Effects of alendronate and vitamin D on metabolomic profiles in a rat model of osteoporosis

By

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Abstract:

Osteoporosis is a progressive bone disease and a significant global medical issue, which places a serious economic and health burden on individuals, families and societies. It is well known that early diagnosis of osteoporosis has a vital role in reducing the burden and complications of excessive bone resorption. It is also known that bisphosphonate drugs play a key role in limiting bone loss. Our study hypothesis was that the identification of metabolites in plasma may serve as a diagnostic mechanism for measuring the extent of bisphosphonate drug suppression of bone loss in patients following long-term bisphosphonate therapy. Our objectives were first, to establish a metabolomic profile in a rat model of osteoporosis that is characteristic of the osteoporosis phenotype by using LC-MS/MS chromatography. Secondly, we wanted to characterize specific metabolites in this rat model that might serve as indicators of bisphosphonate-induced suppression of bone remodeling in osteoporosis. In addition to this work, we also performed Receiver Operator Characteristic (ROC) curve analysis of the metabolomic data to identify potential osteoporosis biomarkers. We also determined the correlation between bone mineral density and specific plasma metabolites in an effort to create an osteoporosis screening tool for use in clinical practice. To perform the metabolomic analysis we used a commercially available metabolomics kit (Biocrates p180), which was run on a Sciex Qtrap 4000 mass spectrometer equipped with an Agilent HPLC system. The study subjects were divided into three experimental groups: control rats dosed with vehicle, rats dosed with 0.12mg/kg alendronate twice weekly, and a third group of rats dosed with a combination of alendronate and vitamin D. Plasma from the three groups of rats was collected at baseline, and then at the 8-week study endpoint and

subjected to metabolomics analysis and *in vivo* micro CT scan measurement to accurately determine bone volume.

Our findings showed that alendronate regulated several key plasma metabolites, notably certain amino acids, a number of lipids, and glucose. These metabolites are likely involved in the processes of bone resorption and bone formation. A distinct metabolite "fingerprint" was detected following the drug treatment between control and treated groups, with distinct metabolic changes seen in the treated groups compared to the control groups. Our results indicated that there was a correlation between 4 metabolites (proline, trans-hydroxyproline, histamine and methionine) and CT-measured bone volume. These findings suggest that the use of metabolomic profiling as a clinical research tool holds great promise for elucidating both the biological activity and the toxicity of bisphosphonate drugs on bone burden. Such an approach would be of significant value in gauging the impact of long-term bisphosphonate drug usage in osteoporosis patients, in assessing the effectiveness of osteoporosis therapy, and the avoidance of bisphosphonate-related side effects such as osteonecrosis of the jaw.

Preface

This thesis is an original work by Amel M. AB Hamza. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "In-vivo" bone imaging using non-invasive imaging modalities", No. AUP00000246, March 2016.

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List of abbreviation

°C	Degree celsius
ALN	Alendronate
ANOVA	Analysis of variance
AUC	Area under the curve
BMI	Body mass index
Bps	Bisphosphonate
DXA	Dual X-ray absorptiometry
ERs	Estrogen receptors
IS	Internal standard
IV	Intravenous
Kg	Kilogram
L	Liter
LC-MS/MS	Liquid chromatography - mass spectrometry-tandem mass spectrometry
mg	Milligram
mL	Milliiter
mM	Millimolar
MRM	Multiple reaction monitoring
NMR	Nuclear magnetic resonance
1,25(OH) 2D	1,25-dihydroxyvitamin D
PCA	Principal component analysis
PLS-DA	Partial least squares discriminant analysis
QCT	Quantitative computed tomography

RANK	Receptor activators of nuclear factor kappa-B ligand
ROC	Receiver operating characteristic
SD	Standard deviation
SC	Subcutaneous
t ½	Terminal elimination phase half-life
TG	Triglycerides
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
Vd	Volume of distribution
VIP	Variable importance in projection
WHO	World health organization
α	Level of significance
αMSH	α -melanocyte-stimulating hormone
μg	Microgram
μL	Microliter

CHAPTER1: INTRODUCTION

1.1 Basic Structure and Function of Bone

Bone is vital for many different physiological and biological roles, including mechanical support, protection of internal organs, transmission of forces generated by muscles and metabolic mineral homeostasis. It is also accepted that bone has an important role in endocrinology as it largely functions as a hematopoietic organ. In particular, the marrow cavity inside bone is a site for hematopoiesis. (1) The endocrine function of bone is mainly responsible for synthesizing two hormones, fibroblast growth factor and osteocalcin. (2) Bone also acts like a protective covering in places like the torso and head where injuries to these locations might otherwise be fatal. The organic constituents of bone include an extracellular matrix and specialized cells responsible for production and maintenance of the matrix. The bone matrix is composed of organic components known as osteoid and ground substance (35%) and mineral components primarily made up of calcium hydroxyapatite (65%). Bone derives its rigidity from the mineral component, which subsequently determines its mechanical behavior. (3)Bone, with the help of numerous hormones, can coordinate several processes in the brain, the pancreas and the kidney including glucose metabolism and skeletal tissue mineralization. (3)

Bone is a dynamic tissue that is built, broken down and rebuilt under a process called bone remodeling. The balance between the activity of osteoblast cells that create the bone and the osteoclast cells that break it down constitutes the Bone

Mass. Bone formation and resorption are initiated by differentiated bone cells (osteoblasts and osteoclasts respectively), with each cell performing a specific function. Bone remodeling is regulated by osteoclasts, which are multinucleated

cells that resorb bone tissue whereas osteoblasts refill the resorption cavities created by osteoclasts. Bone tissue serves as a reservoir for essential minerals such as calcium, phosphate and many biologically active compounds responsible for growth. Osteoblasts play a significant role in building bone tissue whereas osteoclasts maintain homeostasis by releasing calcium from the bone that is used by different parts of the body.

1.2 Types of bone cells

Osteoblasts originate from mesenchymal stem cells. They are located on the surfaces of the bone matrix where they synthesize, transport and assemble components needed by the bone matrix and regulate its mineralization. (4)

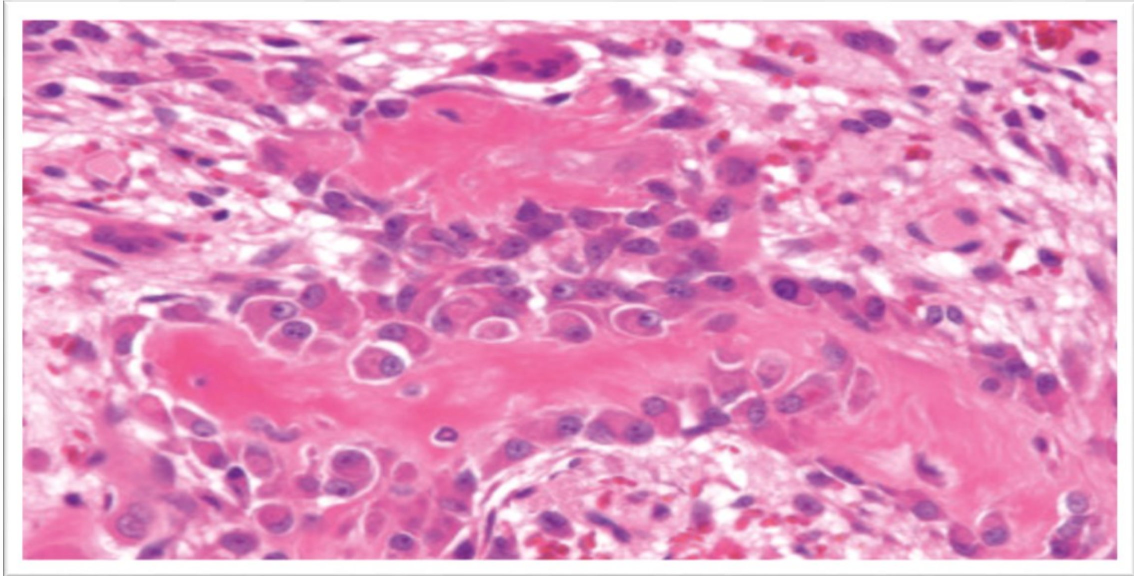


Figure 1: Active osteoblasts synthesizing bone matrix. The surrounding spindle cells represent osteoprogenitor cells. Copyright permission obtained from Robbins Basic Pathology, Tenth edition. (4)

Osteocytes are located within the bone, interconnected by a complex network of cytoplasmic processes through tunnels called canaliculi. Osteocytes translate biological activity via a mechanical force process (mechanotransduction) that in turn regulates bone tissue remodeling, which in turn, regulates plasma calcium and phosphate levels.[5]

Osteoclasts are multinucleated macrophage cells, which were differentiated from a mononuclear phagocytic precursor cell common with macrophages (Figure 3). They attach themselves to the bone matrix to create an acidic environment and secrete neutral proteases for bone absorption. (4)

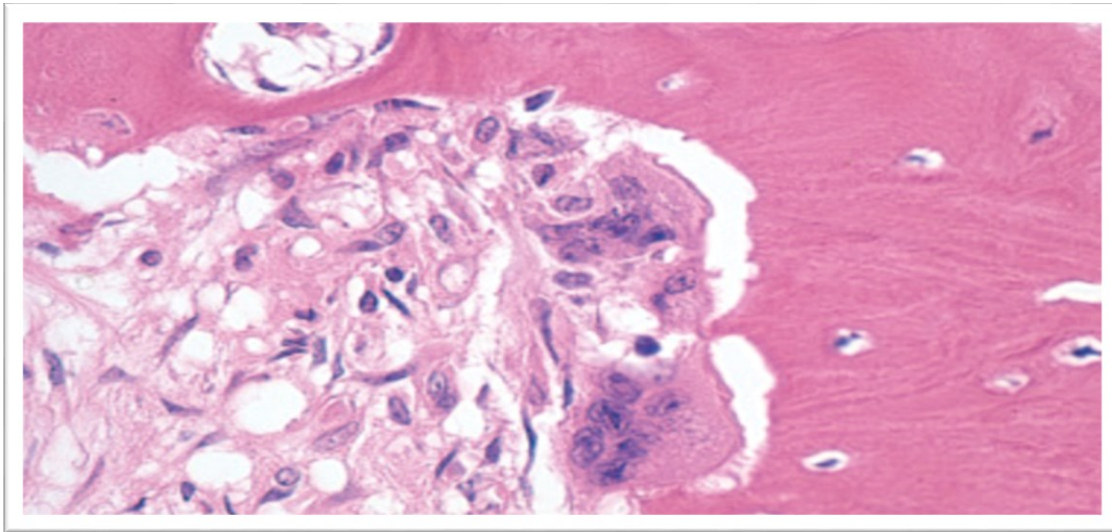


Figure 2: osteoclasts cells indicate resorbing bone. Copyright permission obtained from Robbins Basic Pathology, Tenth edition.. (4)

1.3 Structural Types of Bone

There are only two major types of bones: cortical bone and cancellous/trabecular bone. (5) Adult human bone is composed of 80% cortical bone and 20% trabecular bone.(6)

Both bone types consist of osteons but they differ in their bone structure. Cortical bone is dense and is comprised of Haversian systems (cylindrical multicellular units), which are approximately 40 mm long and 200 mm wide at their base. In contrast, trabecular bone is fragile and resembles a honeycomb. Trabecular bone is composed of plates and rods (about 50 to 400 mm thick) in a structure known as packets. (6) Cancellous bones are only required for mechanical or structural support but cortical bones function in both mechanical support and protection. This physical explanation makes the incidence of thinning of the bone cortex and increased cortical bone porosity during bone modeling more dangerous than for the trabecular kind. (3)

Cortical bones (Figure 3) are prominent in the diaphyses of long and short bones and trabecular bones are seen in the metaphyses of long bones, and in the vertebrae. Cortical bone tissue is the primary component of the long bone shafts but it is also found surrounding the cancellous bone tissue in the vertebrae region. (1)

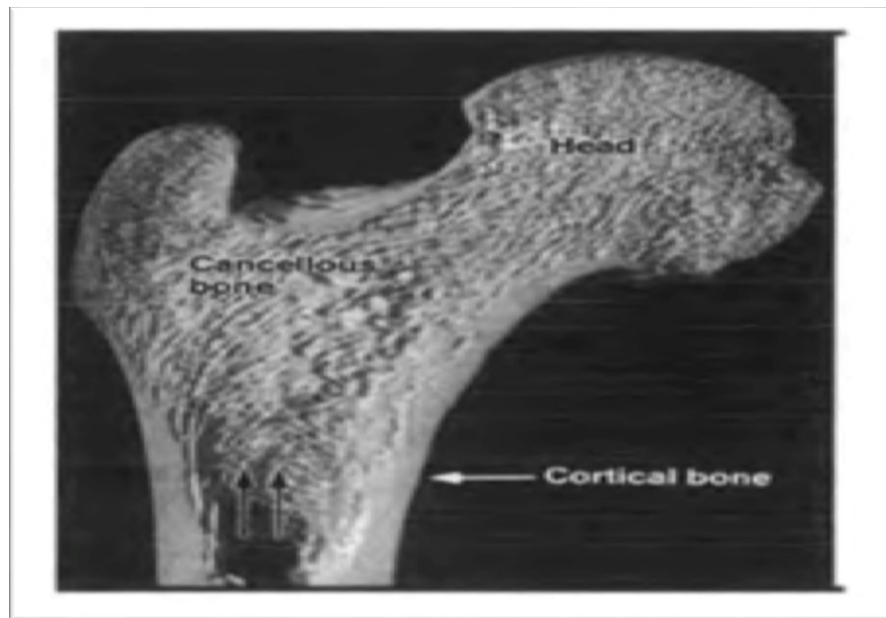


Figure 3: Bone section of the femur in the frontal plane representing cortical and cancellous bone. Double arrows indicate arching design of trabecular. Copyright permission obtained from (Downey and Siegel, 2006) (158)

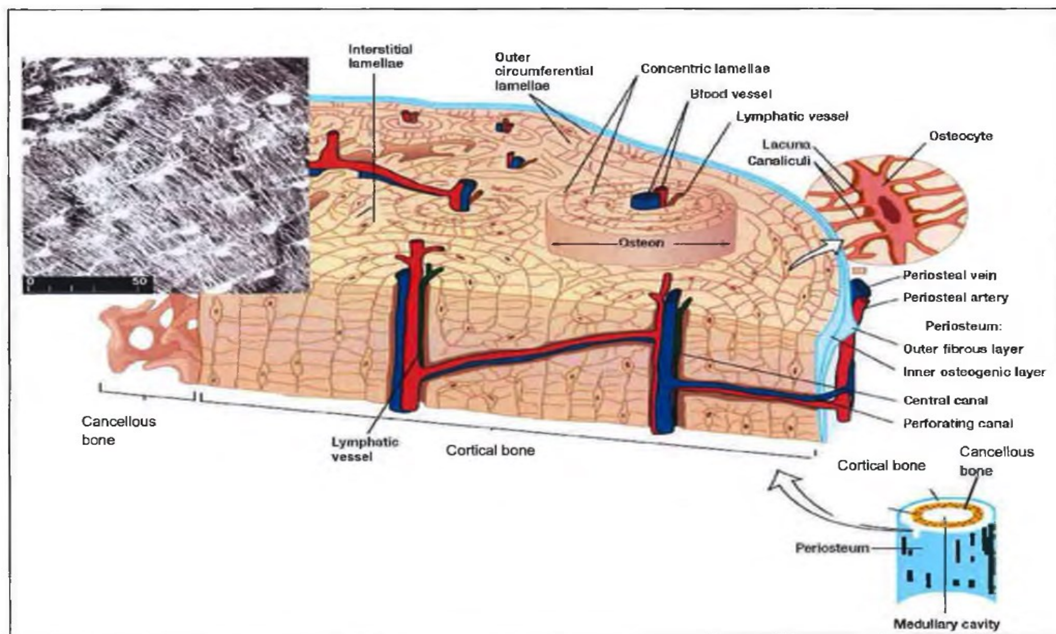


Figure 4: The structure of bone. Copyright permission obtained from (Taylor et al., 2007) (8)

Experimental data indicate that the two major processes of bone remodeling (i.e., bone formation and resorption) are closely regulated and that local factors play a critical role in their control as well. Many studies have also proven that mechanical stimulus has a direct role in the initiation of bone remodeling. (9)

Bone remodeling is referred to as a process where a replenished bone structure is reinstated on the old trabecular surface forming new bone tissue. The remodeling cycle is composed of four consecutive phases that are highly regulated, as mentioned before. Activation precedes resorption, which precedes reversal, which again precedes formation. (10) Twin studies have confirmed that an individual's peak bone density is about 70 percent heritable. Another research study pointed out that most of the bone mass at multiple skeletal locations will be increased by late adolescence (11,12). However, other factors such as dietary habits and exercise during childhood and adolescence may influence peak bone mass for any given individual. (13)

An excessive increase in the number of bone-resorbing sites may lead to a temporary decrease in bone mass. In normal individuals, due to an established coupling effect between bone resorption and bone formation (known as reversal), bone formation will compensate for the resorptive loss and subsequently restore the bone volume balance. Nevertheless, if increased bone resorption results in the physical breakage and separation of trabecular struts in cancellous bones, the template for subsequent bone replenishment is lost. Such disruption in the microstructure of bone results in reduced structural support within the bone, which increases the risk of fragility fracture.

1.4 Scope of Osteoporosis

The word osteoporosis originated from the Greek language where “osteo” means bone and “poros” meant porous. Johann Friedrich George Christian Martin was the first to coin the term “osteoporosis”. The word itself clearly describes the diagnosis and the extent of the effects of the disease. (14)

In 1994, the World Health Organization (WHO) defined osteoporosis as a bone mineral density (BMD) with a score of -2.5 standard deviations (SD) or lower, when compared to the young adult population, measured by the Dual-energy X-ray Absorptiometry (DXA) technique. If the BMD is less than -2.5 SD, a diagnosis can be made of osteoporosis. Accordingly, standardized methods and measurements must be prescribed in the BMD laboratory to ensure precision. (15)

Osteoporosis is a chronic disease that affects the elderly. From many studies, it is evident that the prevalence of osteoporosis has increased significantly in our aging population and will, therefore, affect our society negatively. Moreover, because of the overall burden of illness in this particular age group, it is imperative to improve the diagnosis and the management of this disorder, in an effort to attenuate these outcomes. The 2010 guidelines for the diagnosis and management of osteoporosis in Canada were contained in a report released on the assessment of the risk of fractures and therapies for osteoporosis. This document also discussed the importance of managing osteoporosis. (16)

Osteopenia, which is a pre-disease state of osteoporosis, is characterized by a

BMD value above -2.5 SD but lower than -1 SD compared to the BMD of a young adult population. However, BMD values greater than -1 SD are part of the

normal distribution of bone mass. (17) The T-score compares the BMD of the patient to the BMD of the healthy 30 year old, and is often calculated along with the Z-score where the BMD is compared with age-matched individuals. The risk of fracture is known to increase by two-fold with each SD decrease in the T-score.

Risk for fracture increases to 2.6X for the femoral neck and 2.3X for vertebral fracture. (18,19) Additional complications, such as crushed vertebrae, the lack of medical reference records when the patient was a child and the physical state of a patient can limit the usage of BMD for fear of inaccurate results.

1.5 Osteoporosis

Today we define osteoporosis (OP) as the decrease in the amount of bone with micro-architectural deterioration leading to the potential of a fracture in the hip, spine, waist and shoulder. (6) Osteoporosis is also known as the "silent thief" as the symptoms arising from bone loss or bone "theft" are undetectable at the beginning of the disease.

The annual statistics from Canada indicate that bone fractures from OP are more common than heart attack, stroke and breast cancer combined. In Canada, about 1 in 3 women and 1 in 5 men seem to suffer from osteoporosis. (20)The cost of hospitalization and the frequent need for nursing home care or other assistance for fractures, particularly hip fractures, place enormous burdens on the patient, the family and society. The annual cost of medical care for osteoporosis treatment and osteoporosis fractures was estimated at $3.9 billion (www.osteoporosis.ca).

Statistics show that the most common type of osteoporotic fractures are hip fractures. Hip fractures are an integral part of the OP syndrome. Recently compiled data indicates that 28 percent of women and 37 percent of men who suffer from a hip fracture will die within one year. According to recent studies, osteoporosis is the primary cause of over 80 percent of all fractures in people over the age of 50. Approximately half of the hip fracture patients never recover to their same level of function prior to the fracture, and some lose their independence completely. (www.osteoporosis.ca).

