res 1993;8:567-73.

42. Frederich RC, Hamann A, Anderson S, Lollmann B, Lowell BB, Flier JS. Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. Nat Med 1995;1:1311-4.

43. Thomas T, Gori F, Khosla S, Jensen MD, Burguera B, Riggs BL. Leptin acts on human marrow stromal cells to enhance differentiation to osteoblasts and to inhibit differentiation to adipocytes. Endocrinology 1999;140:1630-8.

44. Martin A, de Vittoris R, David V, Moraes R, Begeot M, Lafage-Proust MH, Alexandre C, Vico L, Thomas T. Leptin modulates both resorption and formation while preventing disuse-induced bone loss in tail-suspended female rats. Endocrinology 2005;146:3652-9.

45. Adami S, Braga V, Zamboni M, Gatti D, Rossini M, Bakri J, Battaglia E. Relationship between lipids and bone mass in 2 cohorts of healthy women and men. Calcif Tissue Int 2004;74:136-42.

46. Brownbill RA, Ilich JZ. Lipid profile and bone paradox: higher serum lipids are associated with higher bone mineral density in postmenopausal women. J Womens Health (Larchmt) 2006;15:261-70.

47. Yamaguchi T, Sugimoto T, Yano S, Yamauchi M, Sowa H, Chen Q, Chihara K. Plasma lipids and osteoporosis in postmenopausal women. Endocr J 2002;49:211-7.

48. Cui LH, Shin MH, Chung EK, Lee YH, Kweon SS, Park KS, Choi JS. Association between bone mineral densities and serum lipid profiles of pre- and post-menopausal rural women in South Korea. Osteoporos Int 2005;16:1975-81.

49. Orozco P. Atherogenic lipid profile and elevated lipoprotein (a) are associated with lower bone mineral density in early postmenopausal overweight women. Eur J Epidemiol 2004;19:1105-12.

50. Serre CM, Farlay D, Delmas PD, Chenu C. Evidence for a dense and intimate innervation of the bone tissue, including glutamate-containing fibers. Bone 1999;25:623-9.

51. Skerry TM, Genever PG. Glutamate signalling in non-neuronal tissues. Trends Pharmacol Sci 2001;22:174-81.

52. Chenu C, Serre CM, Raynal C, Burt-Pichat B, Delmas PD. Glutamate receptors are expressed by bone cells and are involved in bone resorption. Bone 1998;22:295-9.

53. Behl C, Skutella T, Lezoualc'h F, Post A, Widmann M, Newton CJ, Holsboer F. Neuroprotection against oxidative stress by estrogens: structure-activity

relationship. Mol Pharmacol 1997;51:535-41.

54. Singer CA, Figueroa-Masot XA, Batchelor RH, Dorsa DM. The mitogen-activated protein kinase pathway mediates estrogen neuroprotection after glutamate toxicity in primary cortical neurons. J Neurosci 1999;19:2455-63.

55. Zhu X, Liu X, He P, Cao B, Lv Y, Zhang W, Ni X. Metabolomics in serum of ovariectomised rats and those exposed to 17beta-oestradiol and genistein. Gynecol Endocrinol 2010;26:760-7.

56. Ma B, Zhang Q, Wang GJ, A JY, Wu D, Liu Y, Cao B, Liu LS, Hu YY, Wang YL, Zheng YY. GC-TOF/MS-based metabolomic profiling of estrogen deficiency-induced obesity in ovariectomized rats. Acta Pharmacol Sin 2011;32:270-8.

57. Rapuri PB, Kinyamu HK, Gallagher JC, Haynatzka V. Seasonal changes in calciotropic hormones, bone markers, and bone mineral density in elderly women. J Clin Endocrinol Metab 2002;87:2024-32.

58. Frankenfeld CL, McTiernan A, Thomas WK, LaCroix K, McVarish L, Holt VL, Schwartz SM, Lampe JW. Postmenopausal bone mineral density in relation to soy isoflavone-metabolizing phenotypes. Maturitas 2006;53:315-24.

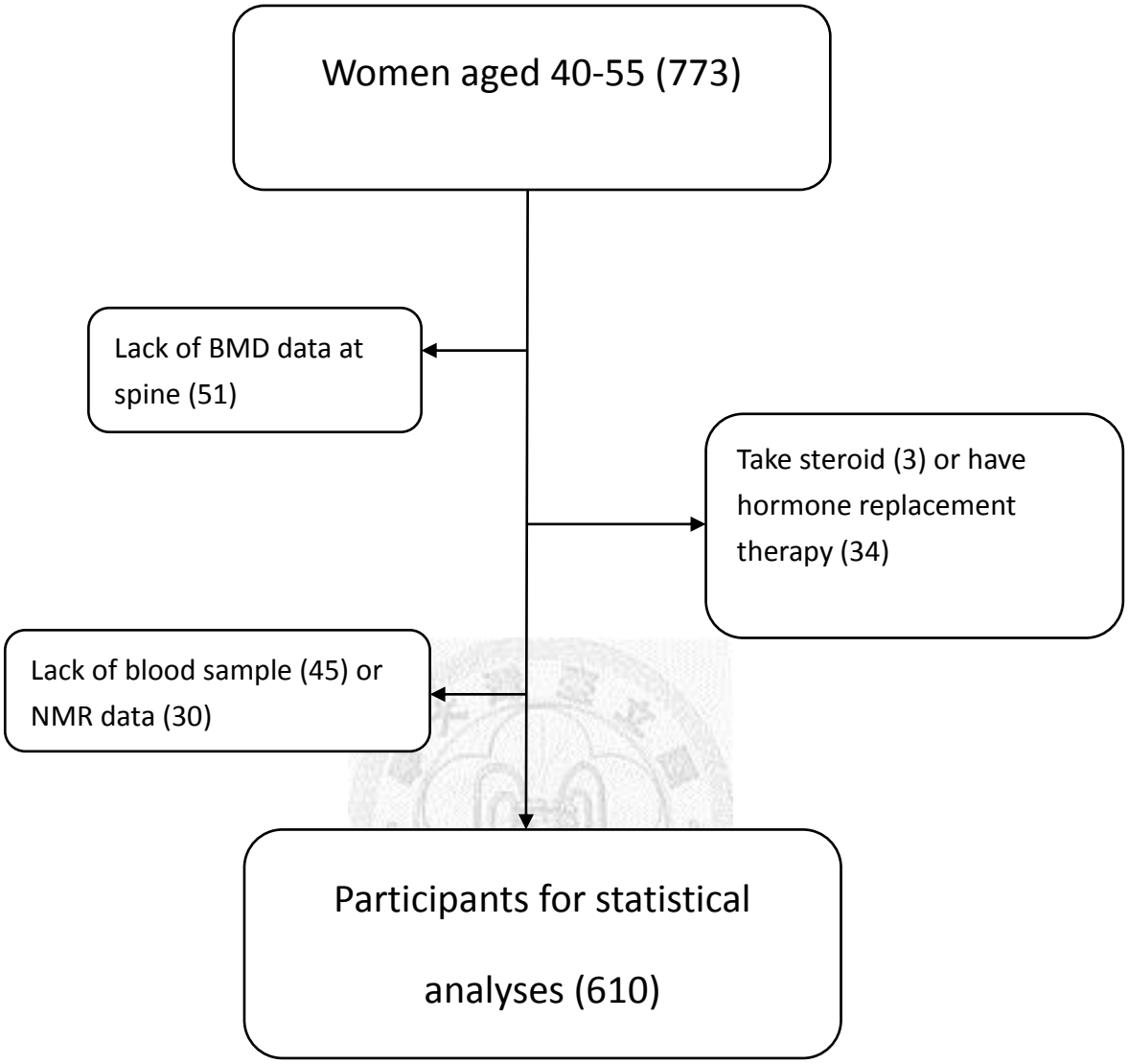


Figure 1. Flowchart of participant recruitment

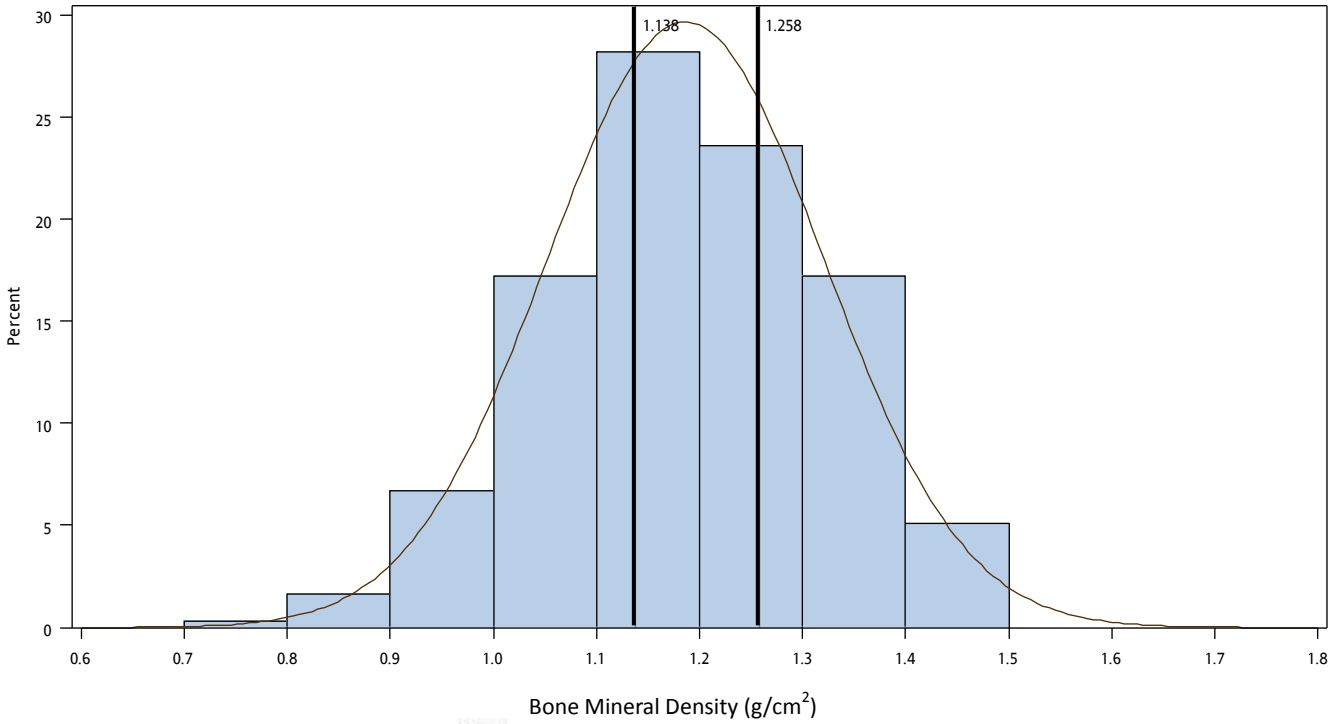


Figure 2. The tertiles of bone mineral density

T1: BMD < 1.138 g/cm^2; T2: 1.138 g/cm^2 ≤ BMD <1.258 g/cm^2; T3: BMD ≥ 1.258 g/cm^2.