1.5.1 Type of Osteoporosis

1.5.1.1 Postmenopausal osteoporosis

Osteoporosis is most prominent in postmenopausal women and will usually manifest as a fracture in the vertebra, or as a Colles' fracture of the wrist, or a fracture of the femur. It is a form of primary osteoporosis and the reason behind the increased susceptibility in older women due to the decline in estrogen levels, which results in increased bone resorption. Estrogen has a primary effect on bone turnover by stimulating the expression of osteoprotegerin that binds to, and represses the action of receptor activators of nuclear factor kappa-B ligand (RANKL), thereby preventing its interaction with its receptor. (6)

Studies suggest that during the lifetime of a woman, she will lose 35 percent of cortical bone mass and about 50 percent of trabecular bone mass. This, in turn, leads to a regional systematic bone loss. Cortical bone losses usually occur only after the age of 40 as opposed to the trabecular bone loss, which starts from the age of 35 years. (6) Estrogen is also known to inhibit osteoclast formation. Thus, increased resorption after menopause leads to an accelerated loss of bone mineral density. (21)

1.5.1.2 Osteoporosis in men

It is well established that the occurrence of osteoporosis in men is less frequent when compared to the rate at which women are affected. Nevertheless, the number of hip fractures in men and women is increasing steadily and it is expected to reach 1.1 million worldwide by 2025. (22) Osteoporosis in men has different risk factors than for women.

These factors include an excess of glucocorticoids, hypogonadism and a variety of other systematic hormonal conditions. Lack of menopause, greater bone mass and shorter life expectancy are other reasons suggesting the reduced occurrence of osteoporosis in the male population. Though hip fractures are more common in women than men, there is a greater chance of femoral fractures in men with osteoporosis. (23)

1.5.1.3 Secondary osteoporosis

Other than primary osteoporosis, another form of osteoporosis is known as secondary osteoporosis. It arises from secondary or indirect causes, including a variety of medical illnesses like serious kidney failure, liver impairment, multiple sclerosis, rheumatoid arthritis, malabsorption syndromes, Cushing's disease, or from drug induced chemical medications and glucocorticoid therapies. These factors may be solely or partially responsible for the individual risk of osteoporosis and fractures. (24)

1.6 Risk factors for osteoporosis

There are various risk factors that determine the possibility of osteoporosis but those patients with a previous history of fracture or low-energy fractures of wrist or hip are at a greater risk for developing OP. (6) Other risk factors are associated with an individual's clinical history, physical profile, dietary profile and lifestyle.

BMD analysis is often undertaken with patients showing one major or two minor risk factors.

Some of the major risk factors for OP include being female, having an age above 65, the presence of a vertebral compression fracture, hypogonadism, malabsorption syndrome, propensity to fall, early menopause, osteopenia, family history of osteoporotic fracture and primary hyperparathyroidism. Additionally, minor risk factors include low dietary calcium, lactose intolerance, rheumatoid arthritis, chronic anticonvulsant therapy, excessive alcohol and caffeine intake, smoking, chronic heparin therapy, and weight gain. (25)

Based on the clinical profiles of osteoporotic women, most are Caucasian or Asian and most are in a postmenopausal state with calcium/estrogen deficiency and a low BMI (Body Mass Index). (26) In men, osteoporosis can be characterized through hypogonadism, neoplasia, osteomalacia and glucocorticosteroid use. The other risk factors common to both genders include alcohol/caffeine intake, an inactive lifestyle, smoking and high protein diets. Lactase deficiency, which is generally seen in black American and Africans can cause osteoporosis due to the low bone mass caused by lack of milk or vitamin D intake in the diet. (6)

Other lesser-known factors for women suggested by Bauer et al (1993) include non-insulin dependent diabetes, thiazide use, estrogen use, increased weight, greater muscle strength, later age of menopause and greater height. (27)

For fracture prediction, clinical risk factors must be considered with care. Fracture prediction models must be validated in multiple population ethnicities, adjusted based on the age and sex of the individual and the type of fracture. Furthermore these parameters must be readily accessible by doctors and intuitive medical care. (25)

Canada’s 2010 osteoporosis guidelines focused on preventing fragility fractures and their outcomes rather than the treatment of low bone mineral density. These guidelines showed that 80 percent of menopausal or post-menopasual women over the age of 50 experience multiple fractures that increase the risk of death. (28). Despite that knowledge, fewer than 20% of women and fewer than 10% of men undergo therapies to prevent the onset of osteoporosis. (29)

Recently, a fracture risk assessment tool FRAX® was developed by the WHO, to calculate the 10-year probability for significant osteoporotic fractures such as the spine, forearm, hip or shoulder fracture. (25). This tool will be vital in controlling the risk involved in this disease.

Table 1 Risk factors for the development of osteoporosis. (25)

Major risk factor	**Minor risk factor**
Age ≥ 65 years	Smoking
Hypogonadism	Weight <57Kg
Family history	Excessive alcohol intake
Fragility fracture after age 40	Excessive caffeine intake
Early menopause	Low dietary calcium
Primary hyperparathyrodism	Rheumatoid arthritis

1.7 Risk Assessment of osteoporosis

Risk assessment is used in decisions broadly categorized by fracture probability. It has three possible conclusions: 1. Requires no treatment. 2. Further assessment is needed. 3. Treatment must commence. To arrive at these judgments, a risk assessment must be derived from a panel of risk factors discussed already. However, none of these risk factors involve patient-specific biomarkers or metabolomic components.

The EPIDOS study documented a risk assessment table with various combinations of risk factors with 10-year hip fracture probabilities in the Swedish population. It is also necessary to consider patients with higher risk factor gradient that enhance sensitivity for a better detection rate. (25)

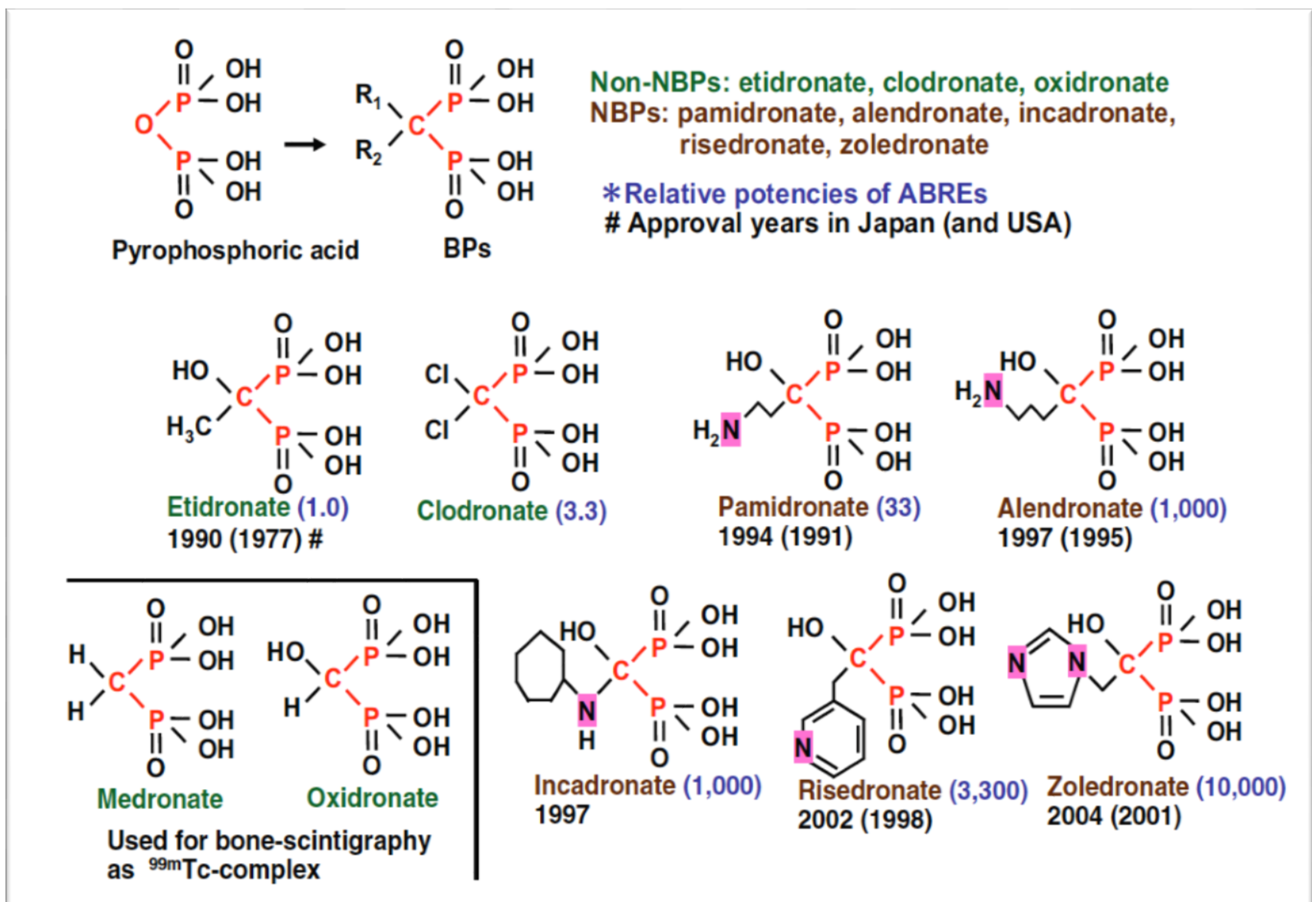


Figure 5: Structures of commonly used non-Nitrogenated BPs (NBPs) and Nitrogen containing BPs. The relative antiresorptive potencies are shown in brackets (30)as is their initial date of clinical approval.

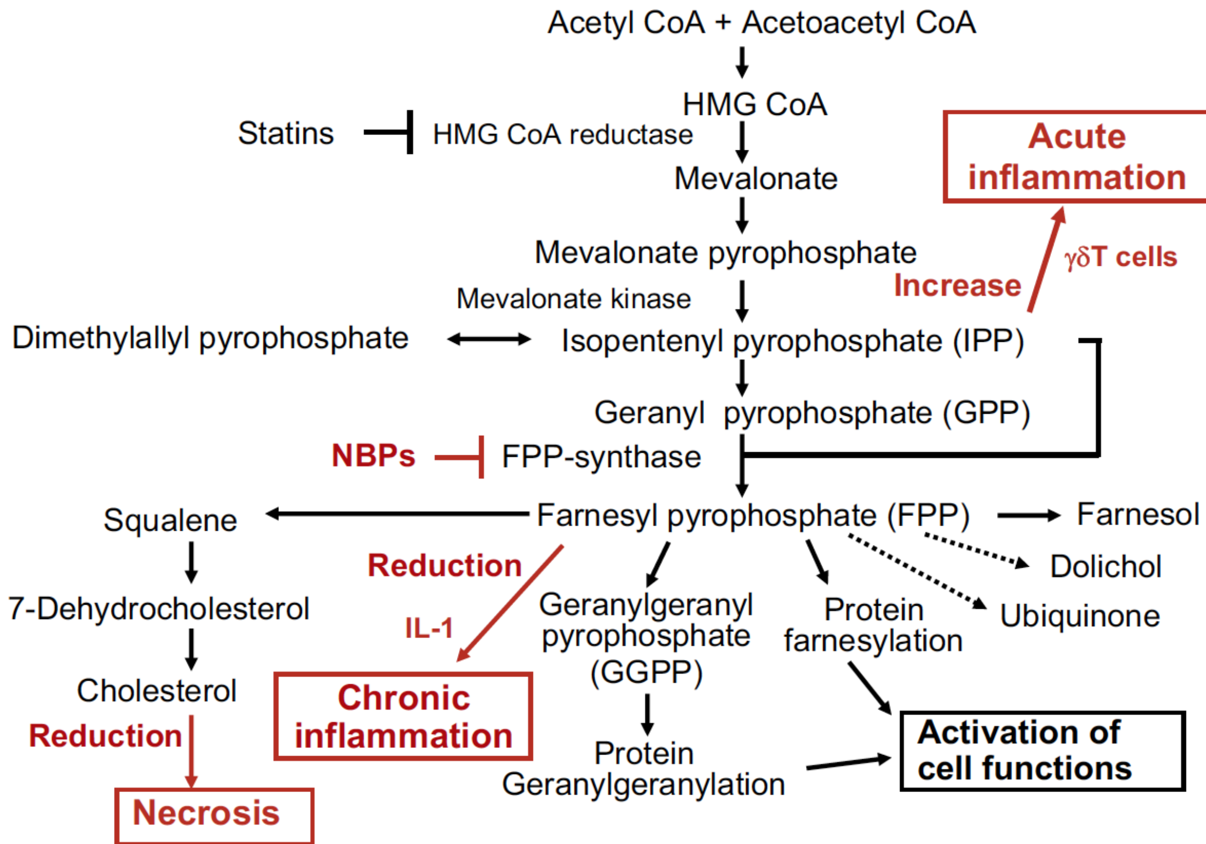


Figure 6: Proposed relation between the known cholesterol biosynthesis pathway and the inflammatory and necrotic actions of Nitrogen-containing BPs (31)

1.8 Mechanism of action for bisphosphonates

Bisphosphonates (BPs) are non-metabolizable analogues of inorganic pyrophosphates. When they are being used as pharmacological agents, BPs have important biological effects on calcium metabolism, impeding bone calcification and resorption. They achieve this effect via two mechanisms of action linked with anti-osteoclastic and anti-angiogenic activities(32). BPs change the bone tissue remodeling mechanism at several stages, impeding bone resorption as well as bone turnover. At the cellular level, they affect the functionality of osteoclasts, their viability, the bioavailability of their progenitors, and their activity on the

bone from the molecular viewpoint. BPs also modulate the function of osteoclasts, by interfering with cellular energy metabolism or with related metabolic enzymes. Due to a chemical structure which closely resembles pyrophosphate, BP interferes with ATP metabolism and induce cell apoptosis. This allows condensation of a BP with an AMP to form an AppCp-type nucleotide instead of an ATP. This ATP analog is similar to ATP in structure but is resistant to breakdown. (33) It has been shown that the mitochondrial ADP/ATP translocase is one of the molecular pathways involved in this osteoclast modifying effect through the activation of caspase-3. (34) Regarding the anti-resorptive activity of BPs, one of the most important factors is the inhibition of osteoclast activity. (35) This function of BPs is connected to their therapeutic action in the treatment of osteoporosis and skeletal cancer metastases. The decrease in bone resorption by bisphosphonates can be best understood by remembering that the bone resorption and bone formation processes are coupled.

Many studies, using a variety of experimental approaches, have shown that BPs were capable of reducing bone turnover by inhibiting osteoclast -mediated bone resorption. (36,37)For instance, Sato and Grasser (1991) reported that BPs work by reducing osteoclast activity via changing the cytoskeletal structure of the cell. (37) Interestingly, Teronen and colleagues (38) reported an alternative theory of bisphosphonate activity based the down-regulation of matrix metalloproteinases. Vitte and colleagues (1996) posted that the BPs work on activation of osteoclast inhibitory factor by osteoblast cells. (39) Recent studies have shown that BPs have direct antitumor activity by inducing apoptosis of tumor cells. (40,41)

Alendronate (ALN) is a nitrogen-containing bisphosphonate, which is administered at a dose of 5 mg/day for the prevention of osteoporosis and 10 mg/day for the treatment of osteoporosis. Lately, a new regimen dose of alendronate as a weekly dose (70 mg) was shown to produce a positive effect on bone mass density when compared to a daily dose regimen of 10 mg. (42)

Many clinical studies have shown the clinical potency of ALN in reducing bone loss and limiting vertebral and non-vertebral fracture in menopausal women. On the other hand, much clinical research has shown the adverse side effects and severe consequences of long-term user of ALN which leads to suppression of bone remodeling. (43) Other than rare cases of esophagitis that have been published, however, Alendronate is known to be well tolerated by most patients. (44)

1.8.1Bisphosphonate pharmacokinetics

Drug pharmacokinetics including the rate and the extent of absorption, volume of distribution (Vd), and the elimination of the drug out of the body (metabolism and clearance) are the major determinants of the concentration of drugs in the body.

In general, using several pharmacokinetic studies on BPs in animals, BPs show poor oral bioavailability. Oral bioavailability means the fraction of an oral dose that reaches systemic circulation, which can be measured by comparing the drug concentration in plasma versus time.

Accordingly, for studies that employ BP drugs, they are usually administered via sub-cutaneous route since they exhibit such poor bioavailability via the oral route. (45,46) Regarding functional groups, the nitrogen-containing bisphosphonates have poor gastrointestinal absorption in humans compared with non-NBPs 0.7 %, 2 – 2.5% respectively. (47) Lin et al. have determined the bioavailability of Alendronate in rats by contrasting oral with intravenous doses where the bioavailability was about 0.9%. (48) Moreover, the oral bioavailability of BP is dose-dependent. For instance a clinical study to estimate the oral bioavailability of etidronate shows 3% for 5 mg/kg and 7% at 30 mg/kg. (49) There was also a proportional increase in drug concentration in the bone when the dose of ALN was increased from 2 mg/kg to 40 mg/kg. (50)Because BP absorption can be affected by food, it is recommended that Alendronate should be taken 30 min before breakfast. (51)

Kinetically, BPs are distributed throughout the body. However, BPs bind preferentially to the bone, but can also be concentrated in soft tissues like the liver, kidney, and spleen. (52) Published studies on the elimination of BPs show that the main route of elimination is renal excretion, where BPs are excreted in an unchanged form, and a smaller percentage by bile. Due to resorption of bone, trapped BPs can be released again in circulation and detected in urine long after the initial dose. Again, the excretion of BPs is correlated with renal function. Therefore, dose adjustment must be considered in patients with poor kidney function. (45,53)

1.9. Role of vitamin D in osteoporosis management

Vitamin D is a fat-soluble micronutrient and a steroid pro-hormone that has important physiological functions in the human body. (52) The vitamin D endocrine system is made up of molecules called calciferols. The essential forms found in food are cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2), while the metabolite 25-hydroxycholecalciferol (25-hydroxyvitamin D3) is found

predominantly in animal products accounting for 10-20% of uptake. (54,55) Vitamin D production can also occur in the skin by sunlight through UVB mediated photochemical isomerization of pre vitamin D from 7-dehydrocholesterol. This process accounts for 80-90% of the total body production. However, low exposure to the sun and use of topical sun-blocking agents can result in vitamin D deficiency. (55,56) Vitamin D_3 is carried to the liver where it is hydroxylated to form the intermediate 25(OH) D, This vitamin D precursor is then further hydroxylated at the kidney, leading to 1α, 25-dihydroxyvitamin D3 (or 1,25$(OH)_2$ D3). This hormone has several important cellular and metabolic effects in skeletal tissues. These include enhancing bone mineralization, remodeling and bone turnover rate, maintaining calcium and phosphate homeostasis, improving muscle strength and preventing osteoporosis. (57)

Vitamin D has well-documented roles in the physiology and development of bone and muscle. Chondrocytes, osteoblasts, and osteoclasts are the cells found in or near bones and contain both the vitamin D receptor and the enzyme CYP27B1.

This cytochrome P450 enzyme is essential for producing the active metabolite of vitamin D - 1,25 dihydroxy vitamin D. (58) 1,25$(OH)_2$ D3 is an essential requirement for bone formation and bone mineralization and it works through several mechanisms on bone tissue. The activation of the vitamin D receptor (VDR) gene will induce the expression of numerous other proteins in the osteoblasts, with the transcription of nuclear factor-kappa B ligand (RANK-L) being the most important. The RANK-L protein is required for the activation and differentiation of the osteoclasts, which then become bone resorption cells.

Another genomically regulated effect of vitamin D on bone tissue is the inhibition of Fas ligand-induced osteoblast apoptosis regulation of bone turnover, thus resulting in longer lived bone-forming cells. (59)

However, vitamin D is more commonly associated with efficient calcium absorption from the intestine. The active vitamin D metabolite, 1,25$(OH)_2$ vitamin D binds to the vitamin D receptor (VDR) in intestinal cells and stimulates the production of Calbindin 9K, a protein that binds calcium and impacts the amount of calcium flowing through calcium channels. Bone mineralization is typically a passive process occurring when sufficient calcium and phosphate is available. Depending on the calcium gradient, passive diffusion of calcium through the intestinal wall also is possible. (60) Parathyroid hormone (PTH) and calcium levels are linked to the action of vitamin D. Dietary calcium consumption impacts the PTH level, and thus, variations in PTH levels can impact the turnover rate of vitamin D metabolites. Low calcium uptake is related with rise in PTH and 1,25$(OH)_2$ vitamin D levels which lead to an abatement in the 1,25$(OH)_2$ vitamin D half-life.