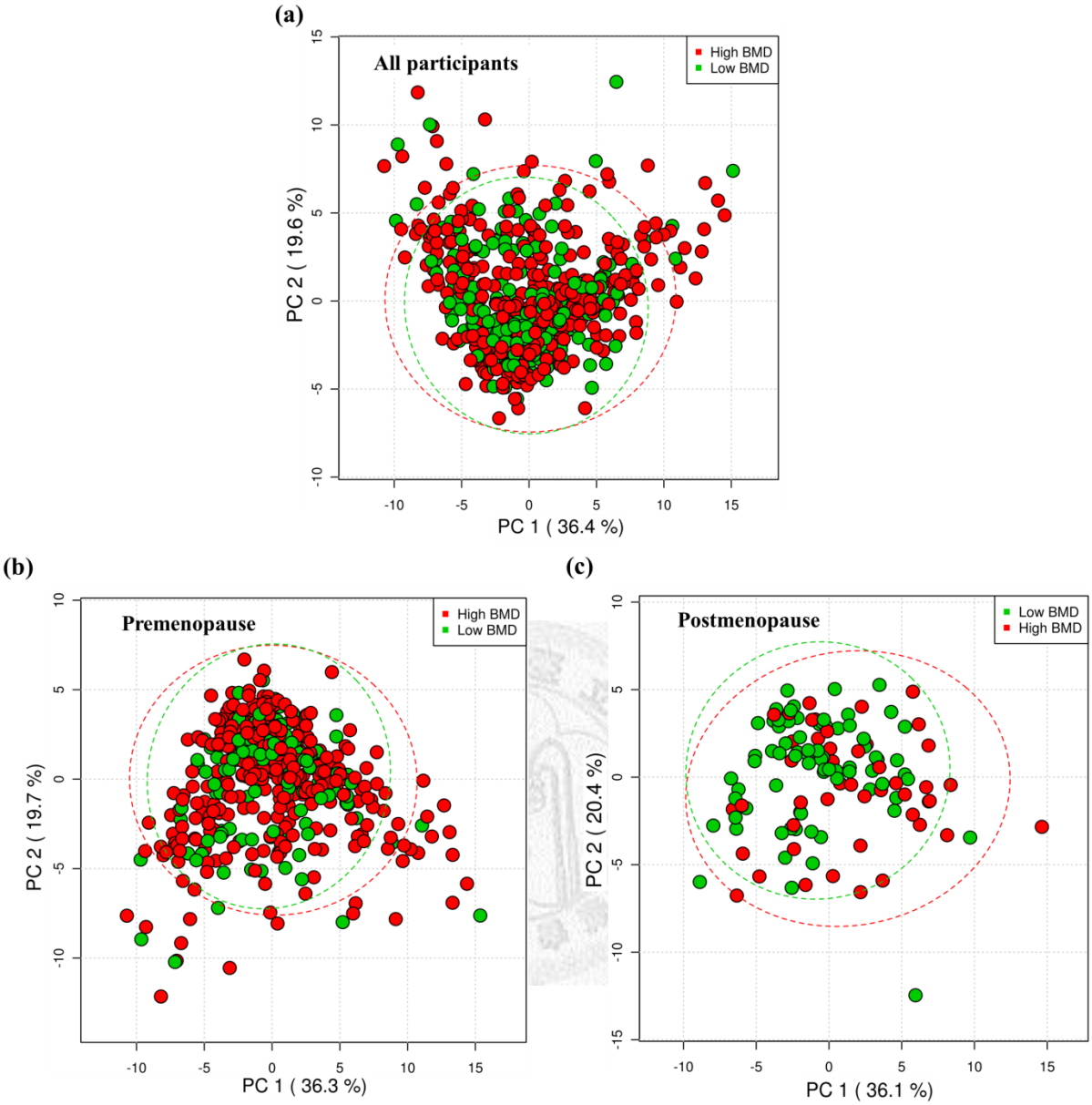


Figure 3. PCA score plots from the analysis of CPMG NMR spectra using women plasma samples

(a) High BMD group: n=399; low BMD group: n=211

(b) Premenopausal women. High BMD group: n=349; low BMD group: n=134

(c) Postmenopausal women. High BMD group: n=45; low BMD group: n=77

High BMD indicates the 2nd and 3rd tertiles of BMD; low BMD indicates the 1st tertile of BMD.

Abbreviations: PCA, principle components analysis; CPMG, Carr-Purcell-Meiboom-Gill; BMD, bone mineral density.

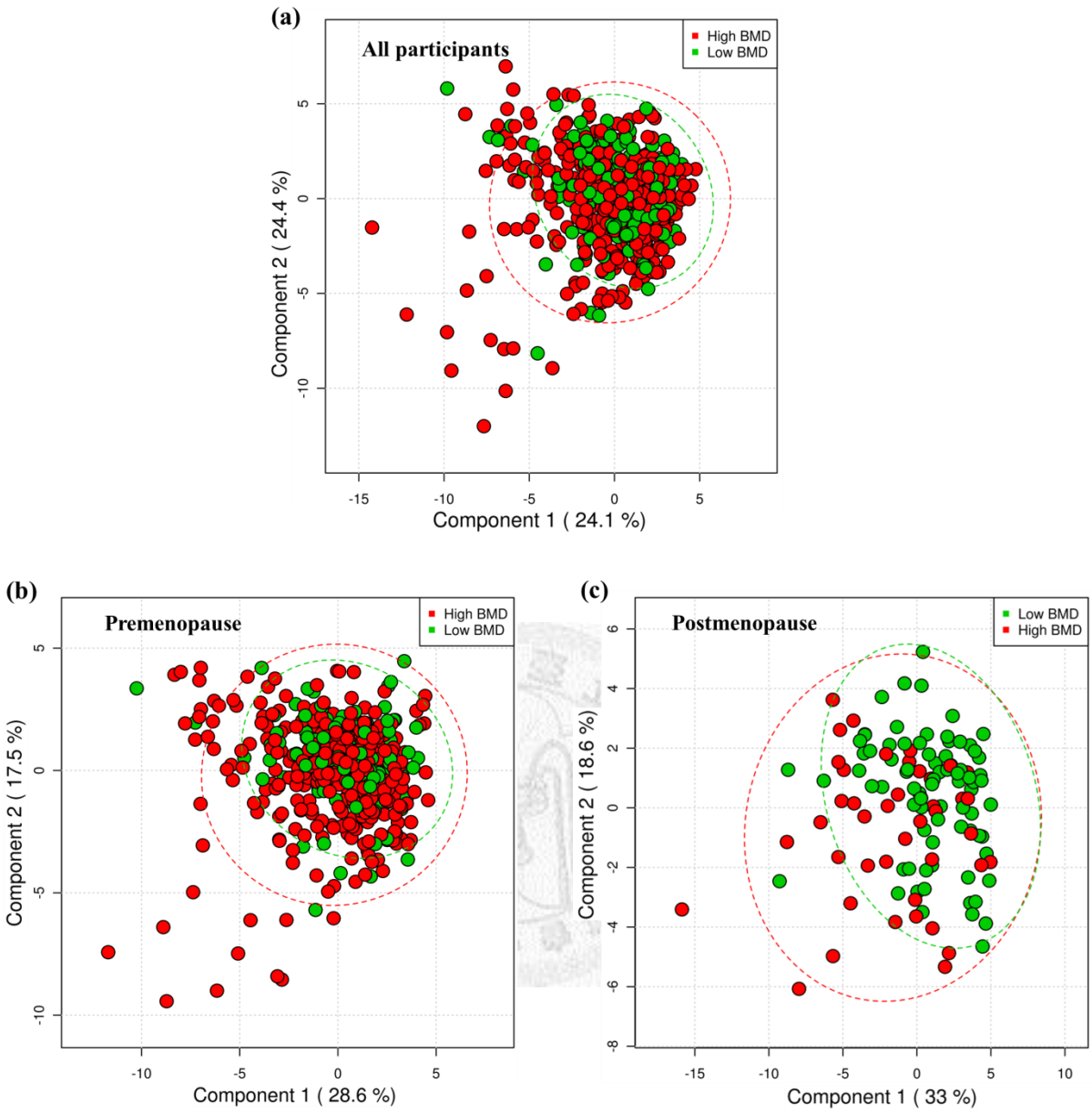


Figure 4. PLS-DA score plots from the analysis of CPMG NMR spectra using women plasma samples

(a) High BMD group: n=399; low BMD group: n=211

(b) Premenopausal women. High BMD group: n=349; low BMD group: n=134

(c) Postmenopausal women. High BMD group: n=45; low BMD group: n=77

High BMD indicates the 2nd and 3rd tertiles of BMD; low BMD indicates the 1st tertile of BMD.

Abbreviations: PCA, principle components analysis; CPMG, Carr-Purcell-Meiboom-Gill; BMD, bone mineral density.