Consequently, calcium insufficiency may exacerbate vitamin D deficiency, although a high calcium consumption may facilitate a vitamin D saving impact. (61)

Sufficient levels of vitamin D have a significant effect on the bone mass of both young and old people. Vitamin D deficiency unfavorably influences calcium metabolism, osteoblast activity, matrix ossification, bone remodeling, and consequently bone mineral density (BMD) . (62) Serum levels of 25(OH) vitamin D are directly associated with BMD with a maximum density attained when the 25(OH) vitamin D level reached 40 ng/ml or more. (63) BMD is the critical parameter for diagnosing osteoporosis and an actual substitute for evaluating how bone tissue reacts to mediations for the amelioration of bone health. Nevertheless, the majority of fragility fractures occur in people who do not have osteoporosis. This suggested that BMD is only one among several signs of bone health and that the evaluation of fracture risk ought to likewise depend on other bone properties. (64,65)

Vitamin D promotes and controls bone health by maintaining the PTH levels in a physiologically healthy level, stimulating osteoblastic activity, and stimulating bone mineralization. Vitamin D also increases muscle strength or musculoskeletal capacity, which help reduce the risk of falls, thereby reducing the risk of fracture. (66)

Secondary hyperparathyroidism and hypophosphatemia can result due to a decrease in circulating 25 (OH) vitamin D levels. These conditions are concomitant with osteoclast development and an increase in bone resorption beyond osteoblast-mediated bone formation. This can precipitate and aggravate into osteopenia and even osteoporosis in adults.

Osteopenia is associated with muscle pain, muscle weakness, primarily in the proximal muscle groups, and a decrease in performance speed. (67)

Vitamin D levels have been widely studied with regard to their effect on bone fracture risk. In particular, low vitamin D levels have been associated with the occurrence of hip fractures in numerous populations, including postmenopausal women. In one particular survey conducted on postmenopausal women with

osteoporosis who were hospitalized because of hip fractures , half were found to have indications of vitamin D deficiency . (68)

Various reasons appear to be behind a greater risk of fracture related to vitamin D deficiency. Failure to absorb adequate quantities of calcium for ideal bone health, an increased vulnerability of falling due to diminished muscle strength, and increased rates of bone damage can increase the danger of fracture. It has also been noted for that lower levels of vitamin D are self-sufficiently related with a higher risk of falling in the elderly. In effect, supplementation with vitamin D has been shown to enhance musculoskeletal capacity and decrease the risk of falling in elderly women. Vitamin D receptors are present in human muscle that may enhance muscle strength and improve steadiness. (69)

Severe vitamin D insufficiency causes rickets or osteomalacia, and osteoporosis. These conditions are characterized by bone loss, mostly from cortical bone and this may add to the pathogenesis of osteoporosis. Consequently, on one hand, severe vitamin D deficiency causes a mineralization complication and osteomalacia; on the other hand, high PTH levels cause a high bone turnover, bone resorption, and osteoporosis. Either way, both mechanisms may result in fractures, particularly hip fractures. (70)

1.10 Metabolomics

The field of metabolomics involves the characterization of metabolites in cells, tissues, biofluids or organs with the help of sophisticated analytical methods such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS).

Metabolomics generates a large quantity of metabolite data and so the application of multivariate statistical methods are important for information extraction and data interpretation. Metabolites are regarded as the endpoints of multiple gene, protein and environmental interactions and so their characterization offers insight into the "chemical phenotype" of an organism. In a more formal sense, a metabolite is any substance or compound that is involved in metabolism, either as its product or cause. As a result, metabolites can include a range of endogenous and exogenous chemical entities such as short peptides, amino acids, nucleic acids, carbohydrates, organic acids, vitamins, polyphenols, alkaloids, minerals and just about any other chemical that can be used, ingested or synthesized by a given cell or organism. (71) Sophisticated multivariate statistical tools including cluster analysis, principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA), as well as pathway mapping, and machine learning for pattern recognition,.

1.10.1. Evolution off Metabolomics

The ancient Greeks in 300 B.C. first recognized that it was necessary to examine body fluids (humor) in order to diagnose diseases. The attraction of ants to glucose in urine was used by traditional Chinese doctors in 300 B.C. to evaluate whether an individual had diabetes. `

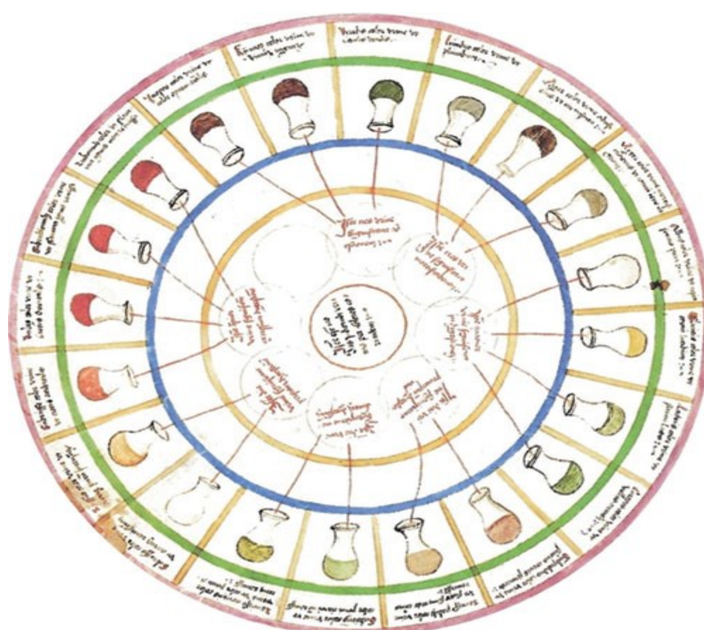


Figure 7: The urine wheel was published in 1506 by Ullrich Pinder, in his book Epiphanie Medicorum. Copyright permission obtained from System Biology Metabolomics. (72)

Santorio Sanctorius, who lived in the 17th century, is widely regarded as the founding father of metabolic studies. In 1614, his published work on "insensible perspiration" in De Statica Medicina. This was the first example of a published work to provide statistical data

and lay down the quantitative basis to pathology with precise studies and instrumentation. However, the identification and measurement of chemicals in tissues and biofluids did not become routine until the 20th century.

This was facilitated by the development analytical instruments such as mass spectrometers and NMR spectrometers, which enabled the precise identification of chemicals.

The first mass spectrometer was developed by J.J. Thomson from the University of Cambridge in 1905. It was used to measure the mass-to-charge ratio of the electron. Throughout the 1950s and 1960s mass spectrometers became sophisticated enough to measure the mass-to-charge ratio of small organic molecules (such as metabolites) and by the 1980s they could measure the masses of macromolecules such as proteins.

NMR spectroscopy emerged in the 1940s and 1950s and by the late 1960s had established itself as the primary method to determine the atomic structure of organic molecules. Chromatographic separation techniques, such as high pressure liquid chromatography (HPLC) and gas chromatography (GC) which were developed in the 1950s and 1960s, also played a pivotal role in the development of metabolomics.

With the necessary instruments in place by the late 1960s there was just a small gap of time until 1971 when Mamer and Horning carried out the first mass-spectrometry based metabolomics experiments(73). Following that, Arthur Robinson and Linus Pauling made important contributions to modern metabolomics by investigating biological variability and various nutritional requirements. (74-76) These early metabolomic studies were called bioanalytical studies (the word metabolomics did not exist until 1999) and required considerable manual effort. Furthermore, they were limited to characterizing only a small number (<20) of molecules at a time.

The field of metabolomics lay dormant for a number of years through the 1980s and 1990s due to the lack of databases and software tools needed to rapidly identify large numbers (100s) of small molecules via NMR or MS. In an effort to develop the resources to enable high throughput metabolomics Dr. David Wishart of the University of Alberta, Canada led The Human Metabolome Project. This project was launched in 2006 with

funding of $7.5 million from Genome Canada. The first draft of the human metabolome database (HMDB) consisted of 2500 metabolites, 1200 drugs and 3500 food components. (77) Subsequent drafts of the HMDB now contain 114,000 metabolites (78) and the

number of drugs contained in the DrugBank database now is >2000 compounds. (78)In developing and verifying the content of the HMDB, the Human Metabolome Project researchers used advanced methodologies such as NMR spectroscopy, mass spectrometry, multidimensional chromatography and machine learning to assist with this work. (79)

1.10.2 Applications of Metabolomics

Over the past few years metabolomics has largely overcome the technical hurdles associated with metabolite identification and analysis. As a result, metabolomics is now being used to identify metabolite biomarkers for disease. (80) This represents a natural extension of what has been done for many decades in clinical chemistry where single metabolites can be used to diagnose or predict disease. For instance, glucose in urine can be used as a biomarker for diabetes, or high cholesterol can be used as a biomarker for susceptibility to heart disease.

What metabolomics offers is the possibility of using multiple metabolites to produce a biomarker "profile". For instance, using metabolomics techniques Bahado-Singh et al. (2014) discovered a set of metabolite biomarkers that can

be used to identify congenital heart defects (CHD) in fetuses using the plasma of expectant mothers in their first trimester of pregnancy. This approach, which overcomes the limitation of genomic or proteomic testing, indicated that there was a significant disturbance in plasma lipid levels in mothers with fetuses affected by CHD including phosphatidylcholine, various sphingolipids and choline metabolites. (81) This results suggests that it is possible to predict, using only a simple blood test, which mothers are expecting children with CHD and to use this knowledge to plan or prepare *in utero* interventions (such as surgery) to repair the heart defect. This example, along with many other recently published examples, shows that the use of metabolomics to predict or enable the early diagnosis of disease holds great promise. (82) Metabolomics also shows considerable promise in its ability to reveal new insights on potential therapeutic interventions and the discovery of novel disease mechanisms. (83)

1.10.3. Metabolomics and osteoporosis

In the field of pharmaceutical research, metabolomics has gained increasing interest as it has been proven to be a fast, more easily accessible, less expensive, more sensitive, and less biased than classical methods used in drug development for characterizing drug safety and drug efficacy. (84,85)

Given their importance in bone development and maintenance, it is widely believed that metabolite measurements might contribute to a better understanding of the progression of osteoporosis after drug treatment.

It is also believed that the use of metabolomics could offer new information into the diagnosis or progression of osteoporosis. Consequently, administration of any medicine will produce metabolic changes that can be associated with either the expected response to the therapy or side effects derived from treatments. (86)

There are more than a half dozen studies that have been published on metabolomics and osteoporosis over the past decade. Interestingly, different metabolite biomarkers have been identified in these studies. This is probably due to the different metabolomics platforms being used and the confounding effects existing in sample type, sample preparation, detection, and data analysis. Lee et al. showed metabolic differences between OVX rats and sham rats based on GC/MS technique of plasma samples. (87) Three potential biomarkers were identified with lower concentrations of phenylalanine, tryptophan and butyric acid

being found in the plasma of OVX rats. Ma et al. (88) compared metabolic disturbances in OVX and sham rats, as measured by GC/MS, Their results showed that some metabolites decreased in the plasma OVX rats such as alanine, malic acid, citric acid, and docosahexaenoic acid while other metabolites increased in the plasma of OVX rats such as arachidonic acid, homocysteine, ethanedioic acid, oleic acid, glyceric acid, uric acid, and octadecadienoic acid. Another GC-MS study by Ma et al. showed that elevated plasma levels of valine, leucine, isoleucine, arachidonic acid, octadecadienoic acid, homocysteine, hydroxyproline, and 3-hydroxybutyric acid were found in OVX rats, and decreased plasma levels of dodecanoic acid, docosahexaenoic acid. (89) A GC-MS study by Lee et al. showed the plasma levels of lysoPC 20:4 , lysoPC 16:0, lysoPC 16:1, lysoPC 18:0, lysoPC 18:3, lysoPC 14:0, and lysoPC 20:5 elevated in OVX rats. Interestingly, almost the same results were also found in Liu's study (90,91) You and colleagues applied a ^{1}H NMR-

based metabolomics approach to plasma to identify potential biomarkers for delineating low bone mass density in postmenopausal women. (92) They found the following metabolites (glutamine, lactate, acetone, glucose, glutamine and lipid.) were higher/lower in women with low bone mass density. In a separate study, Chen et al. used ^{1}H NMR to determine the effect of aldendronate on the metabolomic profile of serum on ovariectomized mice. They were able to identify and quantify 55 metabolites. Differences among treatment groups were ascertained using a Kruskal-Wallis test and principal component analysis (PCA). This work revealed that alendronate, OVX, and sham mice had markedly different serum metabolomic profiles. This indicates that alendronate has large effects that perturb the metabolome significantly away from normal levels. Given that the alendronate group had increased tricarboxylic acid metabolites and significant weight gain, this study demonstrated that alendronate most certainly affects general metabolism. (93)

In summary, these metabolomic studies demonstrate that this technique can be applied to osteoporosis studies to help evaluate side effects as well as the therapeutic effects of treatment.

1.10.4. Importance of metabolomics in osteoporosis

One of the main challenges in controlling osteoporosis is to identify patients at risk for subsequent bone fracture accurately. While there are individual bone turnover markers (e.g., alkaline phosphates, osteocalcin and hydroxyproline), which can be used to monitor bone hemostasis and predict fracture risk, they, alone, are not reliable for diagnosis. (94) For example, some studies reported that elevated plasma homocysteine, which interferes with collagenous-linking in bone, might predict the risk for osteoporosis fracture. (95)

However, other studies did not observe this trend. (96) Rather than attempting to find a single metabolite biomarker for OP or early-stage OP, it might be more fruitful to look for a multi-metabolite "profile" that can more accurately assist in the diagnosis of OP or early stage OP.

1.11. Rationale, Hypotheses, Objectives:

1.11.1. Rationale:

As a progressive bone disease, osteoporosis has become a global medical issue, which poses a serious economic and health burden on patients, families and societies. It is well known that early diagnosis of osteoporosis could play a vital role in decreasing the disease burden and complications of excessive bone resorption. While diagnostic computed tomographic imaging may contain densitometry information useful for osteoporosis screening it is expensive and not suitable for screening. Population studies have shown that a low bone mineral density (BMD), as assessed by DXA and biochemical indicators of bone turnover are not sufficiently accurate to predict fracture risk. (97) Moreover, monitoring changes in biochemical bone markers by immunoassay techniques while more sensitive compared to BMD, is also unsatisfactory due to a lack of sensitivity and diagnostic accuracy. (98)

Therefore, a reliable, fast and cost-effective method for osteoporosis screening needs to be developed to identify those at risk for osteoporotic fractures and to track treatment effects on osteoporosis. Such a test would need to measure multiple biomarkers. These precision biomarkers, if available, could enhance diagnosis and prognosis, guide molecularly targeted therapy, and investigate therapeutic response and outcomes. (99) Metabolite biomarkers as measured via metabolomics present themselves as having the

potential to be relatively economical and high throughput. Therefore the work in this thesis focused on the identification of candidate metabolite biomarkers of osteoporosis and osteoporosis treatment in ovariectomized rats. These metabolite measurements are then further used to interpret the pathological conditions or disease states.

1.11.2. Hypotheses:

1) The detection of metabolic profiles in osteoporosis patients after bisphosphonate drug therapy will indicate which metabolites are associated with therapeutic responses

2) Biomarkers from metabolomics data would give comprehensive information about how osteoporosis develops and potentially lead to early interventions to lower the risk of developing osteoporosis.

1.11.3. Objectives:

1) To delineate the metabolic profile and to determine the significantly altered metabolites, which are responsible for the development of osteoporosis by using LC-MS/MS techniques and identifying, characterizing specific metabolites, which might serve as targets for the treatment or intervention in osteoporosis.

2) To perform Receiver operator characteristic (ROC) curves analysis for potential diagnostic osteoporosis biomarkers.

3) To measure the correlation between bone mineral density and metabolite level changes and determine their suitability for an osteoporosis screening practice.

CHAPTER 2: METHODOLOGY

2.1 Animal study design

The animal use protocol was reviewed and approved by one the University of Alberta's Animal Care and Use Committees in compliance with its Animal Ethics Policy. By definition, ovariectomized (OVX) rats are living animals in which spontaneous or provoked bone loss occurs due to ovarian hormone deficiency. The features of the bone loss and its sequelae parallel those that can be found in postmenopausal women in many respects. According to this definition, such an animal model might serve as a good model of the human condition; therefore it would be useful for studying aspects of bone loss. Indeed, the OVX rat has long been used as a surrogate to study the osteoporosis disease in human biology.

In this study, we used the ovariectomized rat model, with rats aged at about 6 months. The procedure was done according to standard operating procedure of rodent ovariectomy, one month before our experiment via making a midline incision, approximately 1 cm in length, in the mid-dorsum of the animal and using two fine forceps to gently pull apart the ovary and fat pad off of the uterine horn.

In metabolomics, the close chemical similarity between the metabolomes among mammals allows the use of established animal models of various illnesses within a preclinical context. However, small differences in the metabolic fingerprint of study animals compared to humans need to be taken into consideration to prevent errors in the translation of results from preclinical experiments to clinical studies.

2.2. Materials and reagents

Ethanol, methanol, water, acetonitrile, isopropanol (all HPLC grades), phosphate buffered saline (PBS), phenylisothiocyanate, formic acid and pyridine were purchased from Sigma-Aldrich (St. Louis, MO). Polyethylene glycol (HPLC grade) and ammonium acetate were purchased from BDH (Toronto, Canada)). Isoflurane USP was purchased from Halocarbon Products Corporation (River Edge, NJ, USA). The Biocrates p-180 metabolomic assay kits for targeted measurement of the plasma and urine

Metabolomes were obtained from BIOCRATES Life Sciences AG (Innsbruck, Austria).

2.3. Instrumentation and chromatographic conditions

A 4000 QTRAP linear ion trap quadruple LC-MS/MS mass spectrometer (Applied Biosystems/MDS Sciex, Framingham, USA) including a quaternary solvent system pump and an auto sampler with variable injection valve was used for all MS-based metabolomic studies. Chromatograms were recorded using the ABSciex Analyst software (version 1.6.2) in a Windows-based computer system for data collection and processing. Separation of metabolites was performed on a 150 × 4.6 mm i.d., 5 µm particle size Alltima C18 HPLC-column (Alltech, Deerfield Il, USA). The column temperature was set at 50.0 ^{o}C and the injection volume was set at 10.0 µL (for LC assays) and 20 µL (for FIA assays). The mobile phase for the column consisted of 0.2% formic acid in water (solvent A) and 0.2% formic acid in acetonitrile (solvent B). The mobile phase was prepared daily and degassed by ultrasonication for 1 min. The mobile phase was pumped at a gradient flow rate of 0.5 mL/min and 0.45 mL/min for LC and FIA respectively, at room temperature. Analytical run time is 1 min for each sample.

2.4 Plasma collection

At baseline and the end of the experimental (8 weeks) time, the animals were lightly anesthetized with isofurane and 1 mL of blood from the tail vein of each rat was drawn into heparinized Eppendorf tubes and the sample were centrifuged at 3000 rpm for 10 min at 4 ºC. The supernatant (plasma) was transferred to an Eppendorf tube and stored at – 80 ºC until needed.

2.4 Urine collection

At baseline and the end of the experimental (8 weeks) time, the animals were transferred to metabolic cages and allowed free access to water, but the food was withheld to avoid contamination of the urine with particulates. The urine samples were collected in Falcon tubes and stored at -80 ºC until needed.

2.5 Metabolomics assays in rat plasma and urine.

The analysis was accomplished by using a commercial kit from Biocrates Life Science (AbsoluteIDQ kit). Metabolites were extracted from urine and plasma from OVX rats using a 96-well plate system for protein removal, internal standard normalization, and derivatization This lab work was performed according to the AbsoluteIDQ p180 User Manual. Briefly, 10 µL of the biofluid sample and the internal standard were added to the center of the filter on the upper 96-well plate kit per well and dried using a nitrogen flow for 30 min. Subsequently, 50 µL of a 5% solution of phenylisothiocyanate (PITC) was added for derivatization of the amino acids and biogenic amines. After incubating for 20 min, the filter spots were dried again using a nitrogen evaporator for 120 min.

An extraction step was performed by adding 300 μL of a 5-mM ammonium acetate solution in methanol and centrifuged for 30 min at 450 RPM. 150 μL of the sample was transferred into fresh 96-deep well plate and diluted with 150 μL of water for subsequent LC-MS/MS analysis. The extracts of original plate were diluted with 400 μL of the flow injection analysis (FIA) running solvent for further FIA analysis using the QTRAP 4000 mass spectrometer.

One blank sample (no internal standards and no sample added), three zero samples (no sample added) to determine the limit of detection, and three quality control (QC) samples were also added to the Biocrates kit plate. The QC samples were comprised of lyophilized human plasma samples, at defined concentration levels, and were used to validate the performance of the assay and the mass spectrometer. The calibration curves were generated by a seven-point serial dilution which was added to the kit's 96-well plate for the quantification of biogenic amines and amino acids.

The kit included a mixture of internal standards for the quantification of the naturally occurring metabolites. These included chemical homologous as internal standards that were used for the quantification of glycerophospholipids and sphingomyelins. On the other hand, stable isotope-labeled internal standards were used to quantify the other compound classes. Identification and quantification of the metabolites was accomplished using internal standards and multiple reaction monitoring (MRM) detection. A QTRAP® 4000 LC-MS/MS System, (AB Sciex Instruments), with an ion source turbo spray system was used for metabolite detection and operated both in positive and negative modes.

Amino acids and biogenic amines were analyzed using an ACQUITY UPLC System connected to the QTRAP 4000 MS instrument in positive mode. Acylcarnitines, glycerophospholipids, and sphingolipids were analyzed by flow injection analysis (FIA) in the positive mode with the

QTRAP 4000 MS instrument. Hexose (glucose) was analyzed using a subsequent FIA acquisition in negative mode. All files were created with the software Analyst (version 1.6.2).

Data analysis and calculation of the metabolite concentrations analyzed by FIA (acylcarnitines, glycerophospholipids, sphingolipids, and hexoses) is fully automated with the MetIDQ software (Biocrates Life Sciences AG Eduard-Bodem-Gasse, Austria), which is an integral part of the kit. Analysis of peaks obtained by the UPLC runs for the amino acids and biogenic amines was performed and the results were imported into the MetIDQ software for further processing and statistical analysis. Quantified metabolite concentrations were then analyzed using MetaboAnalyst 4.0.

2.6. Standard and stock solutions:

An injectable solution of alendronate (100 □g/mL) was prepared using water for injection; 1 mg of active vitamin D dissolved in 100 mL of a vehicle consisting of water: propylene glycol: ethanol (50:40:10) which gives a concentration of 10 □g/mL. All stock solution stored in the refrigerator(2-8°C).

2.7. Statistical analysis

To determine the alterations in concentration of various metabolites detected by the Biocrates kit we employed multivariate statistical analysis. The data were imported into MetaboAnalyst software (version 4.0) for multivariate data analysis. (100) MetaboAnalyst is an online resource containing a number of bioinformatics tools that provide functional interpretations of lists of metabolites and metabolomics studies. PCA, PLS-DA, ROC curve analysis are tool available

online at http://www.metaboanalyst.ca/. The data were normalized to obtain standard distribution data (Gaussian), which is a critical step before multivariate data analysis. Normalization allows metabolite values more comparable. In this study we used log transformation and auto scaling.

2.8. Micro-CT scanning and imaging

We used *in vivo* micro-Computed Tomography (μCT) imaging to confirm the developing osteoporosis phenotype in all ovariectomized (OVX) rats. Micro-CT measurements detect changes that may occur on bone architecture over time in individual animals. For *in vivo* analysis of tibial trabecular bone volume of OVX rats, the animals were anesthetized by isoflurane gas immediately upon arrival from Charles River supplier (Baseline), and again at the study endpoint (8 weeks). The rat was surrounded by foam material and secured in the imaging bed with medical tape, to prevent movement. The usual scan time was 25 min, and the image quality was checked immediately after each scan to ensure sufficient contrast between bone and soft tissue. All specimens were imaged using the SkyScan 1076 Micro-CT imager (SkyScan® 1076; Bruker-MicroCT, Kontich, Belgium) at x-ray source set at 70 kV with a current of 100 μA and a 1.0 mm aluminum filter to absorb the low energy x-rays before they pass through the specimen. Voxel image resolution was 18 μm, as recommended, to ensure accurate trabecular measurements. We used microCT imaging to determine the changes that occurred to the trabecular bone support after 8 weeks of treatment.

After scanning, the raw images were reconstructed using NRecon reconstruction software (NRecon 1.6.1.5; Bruker-MicroCT, Kontich, Belgium) to set the region of interest and to choose the correct maximum value for identical scaling of all the samples in the experiment. The next step involved analyzing the volume of interest by manually segmenting the trabecular bone from cortical bone in each scan. Next, the bone tissue was separated from the soft tissue using thresholding, and the same threshold value was used for all samples in the project.

CHAPTER 3: RESULTS

3.1. Total body weight

Four weeks after ovariectomization, the body weights of the four groups of rats were examined. The average body weight of each group of rats over the 8-week treatment period is shown in Figure 3.1A. The results were analyzed using repeated-measures 2-way analysis of variance (ANOVA).

During the experimental period, all animals except those in the vitamin D group gained weight, in comparison to their initial weights. At the 2-week time point, it was noted that the rats being dosed with active vitamin D were losing body weight rapidly. After consultation with the University of Alberta veterinary staff, it was determined that the rats were experiencing an adverse drug event (likely hypercalcemia) due to being dosed too frequently with vitamin D. All vitamin D dosing was immediately discontinued, and the rats were permitted to regain their body weight and recover out to the study endpoint.

In our study, the four groups had statistically similar initial body weights ($p > 0.05$). The rat body weights increased steadily throughout the study, except for those of the groups treated with either vitamin D alone or with alendronate combined with vitamin D (alendronate + vitamin D) at week 2.

There was a significant difference in body weight between the ovariectomized control group and the treated groups (Fig. 3.1A). One unexpected observation was the significantly increased body weight in the ovariectomized rats treated with both ALN and vitamin D (OVX-Combo) when compared with that in ovariectomized rats treated with vitamin D alone ($p < 0.05$; Figure 1A). That improved body weight suggested a protective effect of ALN in halting hypercalcemia in the presence of high-dose vitamin D.

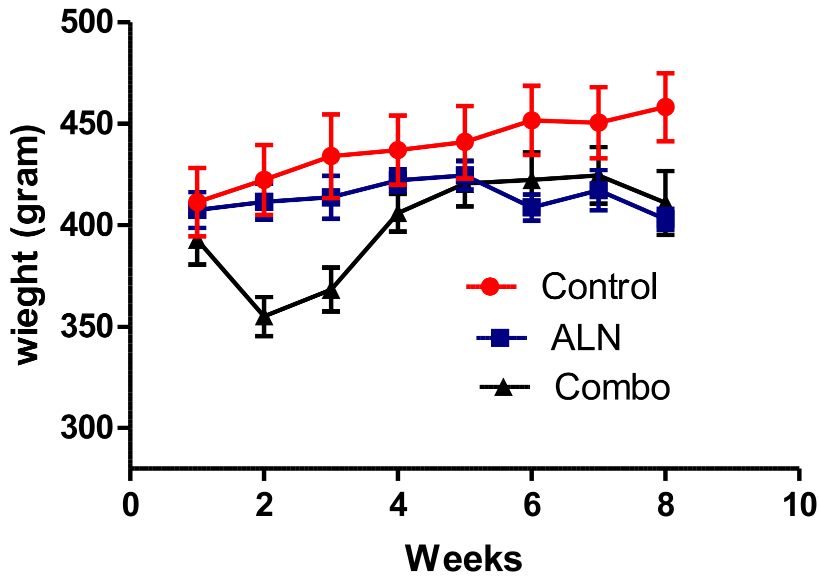


Figure .3.1: Body weight in control ovariectomized rats, ovariectomized rats treated with 0.12 mg/kg of alendronate (ALN), ovariectomized rats treated with vitamin D (vitamin D), and ovariectomized rats treated with a combination of ALN + vitamin D (Combo). Body weight was measured twice weekly during the 8-week experimental period. Values are expressed as means ± SE (n = 10 rats per group) except vitamin D (n = 7 rats)

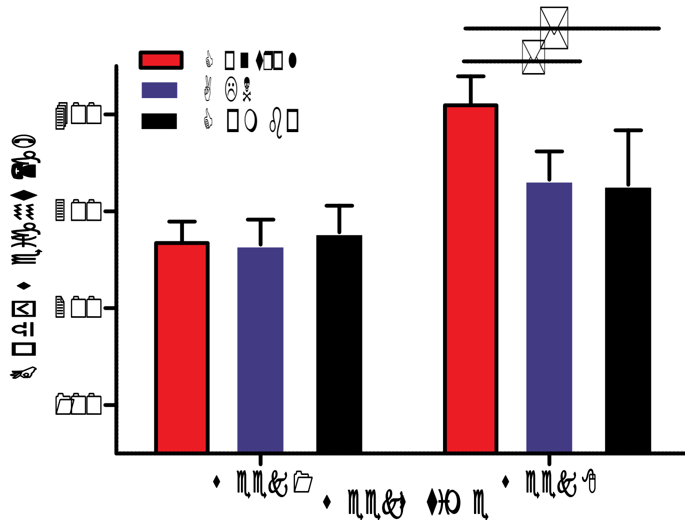
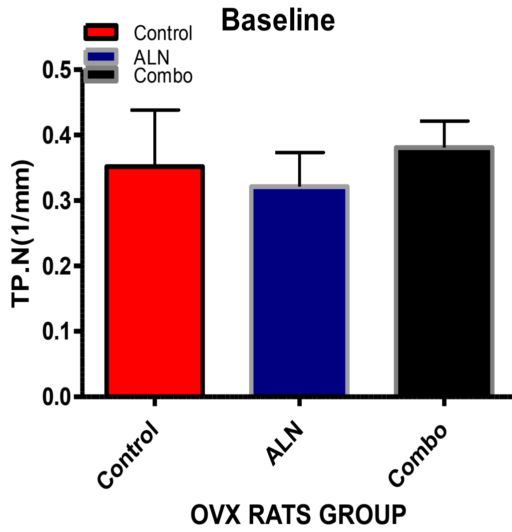


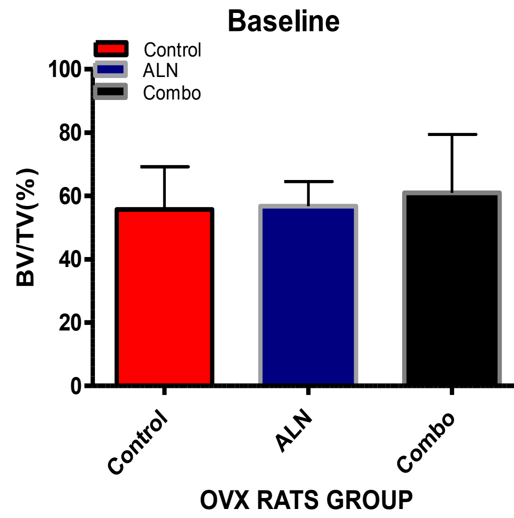
Figure 3.1A: Average body weight of OVX female rats compared with ovariectomized rats with ALN, ovariectomized rats with combo during weeks 0–8. Assessed by analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. Statistical significance is defined as *p>0.05, **p>0.01. Each value is expressed as the mean ±SD.

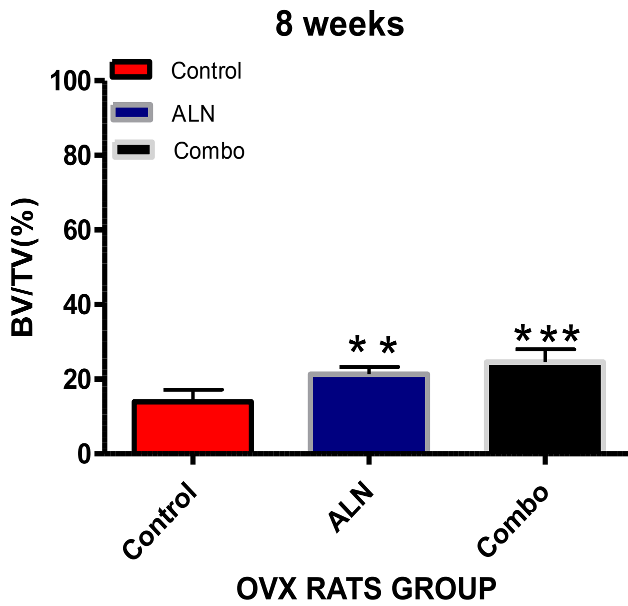
3.2 Micro-Computed Tomography analysis

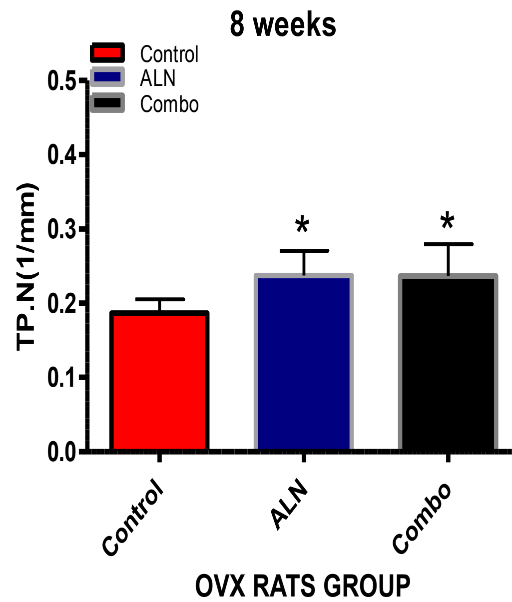
The micro-CT evaluation demonstrated increased trabecular bone volume BV/TV, trabecular number (Tb.N), and trabecular thickness (Tb.Th) as well as decreased trabecular separation (Tb.Sp) in rats treated with 0.12 mg/kg ALN twice weekly or with combination therapy as compared with the OVX control group. In contrast, Tb.Sp ($p < 0.01$) in the proximal femur was significantly increased in the OVX control group, compared with the treated group. Moreover, treating OVX rats with alendronate + vitamin D significantly reversed ($p < 0.05$) the changes in these parameters provoked by ovariectomy, and the treatment could maintain the trabecular bone microarchitecture in the proximal tibia.

Monotherapy with vitamin D caused a slight but not significant change in BV/TV; in contrast, monotherapy with ALN increased BV/TV. Alendronate + vitamin D significantly increased proximal tibial BV/TV, Tb.N, and Tb.Th as compared not only with OVX but also with other monotherapies (ALN alone and vitamin D alone). Those changes in BV/TV by ALN and the combined treatment were also supported by the increased measurements for Tb.N and Tb.Th. In contrast, OVX increased Tb.Sp, indicating a high turnover state and resorption of existing trabecular bone volume, thereby increasing the spacing between the remaining trabeculae. All monotherapy and combined treatments reduced this effect significantly in the proximal tibia. This suppressive effect was prominent in the ALN treatments and was significant in the combined treatments. Figure 3.2A depicts representative micro-CT data measured from trabecular bone in the proximal tibial metaphysis. This figure also shows that all single and combined treatments increased trabecular bone volume and that the combined treatments were more powerful than the individual treatments.


Baseline
Control
ALN
Combo
TP.N(1/mm)
0.5
0.4
0.3
0.2
0.1
0.0
Control
ALN
Combo
OVX RATS GROUP


Baseline
Control
ALN
Combo
BV/TV(%)
100
80
60
40
20
0
Control
ALN
Combo
OVX RATS GROUP


8 weeks
Control
ALN
Combo
BV/TV(%)
100
80
60
40
20
0
**
***
Control
ALN
Combo
OVX RATS GROUP


8 weeks
Control
ALN
Combo
TP.N(1/mm)
0.5
0.4
0.3
0.2
0.1
0.0
*
*
Control
ALN
Combo
OVX RATS GROUP

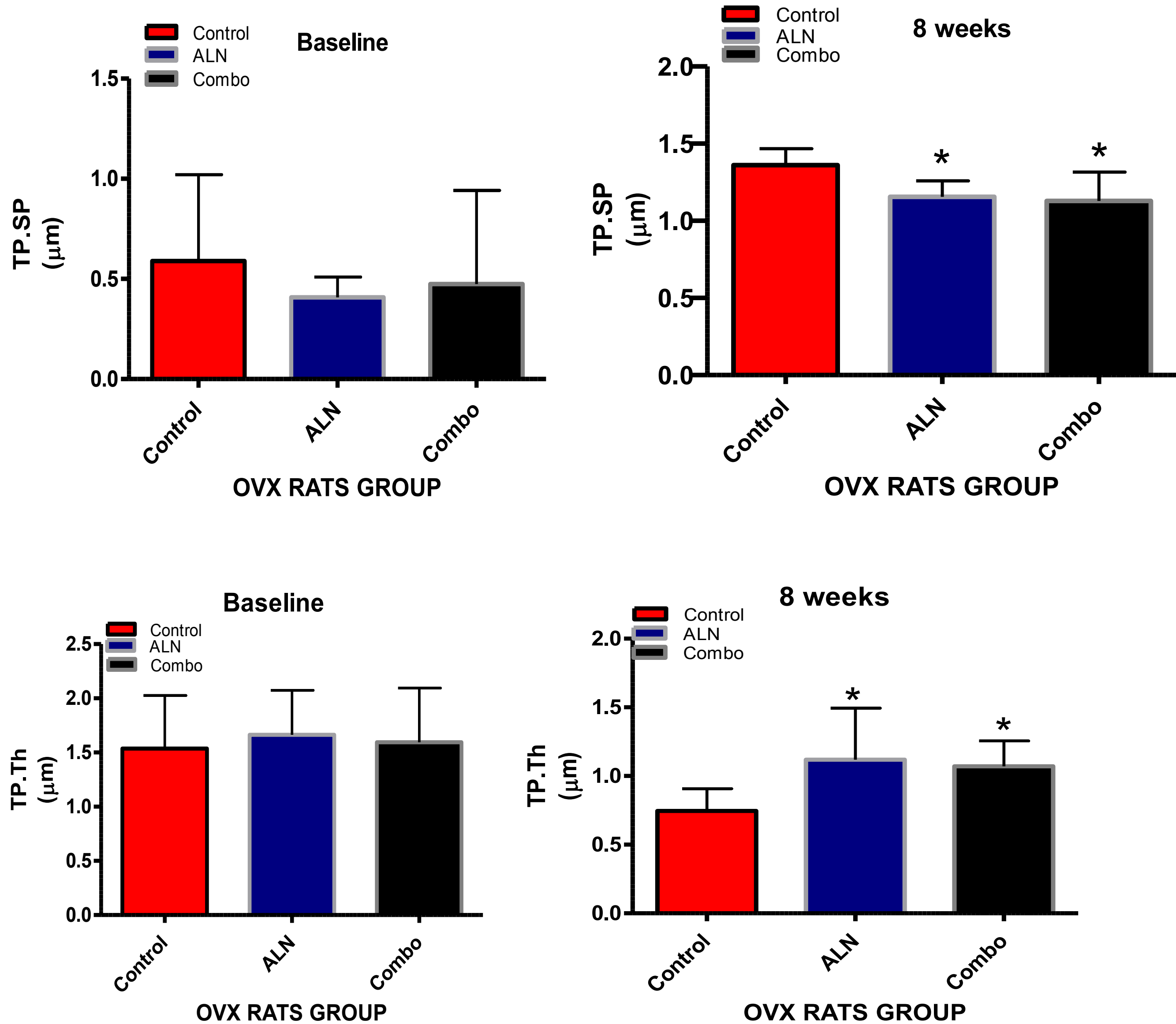


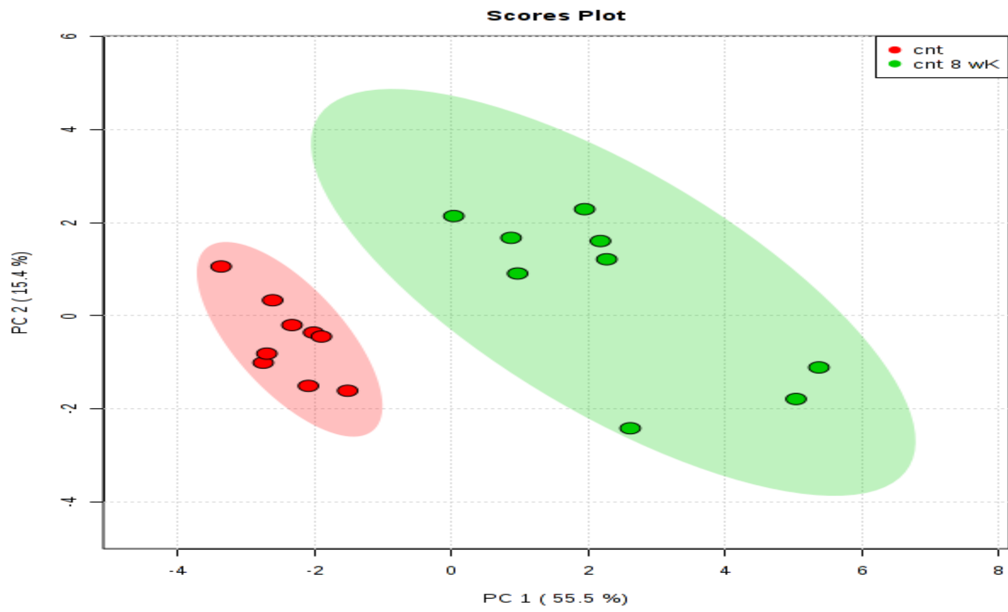
Figure 3.2.A: Micro-CT analysis of Bone volume (BV/TV%), Trabecular number (TP.N), Trabecular thickness (TP.Th), and Trabecular separation (TP.Sp). Data on repeated measures were analyzed by ANOVA, followed by Bonferroni's. Data were analyzed using Prism (Version 11.0). All data are expressed as the mean±S.D.