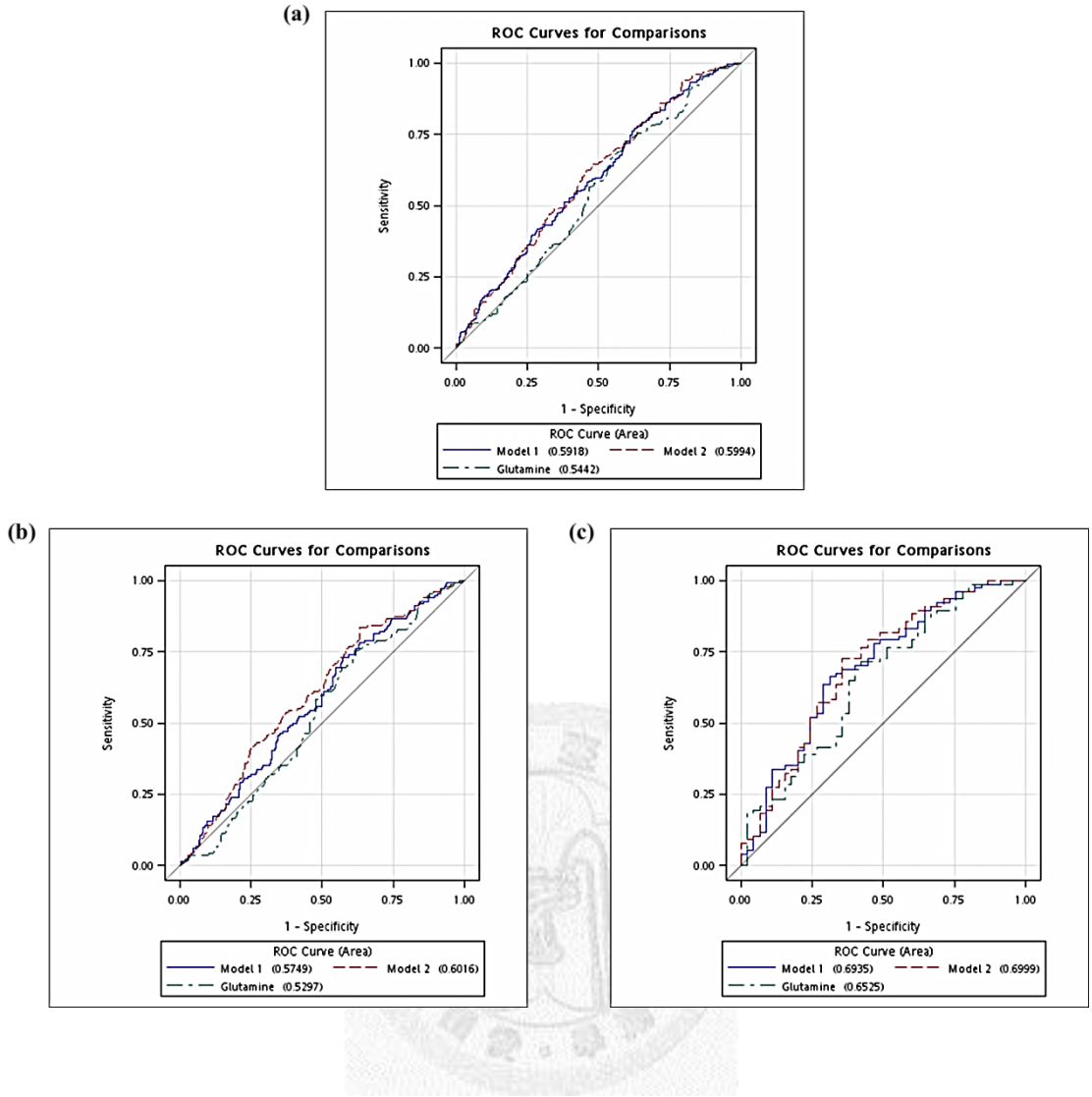


Figure 5. Receiver operating characteristic curves of comparing models for classification of high and low BMD

(**a**) All participants. Model 1: AUC= 0.59 (95% CI=0.55-0.64), Model 2: AUC=0.60 (95% CI=0.55-0.65). Glutamine alone: AUC=0.54 (95% CI=0.50-0.59).

(**b**) Premenopausal women. Model 1: AUC=0.57 (95% CI= 0.52-0.63), Model 2: AUC=0.60 (95% CI=0.55-0.66). Glutamine alone: AUC=0.53 (95% CI: 0.48-0.58).

(**c**) Postmenopausal women. Model 1: AUC=0.69 (95% CI: 0.67-0.72), Model 2: AUC=0.70 (95% CI: 0.67-0.73). Glutamine alone: AUC=0.65 (95% CI: 0.63-0.68).

Model 1: lactate, acetone, lipid, VLDL, and glutamine.

Model 2: Model 1 plus acetate and glucose.

High BMD indicates the 2nd and 3rd tertiles of BMD; low BMD indicates the 1st tertile of BMD.

Abbreviations: AUC, area under the curve; BMD, bone mineral density; CI, confidence interval; VLDL, very low density lipoprotein.

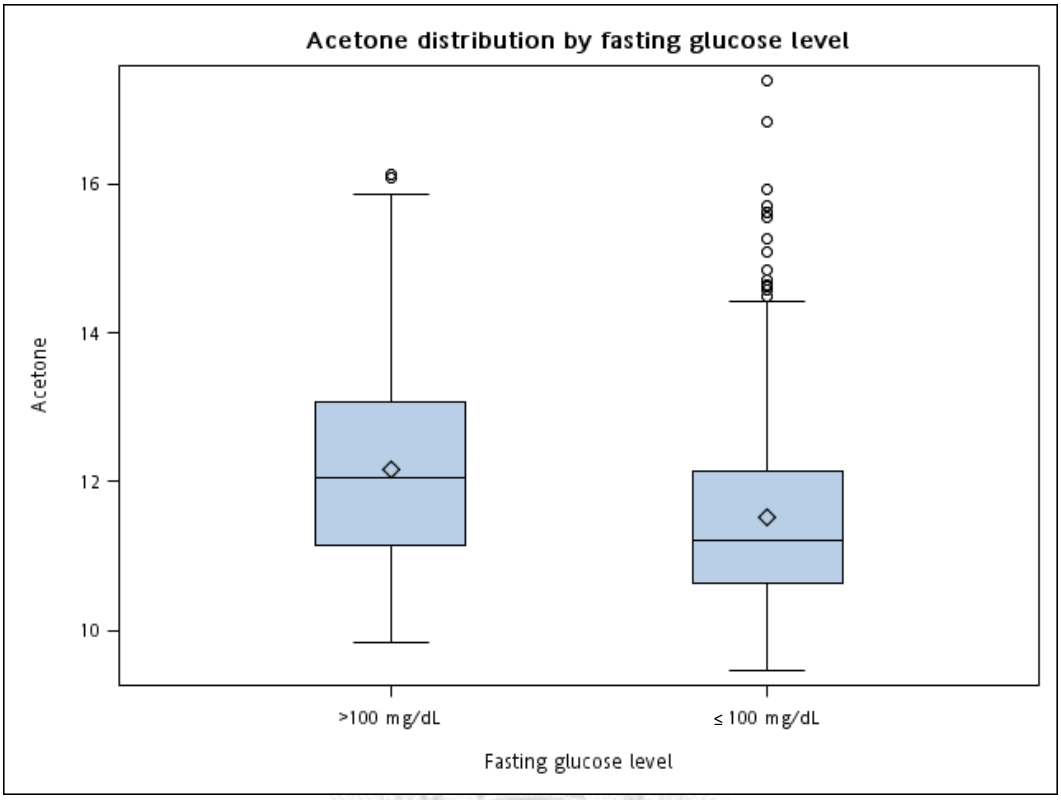


Figure 6. Acetone distribution by fasting glucose level (High: glucose> 100 mg/dL; Low: glucose ≤ 100 mg/dL)

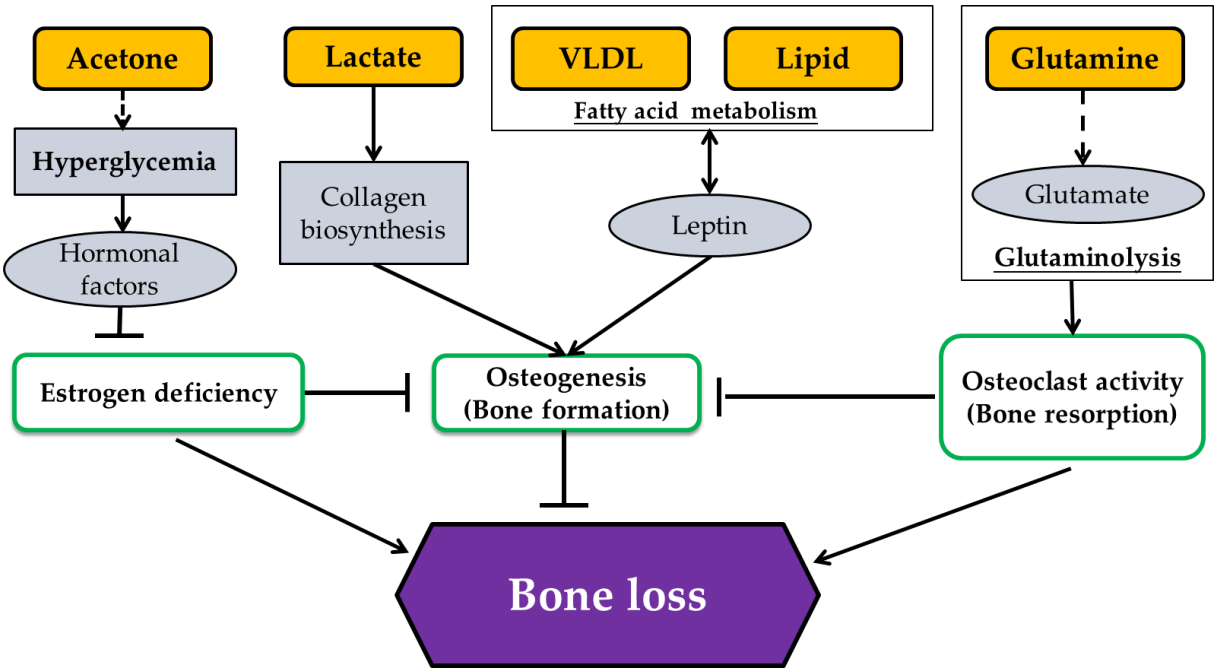


Figure 7. Postulated mechanism relates important metabolites with bone mineral density among postmenopausal women Acetone is referred to ketone bodies which may be generated during diabetic ketosis[36], and then promote to release hormonal factors that suppresses estrogen deficiency and the subsequent bone loss.[37-41] Lactate may against bone loss via regulating collagen biosynthesis during osteogenesis.[34] Lipid and very low density lipoprotein as well as lipid profiles can be reflected by leptin level which is related to differentiation of stromal cells to osteoblasts and thus prevent further loss of bone mass.[42-44] Bone resorption may occur when glutamine interconverts to excess amount of glutamate and then cause bone loss as the composition of osteoclast expresses glutamate receptors.[52]

Table 1. Characteristics of the study population

Variables	BMD		
	High (n=399)	Low (n=211)	*P**
	Mean (S.E.)		
Age (year)	45.4 (0.19)	47.6 (0.34)	**<0.0001**
Body weight (kg)	57.0 (0.45)	53.1 (0.52)	**<0.0001**
Body mass index (kg/m^2)	22.7 (0.16)	21.5 (0.20)	**<0.0001**
Waist circumference (cm)	73.6 (0.35)	71.6 (0.44)	**0.0008**
Diastolic blood pressure (mmHg)	64.9 (0.51)	63.7 (0.64)	0.13
Fasting glucose (mg/dL)	101.4 (1.16)	97.2 (0.61)	**0.01**
Triglycerides (mg/dL)	94.1 (2.70)	87.3 (3.10)	0.10
High-density lipoprotein cholesterol (mg/dL)	69.8 (0.77)	72.7 (1.25)	0.05
Total cholesterol (mg/dL)	198.4 (1.66)	202.7 (2.27)	0.13
Alkaline phosphatase (IU)	60.2 (0.84)	67.3 (1.20)	**<0.0001**
Creatinine (mg/dL)	0.80 (0.005)	0.78 (0.006)	**0.03**
	n (%)		
Menopause	45 (11.4)	77 (36.5)	**<0.0001**
Hypertension	72 (18.1)	30 (14.2)	0.23
Ever smoker	40 (10.4)	16 (8.0)	0.34
Alcohol consumption	15 (4.0)	14 (7.3)	0.09
Calcium supplement	213 (53.9)	120 (56.9)	0.49
Regular exercise (≥2 times/week)	168 (45.7)	91 (49.2)	0.43

Abbreviation: BMD, bone mineral density.