3.3. Metabolomics results

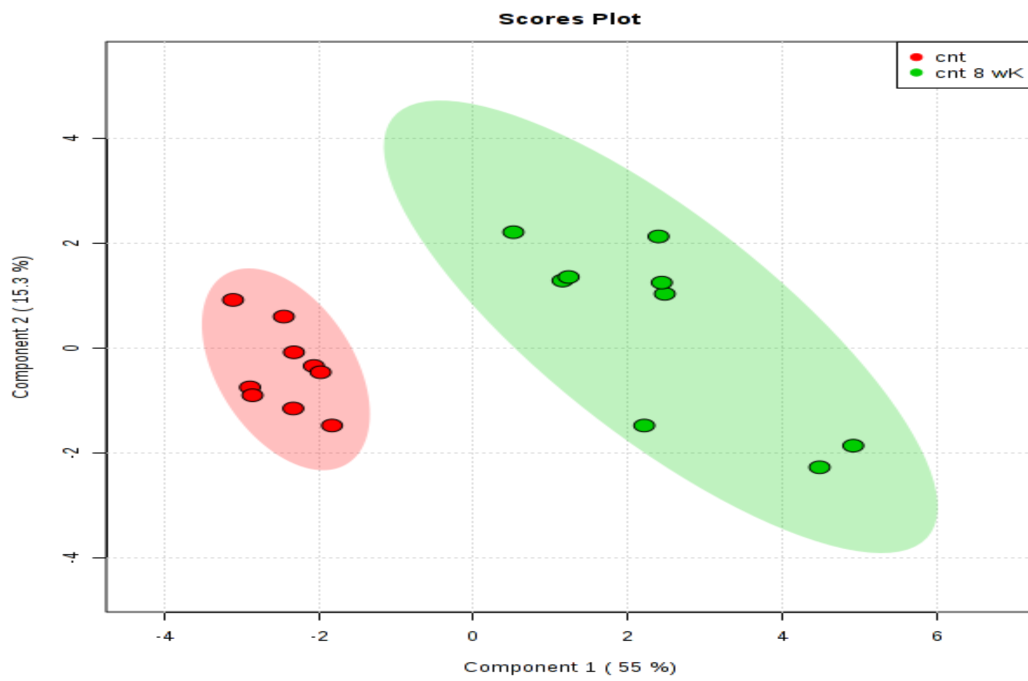
3.3.1. Control group at baseline versus 8 weeks

The control groups were compared at baseline and at 8 weeks to verify metabolite differences at each time point. At 8 weeks, the metabolites of the same groups were differentiated from each other, as shown by the PLS-DA plots (Fig. 3.3) At 8 weeks, the control groups showed significant differences in 24 metabolites: (amino acids) proline, arginine, leucine, glutamine methionine sulfoxide, glucose, (glycerophospholipids) PC ae C34:3, PC ae C38:2, PC ae C36:2, PC ae C34:2, PC ae C32:2, PC ae C34:0, PC aa C40:2, PC aa C42:4, PC aa C38:4, PC aa C36:3, PC aa C28:1, and (sphingolipids) SM C24:0, SM C18:0, SM C16:0 and carnitine C0.

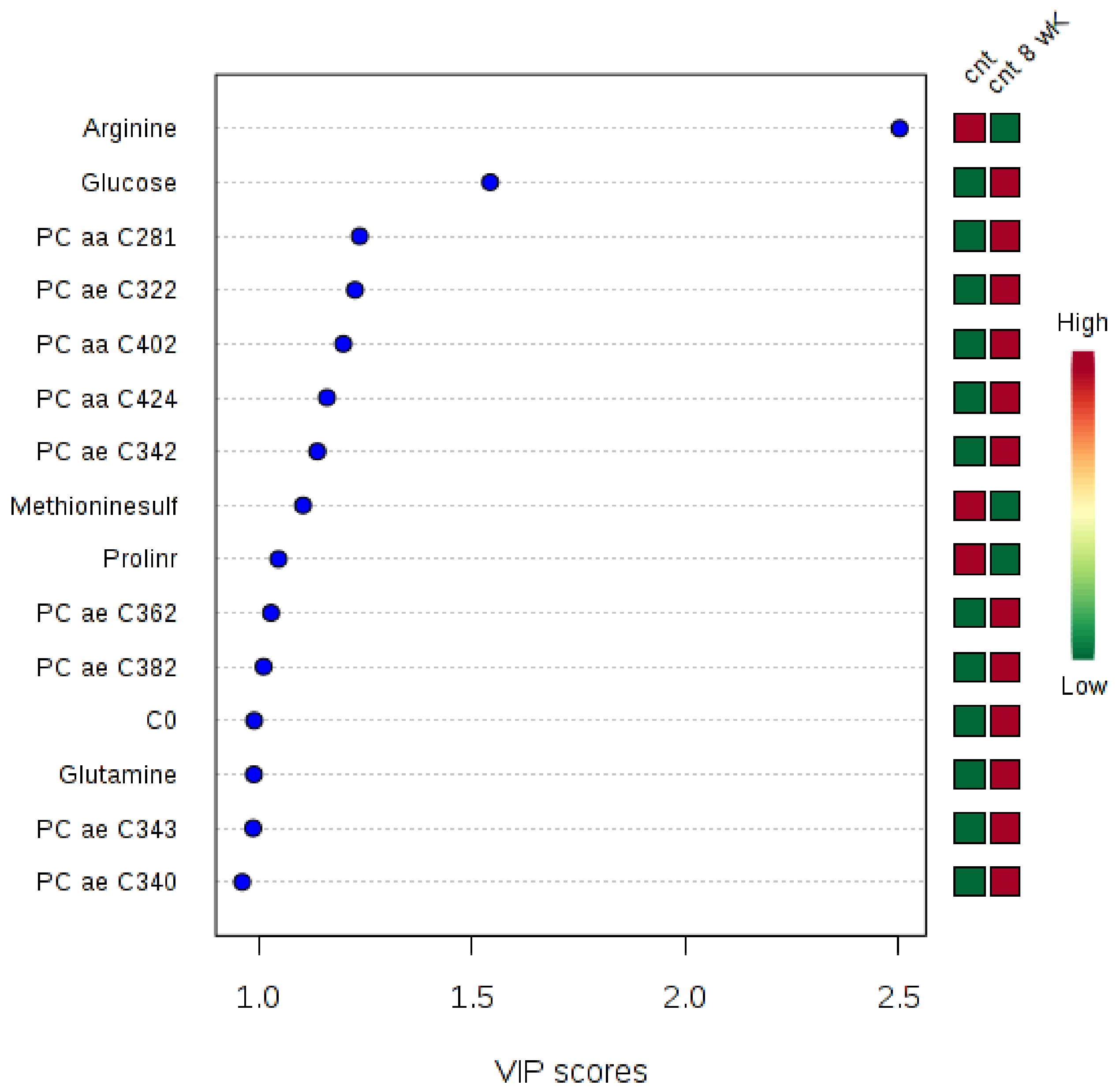
Box and whisker plots of the metabolite concentration ranges were constructed for the control groups using MetaboAnalyst. The plots confirmed our results regarding the altered metabolites and the effect of the treatments. The concentration of certain metabolites such as arginine was lower in the OVX control group at 8 weeks, whereas in the ALN group, there were increased levels of these metabolites, benefiting bone. The glucose level was significantly lower in the alendronate-treated group. In this thesis, we will discuss the relation of these metabolites to bone health.



(A) PCA



(B) PSL-DA

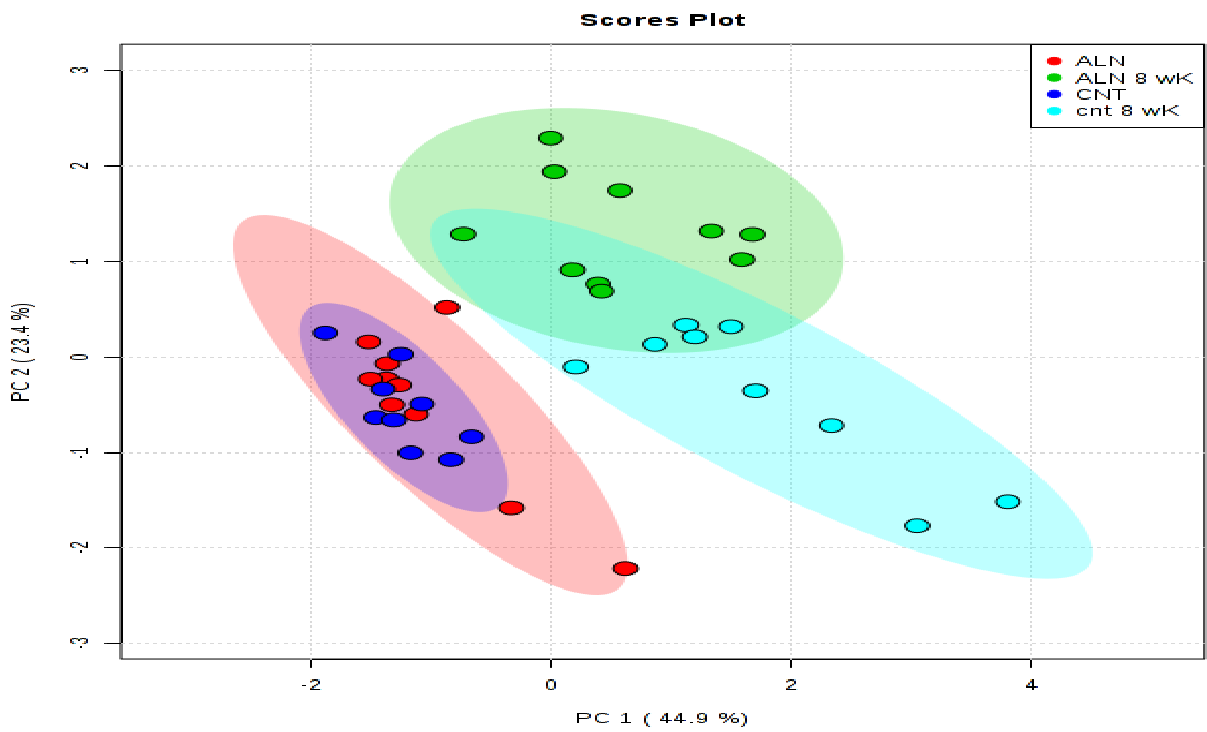


(C) VIP score

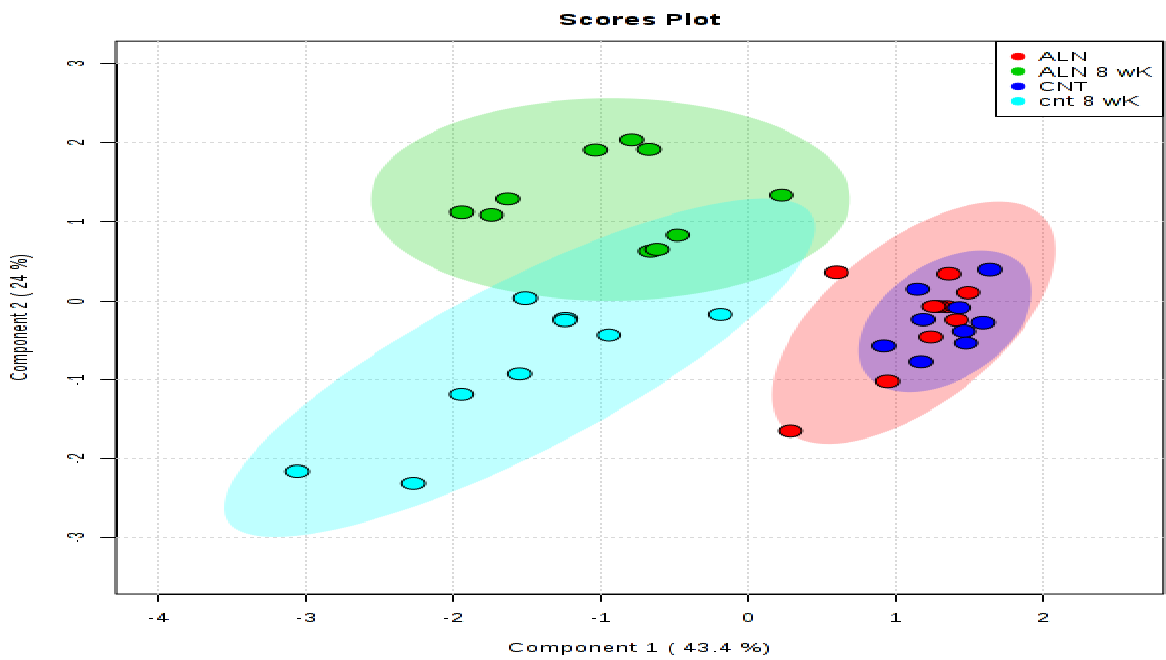
Figure .3.3 A: metabolic changes in plasma from control group overtime based on Principal component analysis (PCA) , (A) based on partial least squares discriminant analysis (PLS-DA) score plots (B) and variable importance in the projection (VIP) values (C)

3.3.2. Alendronate group versus control group over time

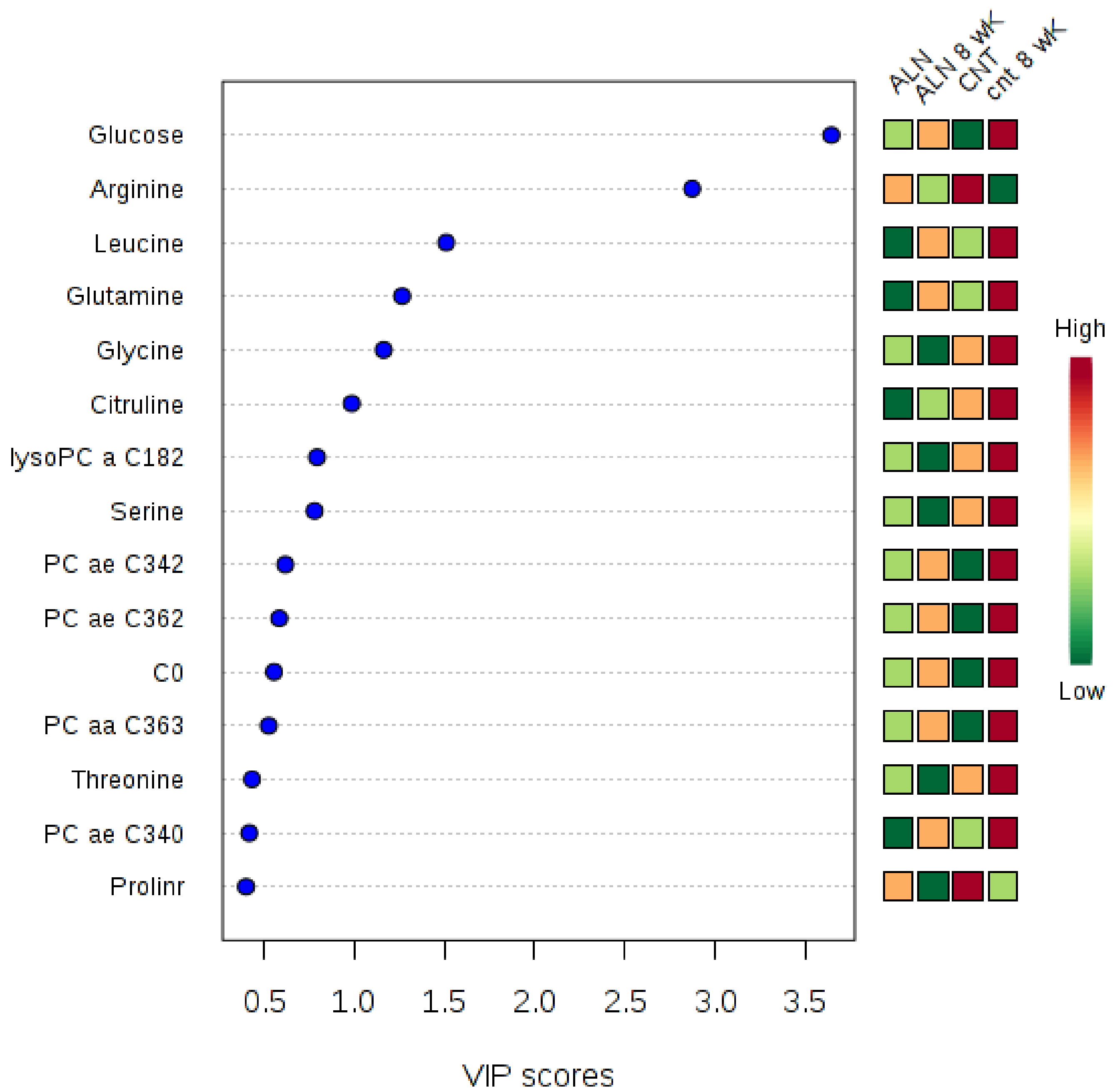
The metabolic profile of plasma samples collected from the OVX+ alendronate, and control group at baseline and 8 weeks were quantified using DI-LC-MS/MS. PCA and Partial least squares discriminant analysis (PLS-DA) of the control versus treatment groups at 8 weeks showed a clear separation of the metabolic profiles at 8 weeks. Our comparison showed significant alterations in 12 metabolites (Fig. 3.3), including glucose ($p < 0.01$), phosphatidylcholine diacyl (PC aa) C40:2 ($p < 0.01$), PC acyl-alkyl (PC ae) C36:2 ($p < 0.011$), PC ae C34:2 ($p < 0.01$) sphingomyelin (SM) C24:0 ($p < 0.01$), SM C24:1 ($p < 0.01$), hydroxysphingomyelin [SM(OH)] C22:1 ($p < 0.03$), the amino acids proline ($p < 0.02$), arginine ($p < 0.01$), and serine ($p < 0.01$), methionine sulfoxide ($p < 0.01$), trans-hydroxyproline ($p < 0.05$) and glucose ($p < 0.01$). Whereas, almost no difference was observed in plasma metabolite levels between the groups at week zero. For the PLS-DA calculations, the R2, Q2 and permutation test parameters were calculated to assess the model evaluation, fitness and prediction power. The values were R2=0.7, Q2=0.3 and P <0.001 respectively. This is indicating that PLS-DA is very robust and not over trained. In the permutation test a total of 1000 resamplings were performed and calculated to determine the statistical significance.



(A) PCA

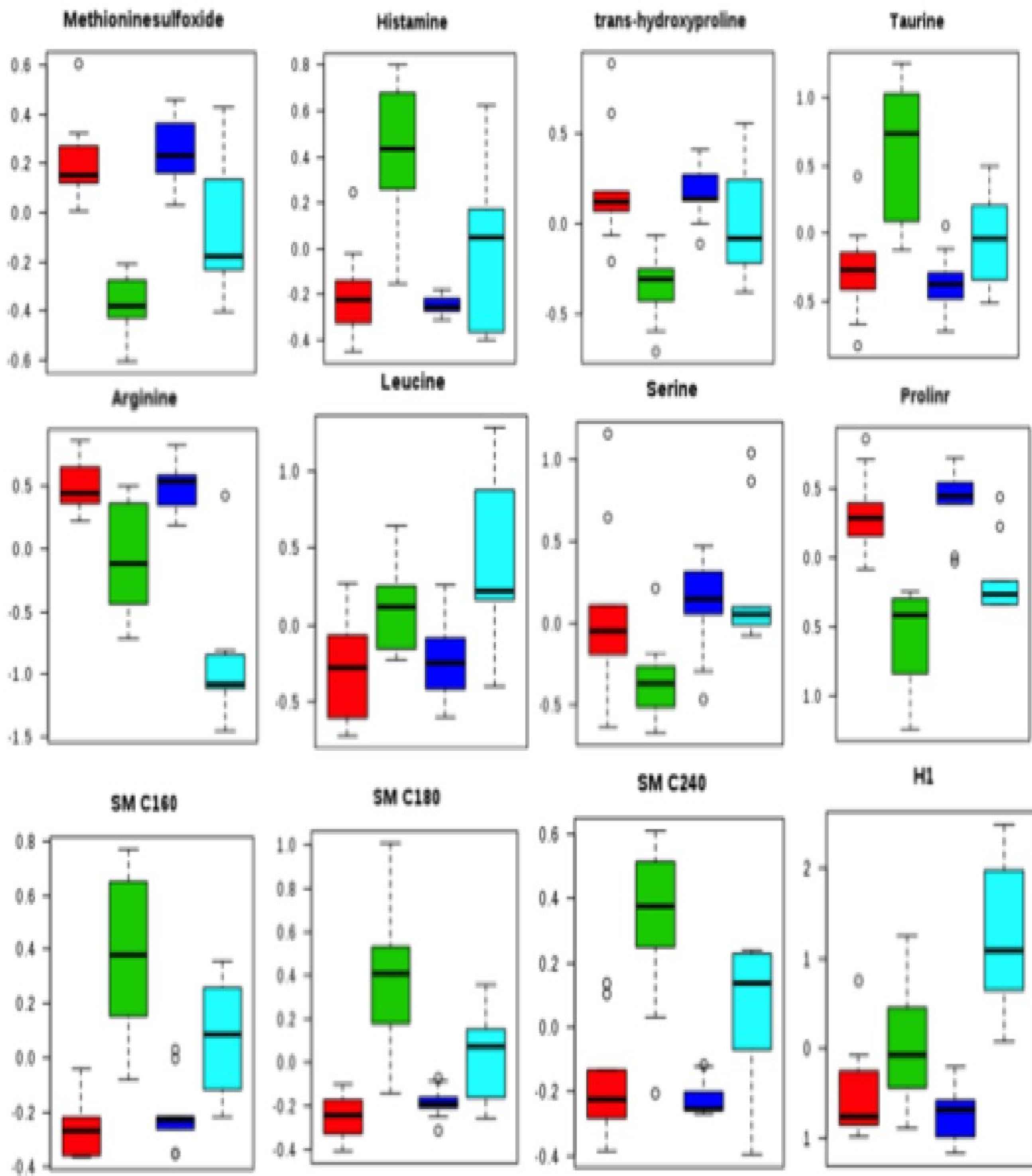


(B) PLS_DA



(C) VIP score

Figure .3.3 B: Global metabolic changes in plasma from control group and Alendronate group based on Principal component analysis (PCA), (A) based on partial least squares discriminant analysis (PLS-DA) score plots (B) and variable importance in the projection (VIP) values (C)


Methioninesulfoxide
Histamine
trans-hydroxyproline
Taurine
Arginine
Leucine
Serine
Prolinr
SM C160
SM C180
SM C240
H1

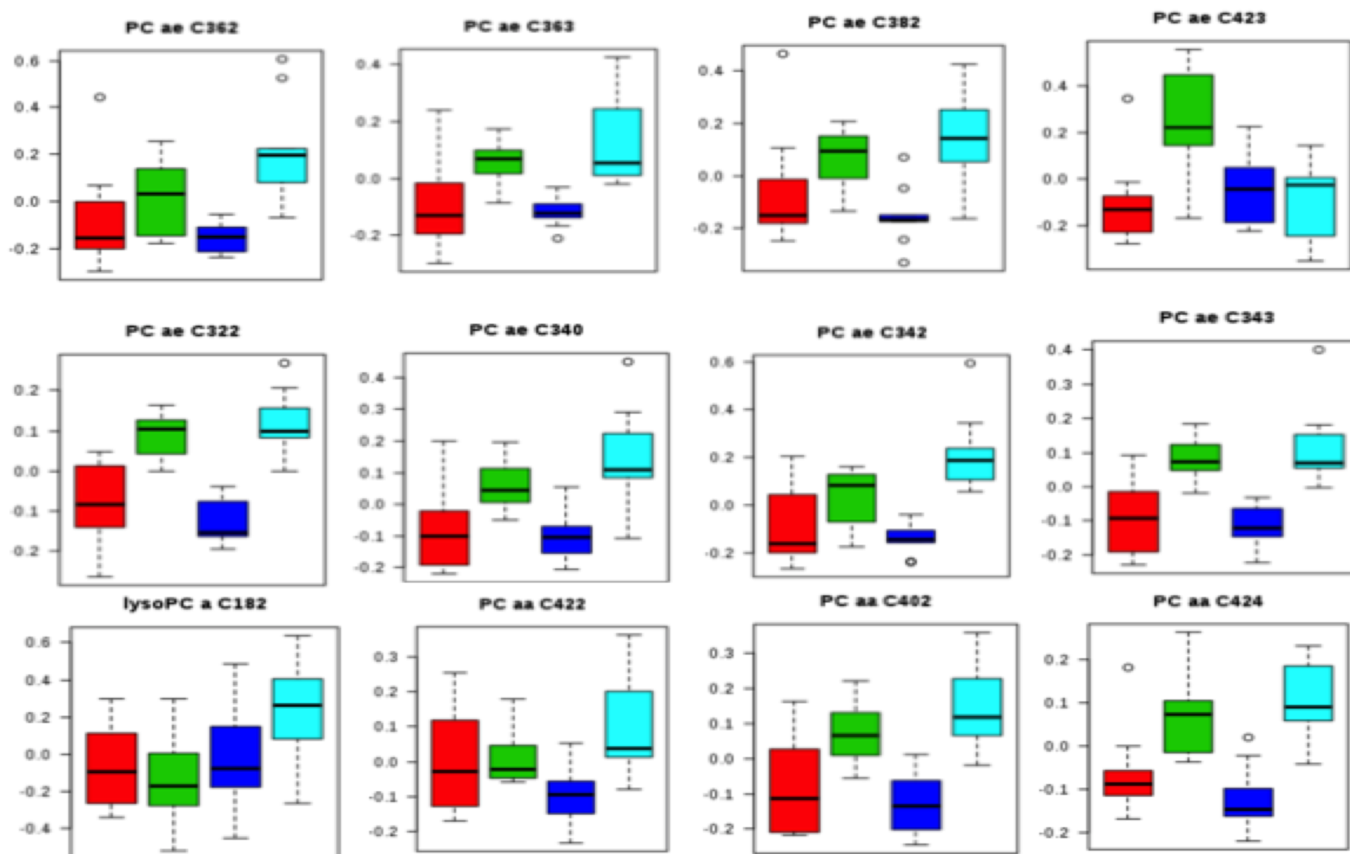
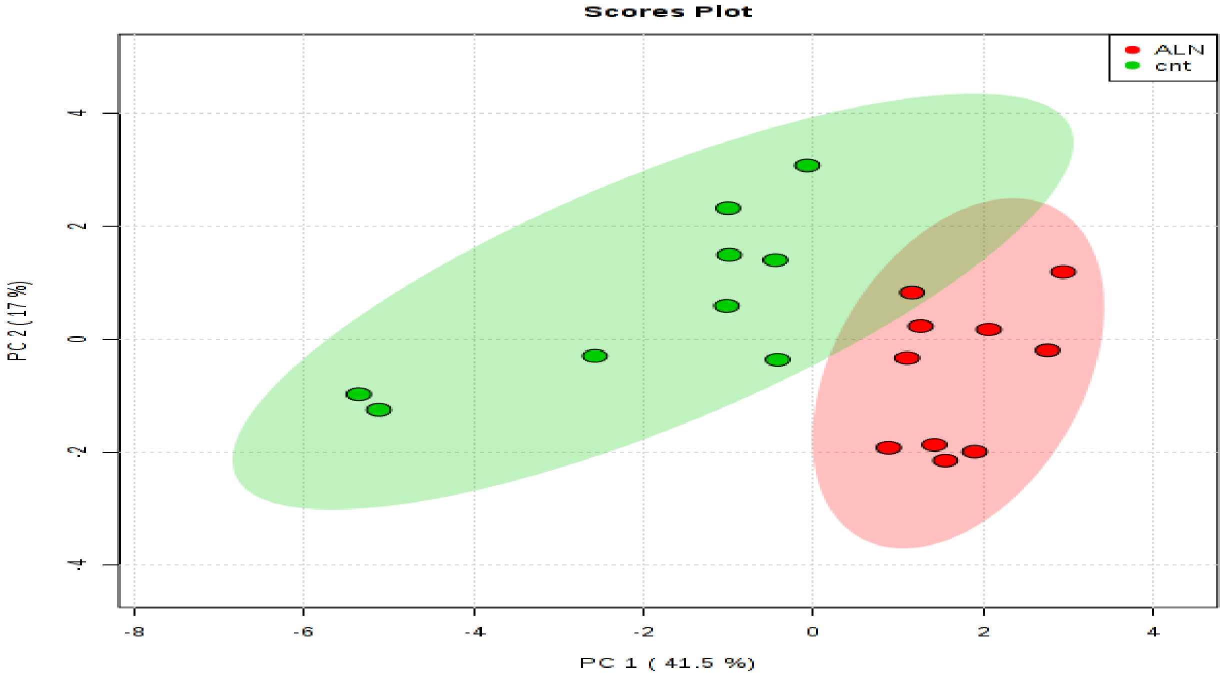


Figure .3.4: Box and whisker plot analyses of plasma metabolites from LC MS/MS data between the two different rat groups at baseline and 8 week time points including: Amino acids; organic acids; sugars and; fatty acids. CNT, control OVX, ovariectomized rat without treatment at baseline; ALN, ovariectomized rat treated with alendronate at baseline, CNT 8 weeks, control OVX, ovariectomized rat without treatment at 8 weeks. ALN, ovariectomized rat treated with alendronate at 8 weeks. $P < 0.05$ compared to the OVX group.

3.3.3. Alendronate group versus the control group

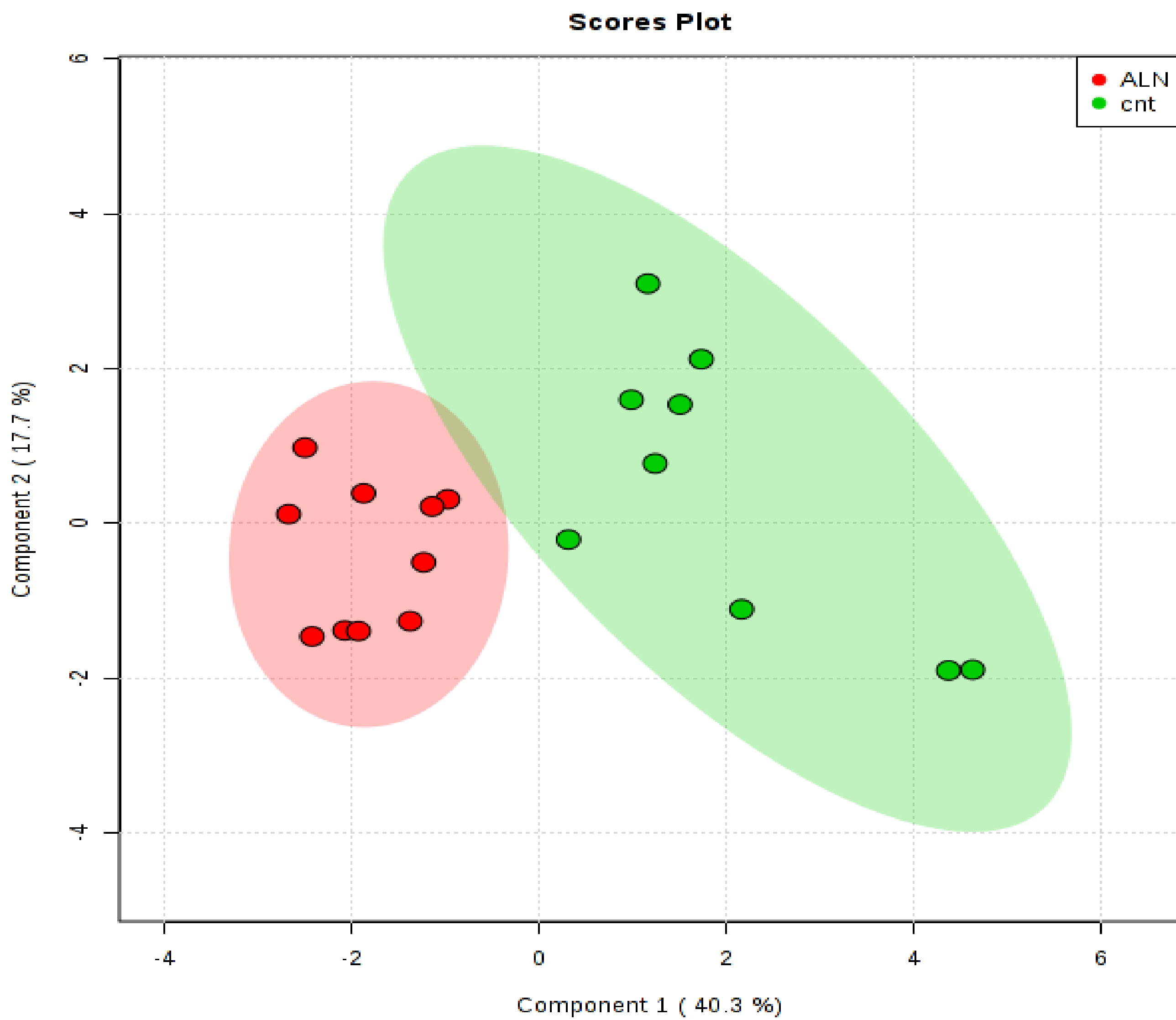
With regards to the ALN treatment group, the PLS-DA plot showed separation versus the control group at 8 weeks (Fig. 3.5) Statistical analysis also showed significant differences in the concentration of a number of endogenous metabolites, including glucose ($p < 0.002$), PC aa C42:2 ($p < 0.05$), PC aa C40:2 ($p < 0.05$), PC aa C40:0 ($p < 0.05$), PC ae C34:2 ($p < 0.05$), PC ae C36:2 ($p < 0.05$), PC ae C34:0 ($p < 0.05$), amino acids proline ($p < 0.05$), arginine ($p < 0.05$), serine ($p < 0.05$), methionine sulfoxide ($p < 0.05$), histamine ($p < 0.05$)

and trans-hydroxyproline ($p < 0.05$). The ROC curve (Figure **3.11.1**) indicates that 10-metabolite combination was a highly significant predictor of bone status: AUC.0.955 (95% CI, 0.75–1).

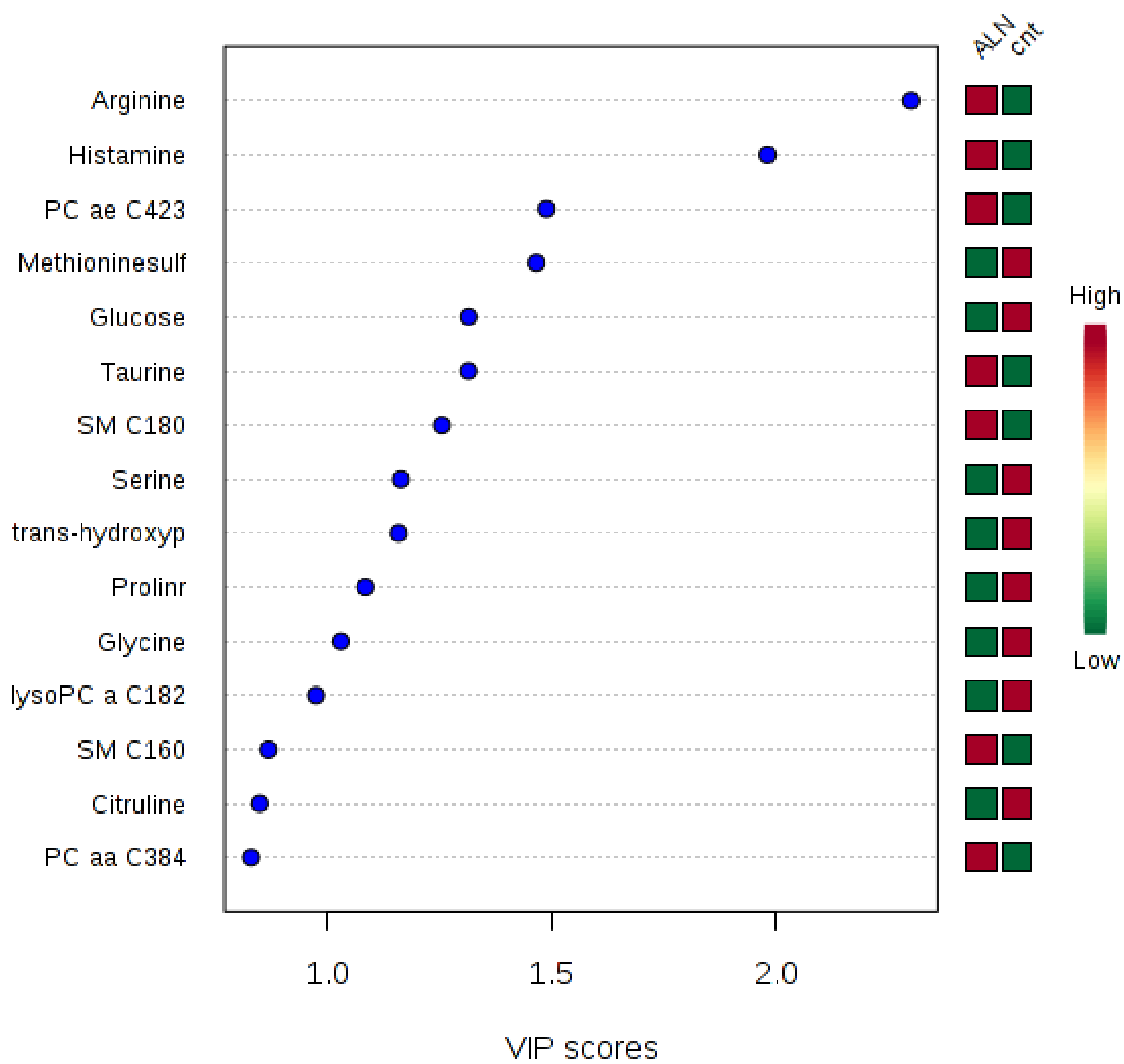


(A) PCA

(B)



(B) PLS-DA



(C) VIP score

Figure 3.5: metabolic changes in plasma from control group and Alendronate group at 8 weeks time points based on Principal component analysis (PCA) , (A) based on partial least squares discriminant analysis (PLS-DA) score plots (B) and variable importance in the projection (VIP) values (C)