**P*-values were obtained from Student's t tests (normally distributed continuous variables) and Chi-square tests (categorical variables) by comparing participants with high and low BMD.

Number in bold indicates statistically significant finding.

Table 2. The PLS-DA parameters and permutation test for differentiating high and low BMD levels

Comparison Groups	PLS-DA parameters			$P_{permutation\ test}$ [c]
	No.[b]	R^2	Q^2†	
High BMD－Low BMD[a]	2	0.03	-0.002	0.07
Premenopause				
High BMD－Low BMD	1	0.01	-0.02	0.76
Postmenopause				
High BMD－Low BMD	1	0.12	0.04	**0.03**

Abbreviations: PLS-DA, partial least squares-discriminant analysis; BMD, bone mineral density.

[a] High BMD indicates the 2nd and 3rd tertiles of BMD; low BMD indicates the 1st tertile of BMD.

[b] The number of components based on Q^2 indicates the best classifier of PLS-DA using 10-fold cross-validation method.

[c] Distribute 1000 permutations.

†Predictive capability.

Number in bold indicates statistically significant finding.

Table 3. The change of plasma metabolites in postmenopausal women to distinguish high and low BMD

Metabolites	Multiplicity[a]	Signal assignment[b]	δ H (ppm)	VIP[c]	High BMD[d]	Low BMD[c]	Pathways*
Lactate	d	CH_3	1.33	1.74-3.31	↑	↓	Glycolysis/Gluconeogenesis
Acetone	s	CH_3	2.22	1.84-3.16	↑	↓	Synthesis and degradation of ketone bodies
Acetate	s	CH_3	1.91	1.56-1.82	↓	↑	Pyruvate metabolism
Lipid	m	$CH_3CH_2CH_2$, $(CH_2)_n$ $CH_3CH_2(CH_2)_n$,	1.20-1.26	2.59	↑	↓	Fatty acid metabolism
VLDL	m	CH_2CH_2CO	1.53-1.6	1.63-2.49	↑	↓	
Glucose	ddd	Histone H2, H3, H4, H5	3.37-3.57	2.75	↑	↓	Carbohydrate metabolism
Glutamine	m	half γ -CH_2	2.42-2.48	1.55-2.09	↓	↑	D-Glutamine and D-glutamate metabolism

Abbreviations: BMD, bone mineral density; VLDL, very low density lipoprotein; δ H, chemical shift; ppm, parts per million.

[a] s,singlet; d, doublet; t, triplet; m, complex multiplet; ddd, doublet of doublets of doublets.

[b] Signal assignment provides precise bioanalytical and dynamic information.

[c] All metabolites with variable importance in projection (VIP) score >1.5.

[d] High BMD indicates the 2nd and 3rd tertiles of BMD; low BMD indicates the 1st tertile of BMD.

*Pathway information was obtained from KEGG PATHWAY Database (http://www.genome.jp/kegg/).

Table 4. Association between plasma metabolites and bone mineral density (T1 vs. T2 + T3)

Metabolites[a]	High BMD[b] (n=399)	Low BMD[b] (n=211)			
	OR	OR (95% CI)	*P* value	AOR (95%CI)[c]	*P* value
Lactate	1.00	0.92 (0.81-1.04)	0.16	0.90 (0.76-1.05)	0.18
Acetone	1.00	0.90 (0.79-1.02)	0.09	0.87 (0.74-1.03)	0.11
Acetate	1.00	1.40 (0.97-2.03)	0.07	1.32 (0.83-2.10)	0.24
Lipid	1.00	1.08 (0.44-2.66)	0.87	0.72 (0.24-2.18)	0.56
VLDL	1.00	0.88 (0.75-1.04)	0.13	0.85 (0.69-1.04)	0.11
Glutamine	1.00	**1.47 (1.07-2.03)**	**0.02**	**1.55 (1.03-2.33)**	**0.04**
Glucose	1.00	0.96 (0.91-1.02)	0.18	0.99 (0.93-1.06)	0.83

Abbreviations: AOR, adjusted odds ratio; BMD, bone mineral density; VLDL, very low density lipoprotein; CI, confidence intervals.

[a] All metabolites with variable importance in projection (VIP) score >1.5.

[b] High BMD indicates the 2nd and 3rd tertiles of BMD; low BMD indicates the 1st tertile of BMD.

[c] All models were adjusted for age (continuous), body weight (continuous), height (continuous), waist circumference (continuous), menopausal status (yes/no), creatinine (> median, 79 mg/dL, yes/no), regular exercise (≥2 times/week, yes/no), serum alkaline phosphatase (≥ median, 60 IU, yes/no).