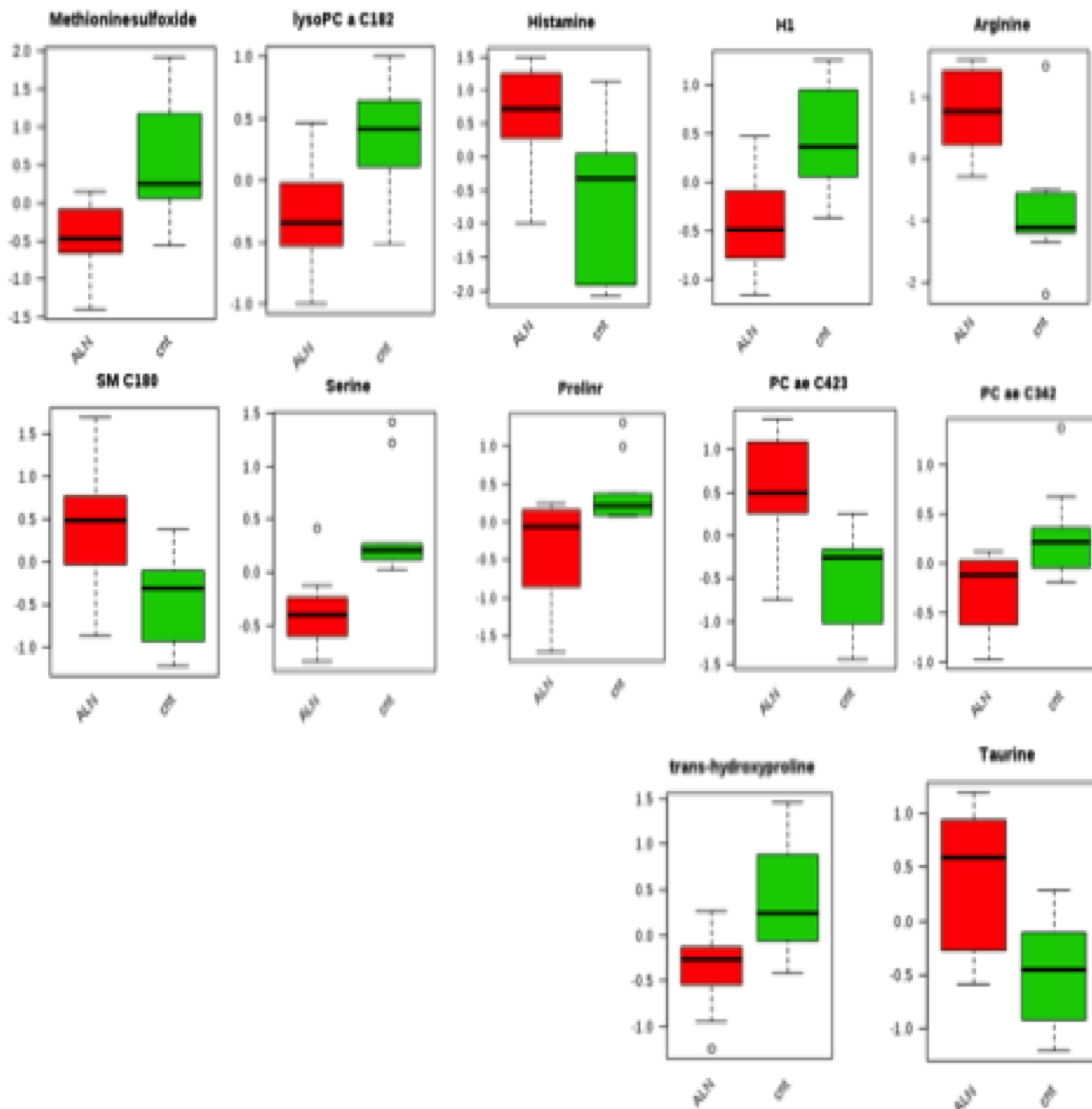
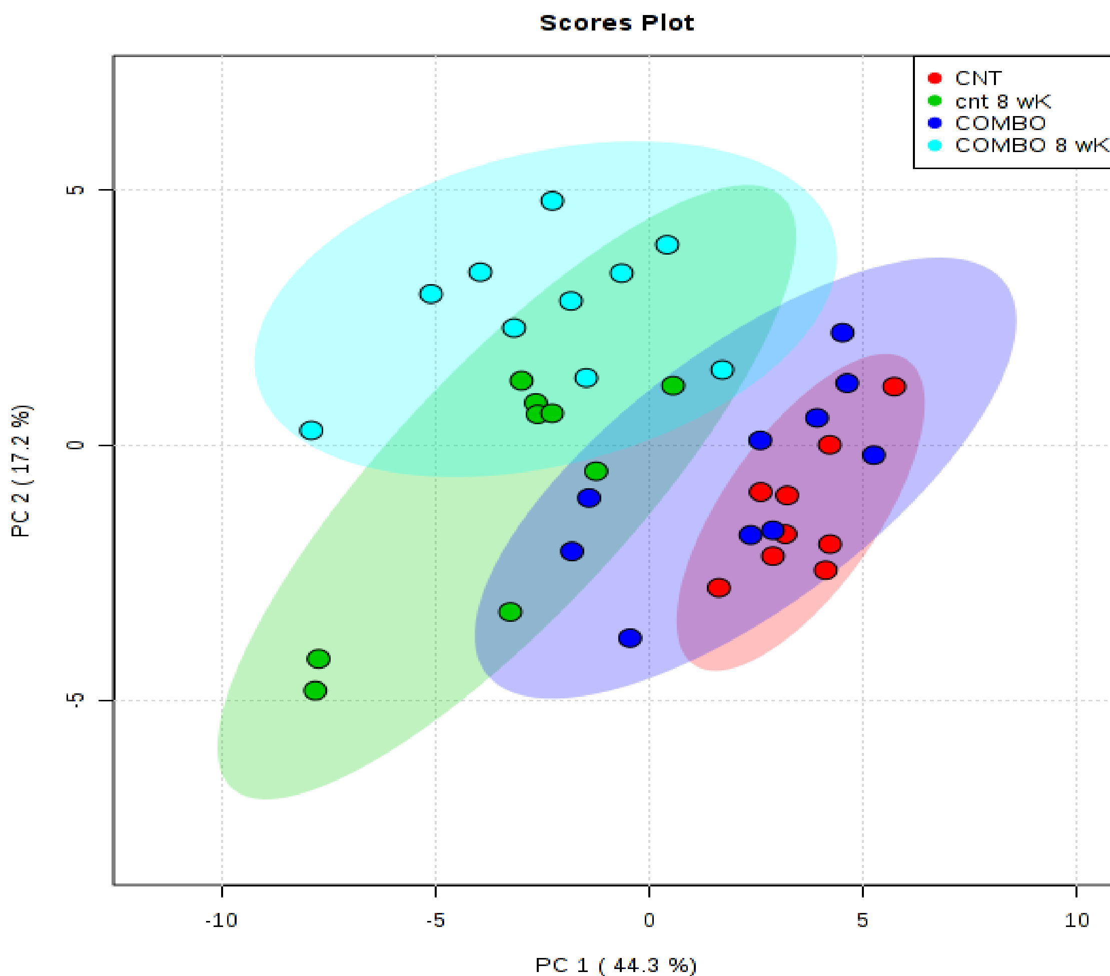


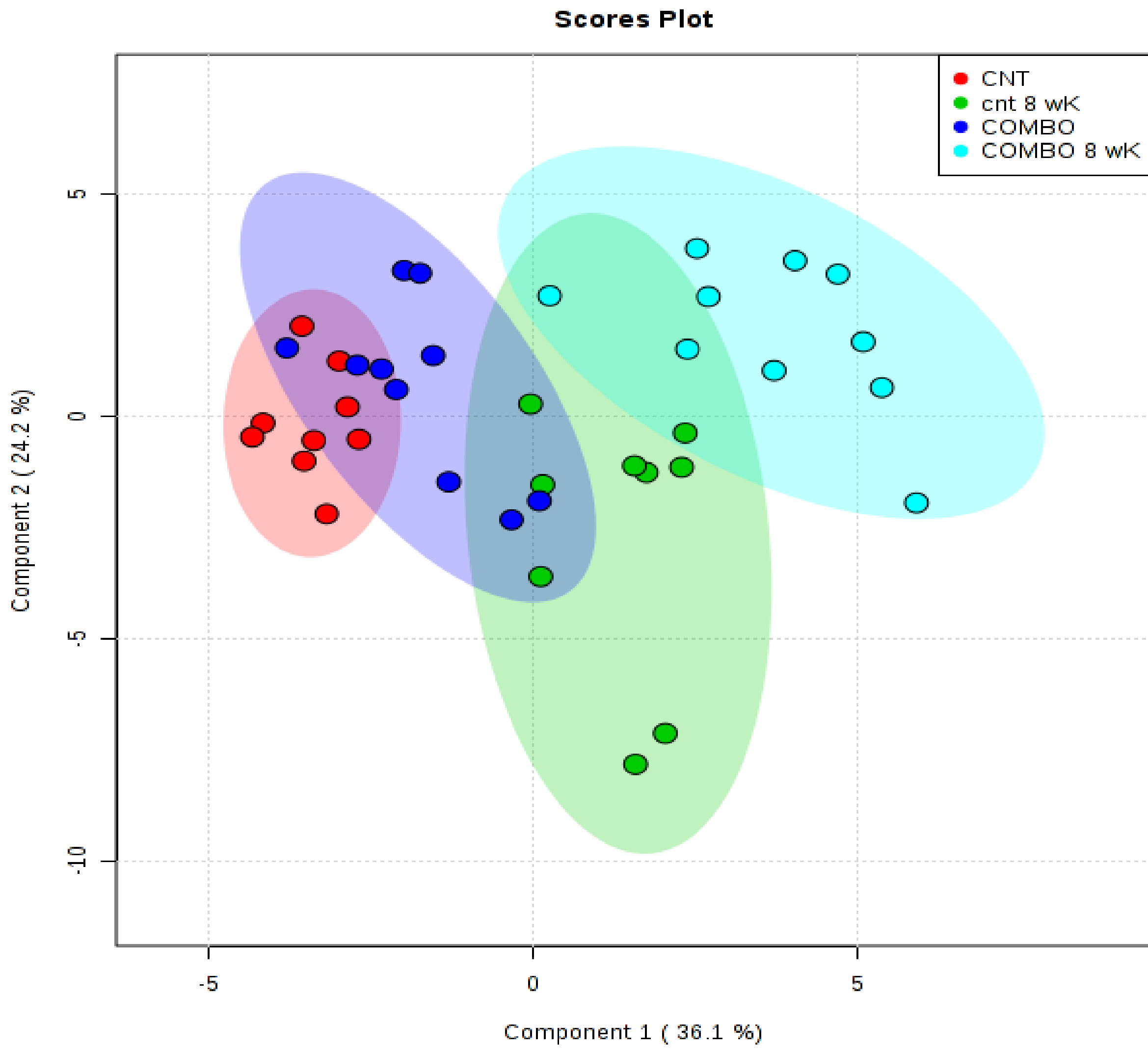
Figure 3.6: Box and whisker plot analyses of plasma metabolite levels from LC MS/MS data between the two different rat groups at 8 weeks including: amino acids; organic acids; sugars and; fatty acids. CNT, control OVX, ovariectomized rat without treatment at baseline; ALN, ovariectomized rat treated with alendronate at baseline, CNT 8 weeks, control OVX, ovariectomized rat without treatment at 8 weeks. ALN, ovariectomized rat treated with alendronate at 8 weeks. P < 0.05 compared to the OVX group.

3.3.4. Combined (ALN+vitamin D) group versus control group over time

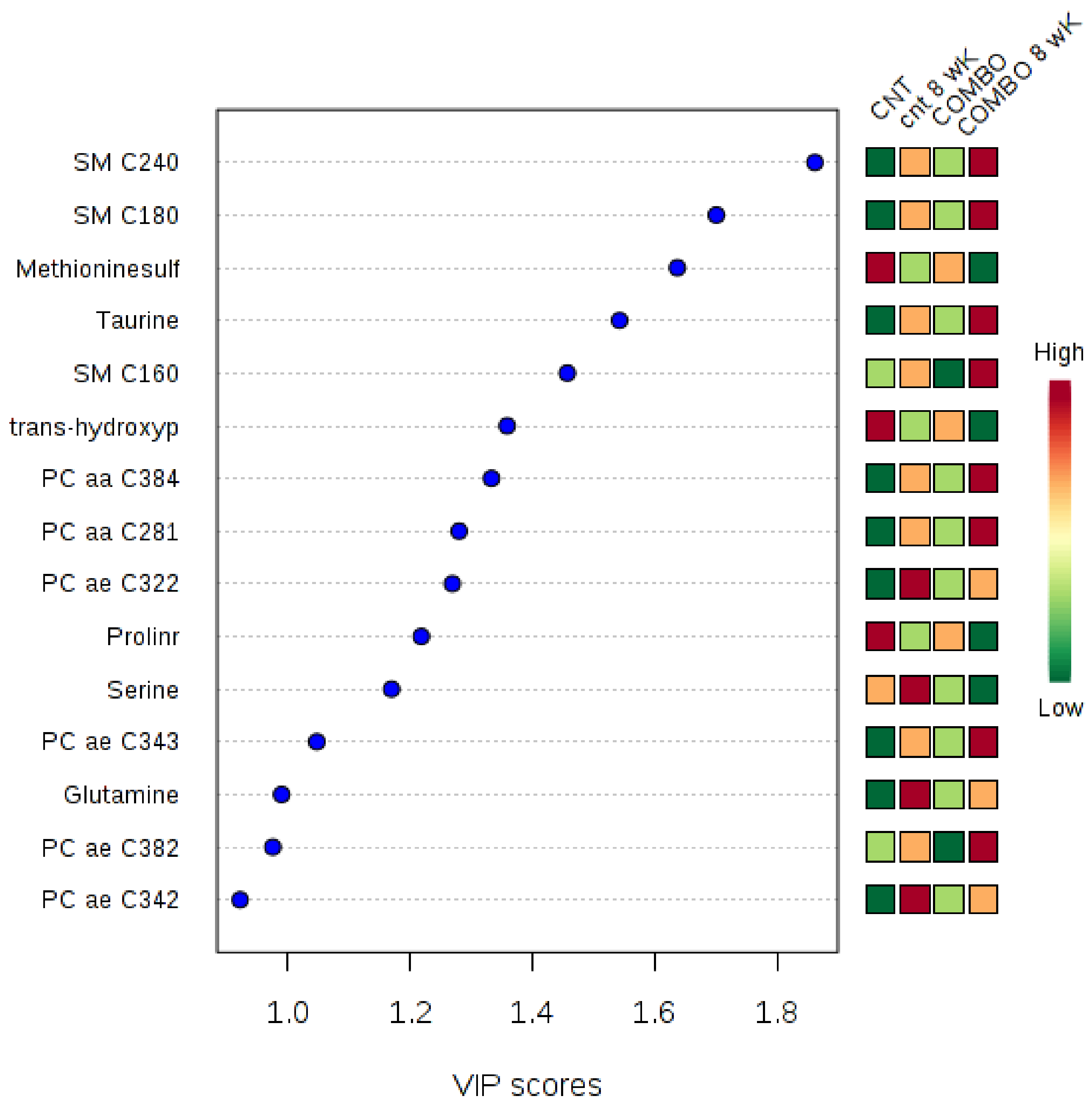
Distinct differences in the major metabolites levels between the OVX control and OVX treated groups were observed in the PCA and PLS-DA plots, shown in Figure 3.7. For the PLS-DA evaluation, the R2, Q2 and permutation test parameters were calculated for the model evaluation, fitness and prediction power. The R2 was 0.9, the Q2 was 0.7 and the permutation test was P = < 0.001. For the permutation test a total of 1000 resamplings were performed and calculated to determine the statistical significance.



(A) PCA



(B) PLS-DA



(C) VIP score

Figure 3.7: Global metabolic changes in plasma from the control group and alendronate with vitamin D group based on principal component analysis (PCA), (A) based on partial least squares discriminant analysis (PLS-DA) score plots (B) and variable importance in projection (VIP) values (C)

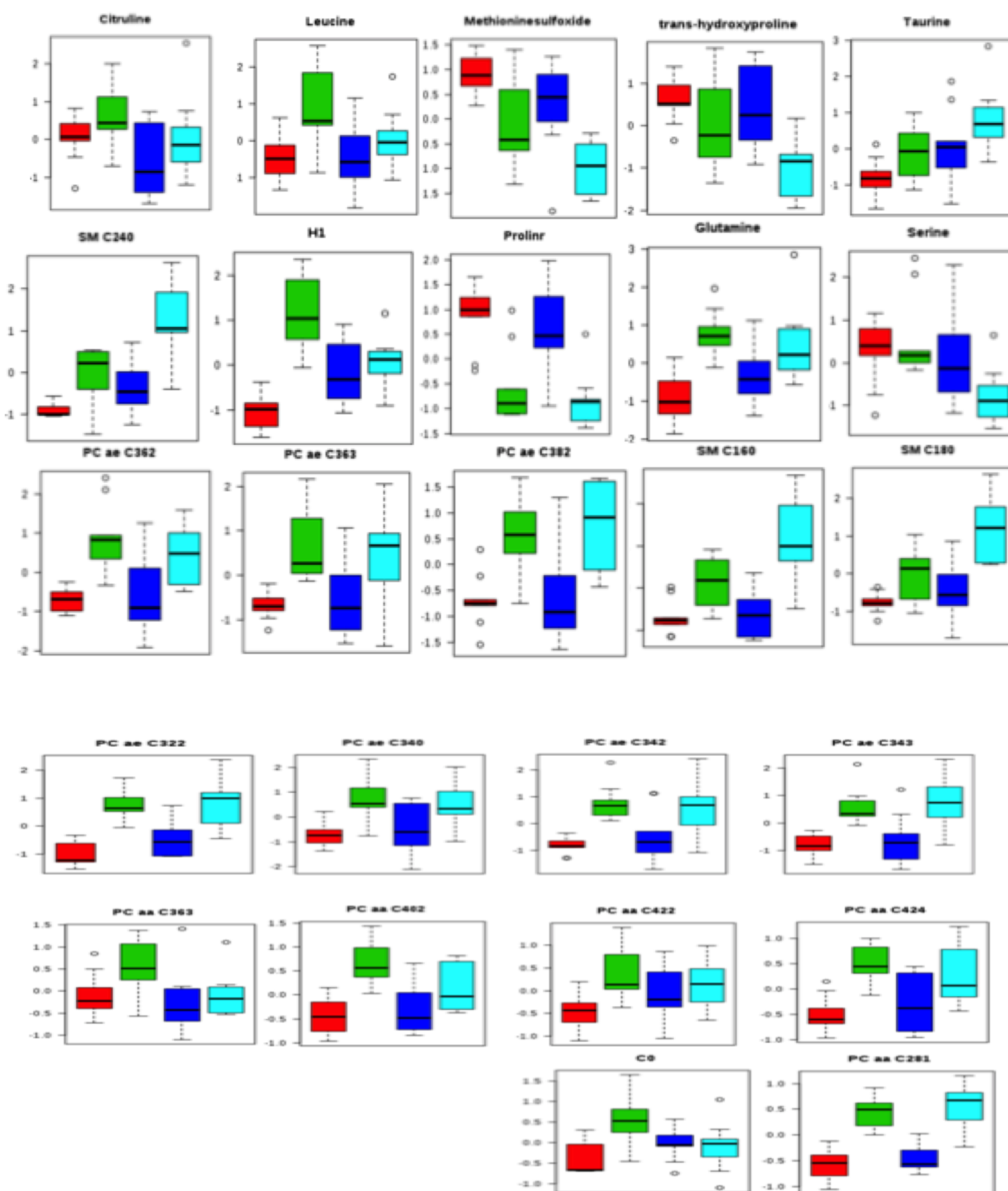
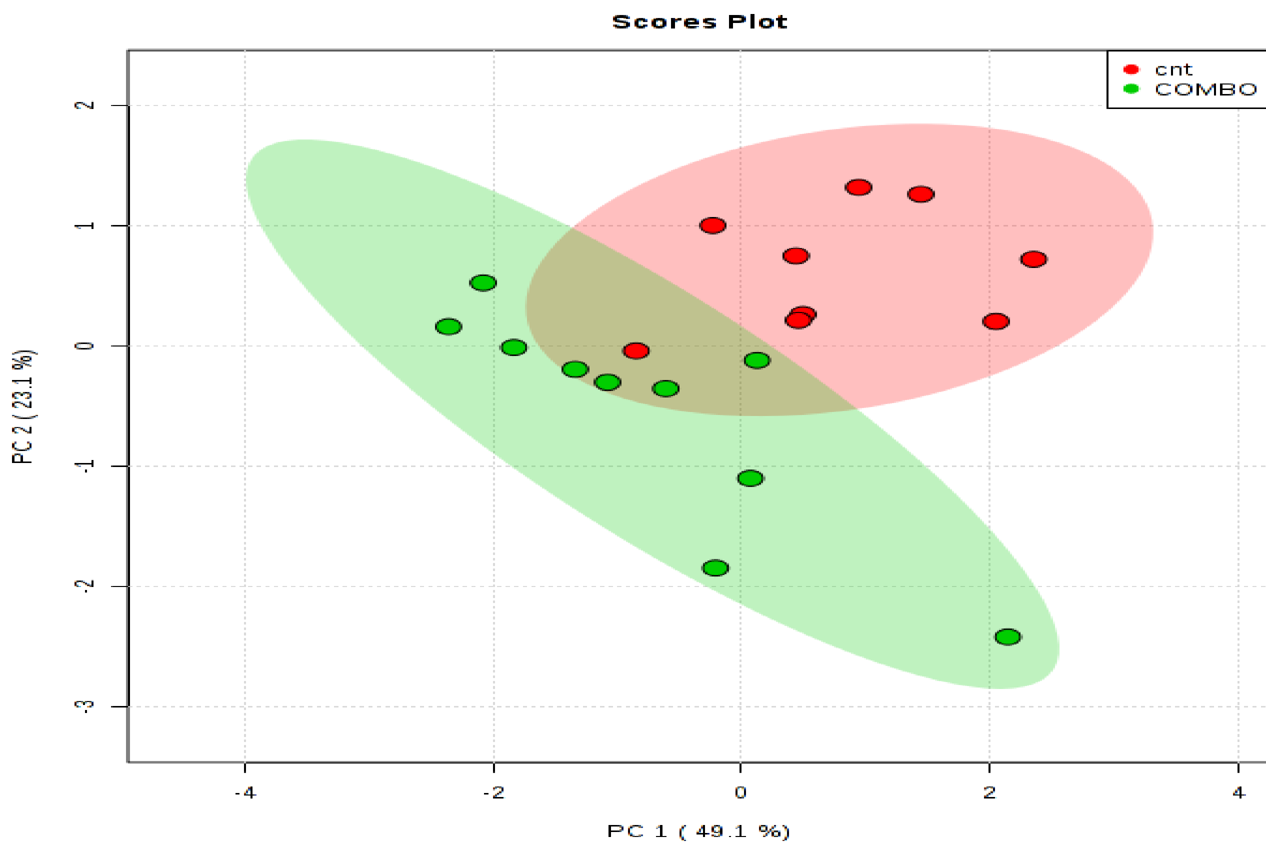