Number in bold indicates statistically significant finding.

Table 5. Association between plasma metabolites and bone mineral density stratified by menopausal status (T1 vs. T2+T3)

Metabolites[a]	Premenopause		Postmenopause		*p interaction*
	Low BMD/High BMD[b] (134/349)		Low BMD/ High BMD (77/45)		
	OR (95% CI)	AOR (95%CI)[b]	OR (95% CI)	AOR (95%CI)[b]	
Lactate	0.96 (0.83-1.11)	1.01 (0.85-1.21)	**0.65 (0.49-0.86)**	**0.55 (0.33-0.92)**	**0.004**
Acetone	0.90 (0.77-1.06)	0.97 (0.81-1.17)	**0.64 (0.48-0.86)**	**0.51 (0.31-0.85)**	**0.01**
Acetate	1.13 (0.72-1.75)	1.01 (0.60-1.68)	**4.02 (1.67-9.67)**	3.31 (0.83-13.19)	**0.02**
Lipid	0.97 (0.33-2.89)	1.32 (0.38-4.56)	**0.12 (0.02-0.98)**	**0.04 (0.001-0.91)**	**0.04**
VLDL	0.89 (0.73-1.08)	0.95 (0.76-1.18)	**0.62 (0.43-0.88)**	**0.49 (0.27-0.90)**	**0.02**
Glutamine	1.32 (0.90-1.95)	1.24 (0.78-1.96)	**2.88 (1.45-5.72)**	**6.04 (1.57-23.21)**	**0.04**
Glucose	0.96 (0.90-1.03)	0.97 (0.90-1.04)	0.95 (0.85-1.06)	1.14 (0.92-1.40)	0.38

Abbreviations: AOR, adjusted odds ratio; BMD, bone mineral density; VLDL, very low density lipoprotein; CI, confidence intervals.

[a] All metabolites with variable importance in projection (VIP) score >1.5.

[b] High BMD indicates the 2nd and 3rd tertiles of BMD; low BMD indicates the 1st tertile of BMD.

[c] All models were adjusted for age (continuous), body weight (continuous), height (continuous), waist circumference (continuous), menopausal status (yes/no), creatinine (> median, 79 mg/dL, yes/no), regular exercise ≥2 times/week (yes/no), serum alkaline phosphatase (≥ median, 60 UI, yes/no).

Number in bold indicates statistically significant finding.

Table 6. Receiver operating characteristic contrast tests of pairwise comparison between different models to classify high and low BMD

Contrast models	All participants	Premenopause	Postmenopause
	P value*		
Model 2 vs. Mode 1[a]	0.38	0.16	0.74
Glutamine alone vs. Model 1	**0.05**	0.12	**0.03**
Glutamine alone vs. Model 2	**0.03**	**0.02**	**0.01**

[a] Model 1 included lactate, acetone, lipid, VLDL, and glutamine; model 2 included variables in model 1 plus acetate and glucose.

**P*-values were obtained from Chi-square tests.

Number in bold indicates statistically significant finding.

Table 7. Model comparisons for the association between plasma metabolites and bone mineral density (T1 vs. T2+T3)

	Lactate	Acetone	Acetate	Lipid	VLDL	Glutamine	Glucose
	Low BMD[a] (399) / High BMD[a] (211)						
	AOR (95% CI)						
Model 1[b]	0.92 (0.81-1.04)	0.90 (0.79-1.02)	0.42 (0.97-1.03)	1.08 (0.44-2.66)	0.88 (0.75-1.04)	**1.47 (1.07-2.03)**	0.96 (0.91-1.02)
Model 2[c]	**0.86 (0.76-0.99)**	**0.82 (0.71-0.95)**	**1.55 (1.05-2.30)**	0.56 (0.21-1.48)	**0.80 (0.67-0.95)**	**1.67 (1.19-2.35)**	0.96 (0.91-1.02)
Model 3[d]	0.99 (0.86-1.15)	0.95 (0.82-1.10)	1.07 (0.70-1.63)	1.04 (0.37-2.90)	0.93 (0.77-1.11)	1.22 (0.84-1.76)	0.99 (0.93-1.06)
Model 4[e]	0.94 (0.80-1.09)	0.92 (0.78-1.08)	1.17 (0.74-1.83)	0.85 (0.29-2.51)	0.89 (0.74-1.09)	1.40 (0.94-2.09)	1.00 (0.93-1.07)
Model 5[f]	0.90 (0.76-1.05)	0.87 (0.74-1.03)	1.32 (0.82-2.10)	0.72 (0.24-2.12)	0.85 (0.69-1.04)	**1.55 (1.03-2.33)**	0.99 (0.93-1.06)

Abbreviations: AOR, adjusted odds ratio; BMD, bone mineral density; VLDL, very low density lipoprotein; CI, confidence intervals.

[a] High BMD indicates the 2nd and 3rd tertiles of BMD; low BMD indicates the 1st tertile of BMD.

[b] Model 1: Unadjusted model.

[c] Model 2: Adjusted for age (continuous), menopausal status (yes/no).

[d] Model 3: Adjusted variables in model 2 plus body weight (continuous) and height (continuous)

[e] Model 4: Adjusted variables in model 3 plus serum alkaline phosphatase (≥ median, 60 IU, yes/no) and regular exercise (≥2 times/week, yes/no)

[f] Model 5: Adjusted variables in model 4 plus waist circumference (continuous) and creatinine (> median, 79 mg/dL, yes/no).