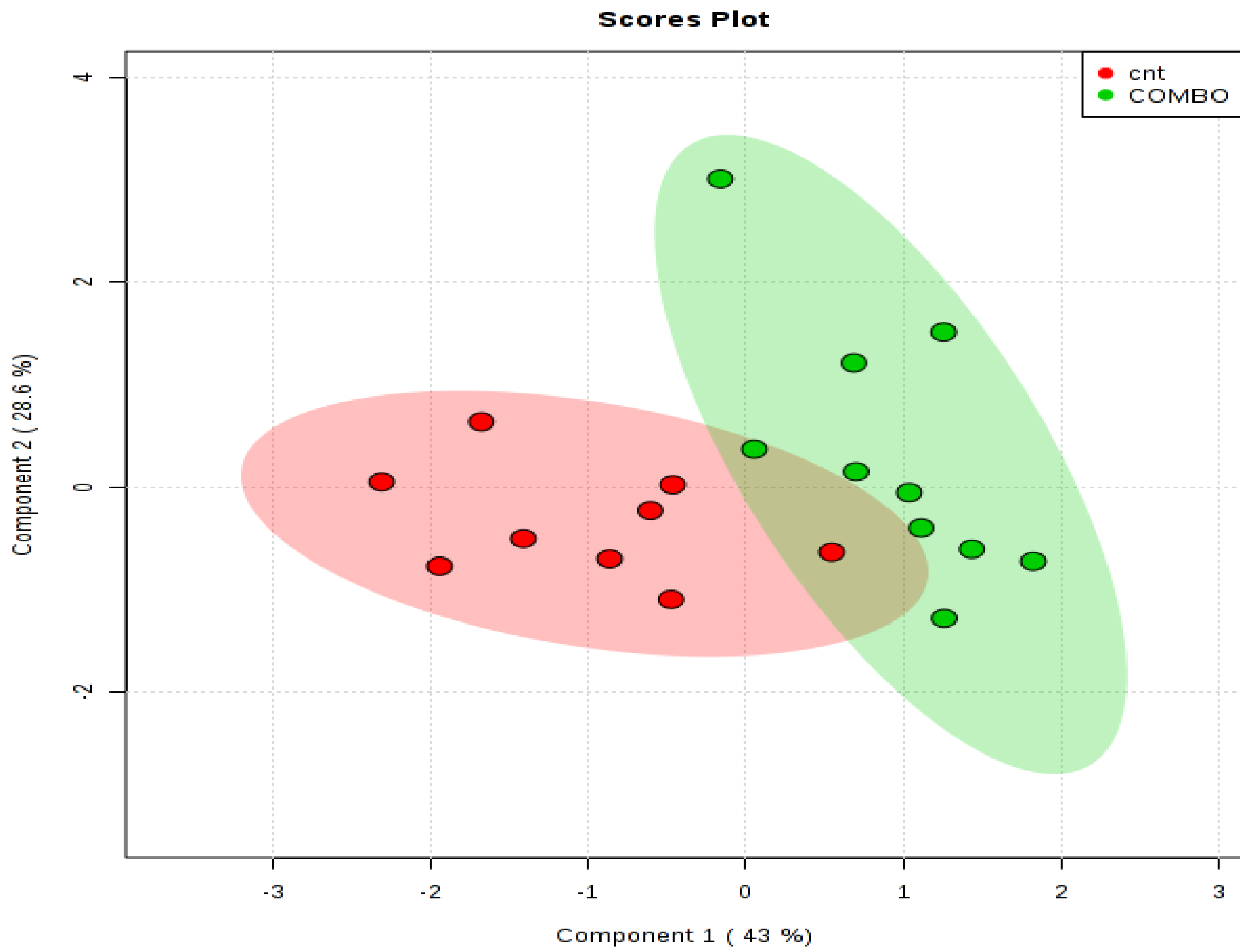
Figure 3.8: Box and whisker plot analyses of metabolites from LC MS/MS data for the two different rat groups (ALN+vitamin D vs. control) at baseline and 8 weeks time points including: amino acids; organic acids; sugars and; fatty acids. CNT, control OVX, ovariectomized rats without treatment at baseline; COMBO, ovariectomized rats treated with alendronate with vitamin D at baseline, CNT 8 weeks, control OVX, ovariectomized rat without treatment at 8 weeks. COMBO 8 week, ovariectomized rat treated with alendronate with vitamin D at 8 weeks. $P < 0.05$ compared to the OVX group.

3.3.5. Combined (ALN+vitamin D) group versus control group at 8 weeks

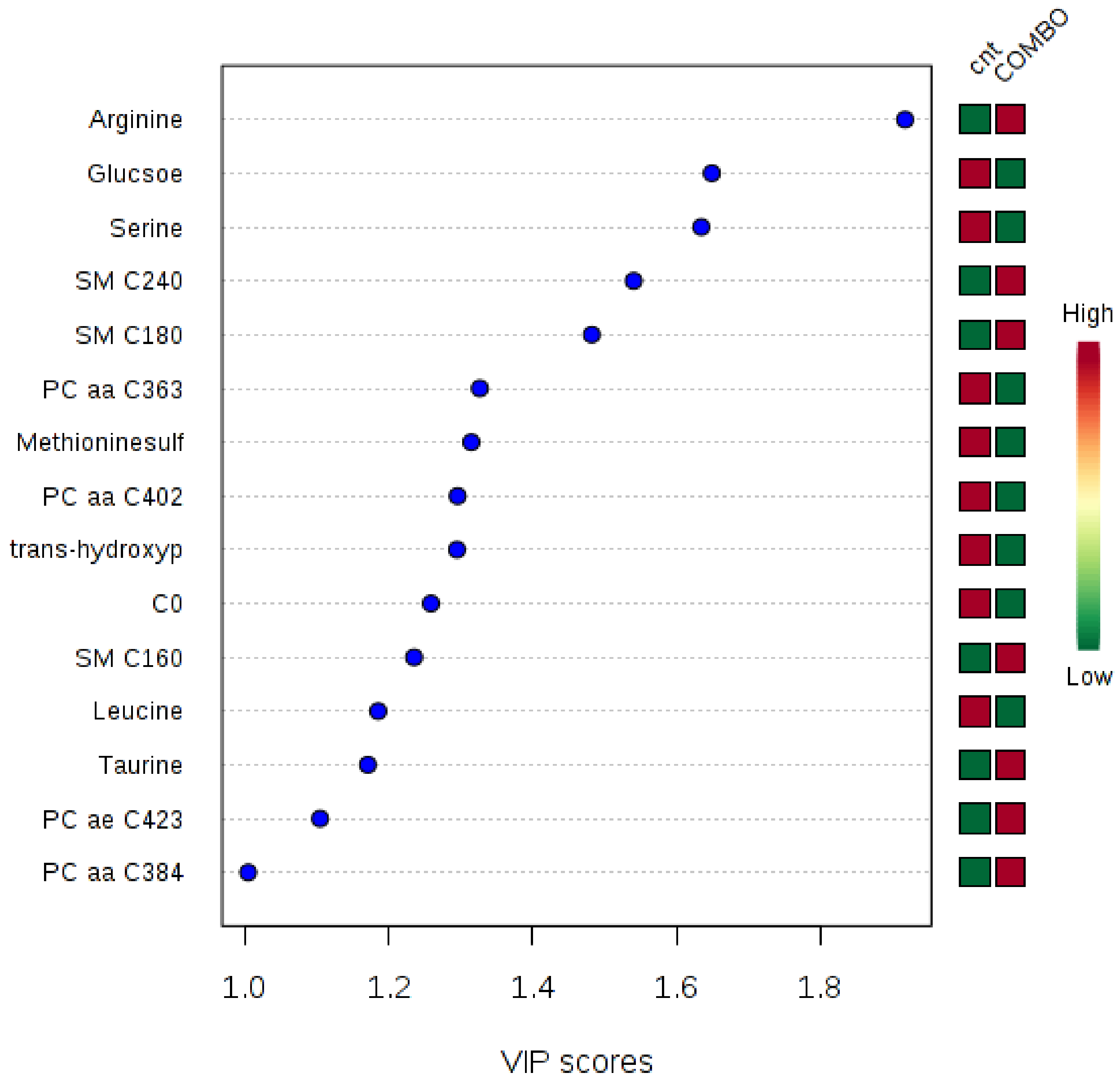
The results show the same significant metabolites as seen in the alendronate group compared with the control group. The ROC curve (Figure 3.12) indicates that a 10-metabolite combination was a highly significant predictor of bone status: AUC 0.955 (95% CI, 0.75–1).



(A) PCA



(B) PSL-DA



(C) VIP score

Figure 3.9: metabolic changes in plasma from the control group and the alendronate with vitamin D group at 8 weeks time points based on principal component analysis (PCA), (A), based on partial least squares discriminant analysis (PLS-DA) score plots (B) and variable importance in projection (VIP) values (C)

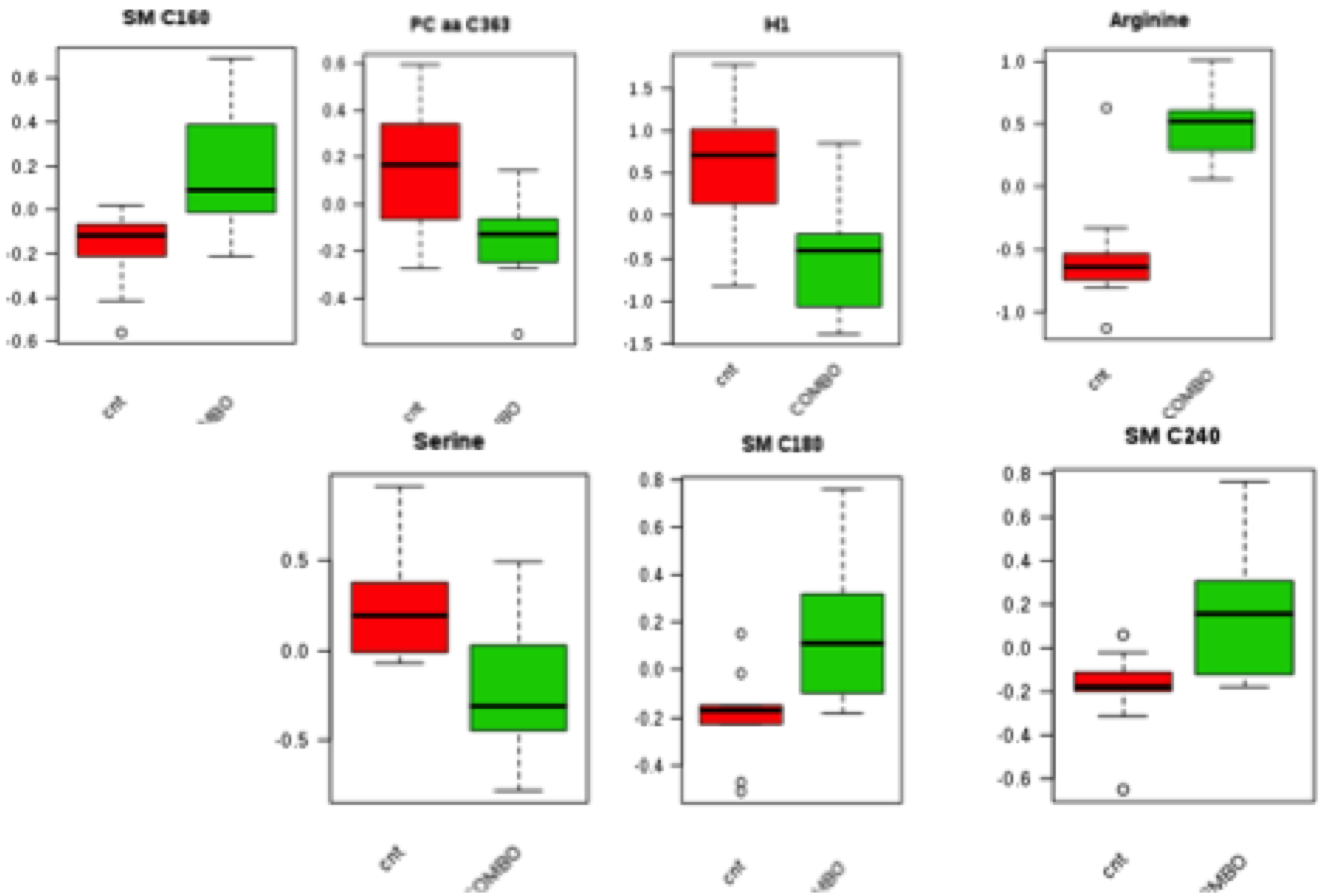


Figure 3.10: Box and whisker plot analyses of metabolites from LC MS/MS data collected for the two different rat groups (ALD+vitamin D vs. control) at 8 weeks including: Amino acids; organic acids; sugars and; fatty acids. CNT, control OVX, ovariectomized rats without treatment at 8 weeks; combo, ovariectomized rats treated with alendronate with vitamin D at 8 weeks. All illustrated metabolites are significant ($P < 0.05$) compared to the OVX group.

3.3.6. Vitamin D group versus control group at 8 weeks

Metabolomic analyses were not conducted for the vitamin D monotherapy group as those rats experienced an adverse drug event from an excessively high dose of vitamin D, as previously mentioned. Consequently, those results were not be related to our thesis objectives…

3.4. Receiver operating characteristic (ROC) curve

Important characteristics of any diagnostic test are high specificity and sensitivity, that is, the ability to accurately separate the group being assayed into those with and without treatment in question. These features can be assessed by measuring the area under the ROC curve. By definition, the ROC curve is plotted with the true positive rate (sensitivity values) on the Y-axis with the false positive ratio (1 - specificity) on the X-axis. An area under the ROC curve (AUC) of 1 represents a perfect test that generates no false positives or false negatives; an AUC of 0.5 represents an untrustful diagnostic test, as it can be produced by chance. The area under the curve can measure the accuracy of a diagnostic test.

3.4.1 Receiver operating characteristic curves of the alendronate treated group

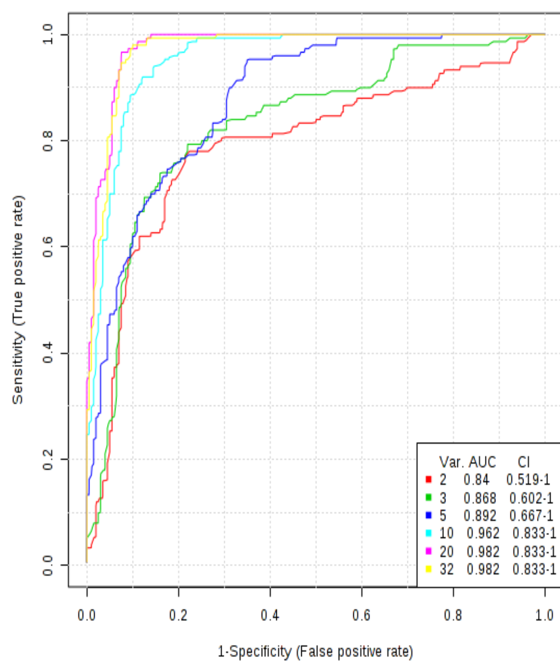
To determine the potential impact of the use of metabolites as markers of bone loss, the plasma data for these metabolites were used to generate ROC curves. The ROC curve was utilized to distinguish between the alendronate group from the control group (Figure 3.12.A) and to ascertain which ROC curve had the greatest AUC values and the minimum number of metabolites. Receiver operator characteristic (ROC) curves were generated using the MetaboAnalyst web-based statistical package(100). Multivariate ROC curve based exploratory analysis was conducted and based on a support vector machine (SVM) model for selecting and weighting metabolites. The generated ROC curves were then used to calculate the area under the curve (AUC). The 95% confidence interval

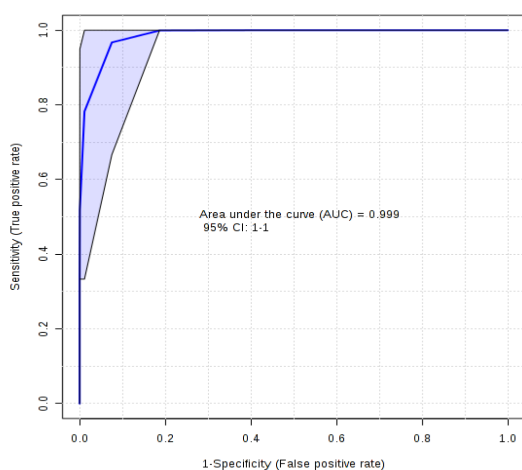
(CI) and P values for the AUC curves were measured. Permutation testing was also conducted to determine the probability that the AUC obtained was due to chance.

The ROC curves were then used to calculate the AUC values of the plasma samples for the Control group and alendronate group (Figure 3.11.), (AUC.0.982 (95% CI, 0.83–1); 95% confidence for 10 metabolites 0.99, permutation test P<0.05), and ROC curve of plasma sample of Control group and Combo group (Figure 3.12.) (AUC: 0.955 (95% CI, 0.75–1); 95% confidence for 10 metabolites 0.917, permutation test P<0.05)

The ROC curve of ten metabolites was found to have the greatest power to discriminate OVX rats that have a slight reduction in trabecular bone volume (i.e. those classified as a treated group) from rats without treatment.

Assessment of Receiver operative characteristic model was performed with Permutation testing (Figure 3.12, 3.12) for the optimal model was performed using 2000 random samples and showed a low probability that the diagnostic accuracy represented by the area under the ROC curve was due to chance, P <0.05. This result is supportive of the concept that a panel of biomarkers may be more appropriate for clinical use rather than relying on a single metabolite in isolation as a panel may offer increased specificity and sensitivity.





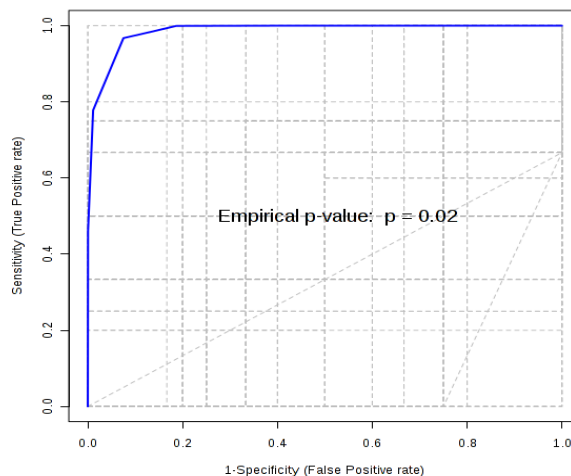


Figure 3.11: ROC curves of metabolite models for osteoporosis diagnosis in rats. (A) All Models: AUC.0.982 (95% CI, 0.83–1); (B) 95% confidence for 10 metabolites 0.99, (C) permutation test P<0.05, ROC: receiver-operating characteristic curve; AUC: area under the curve; CI: confidence interval.

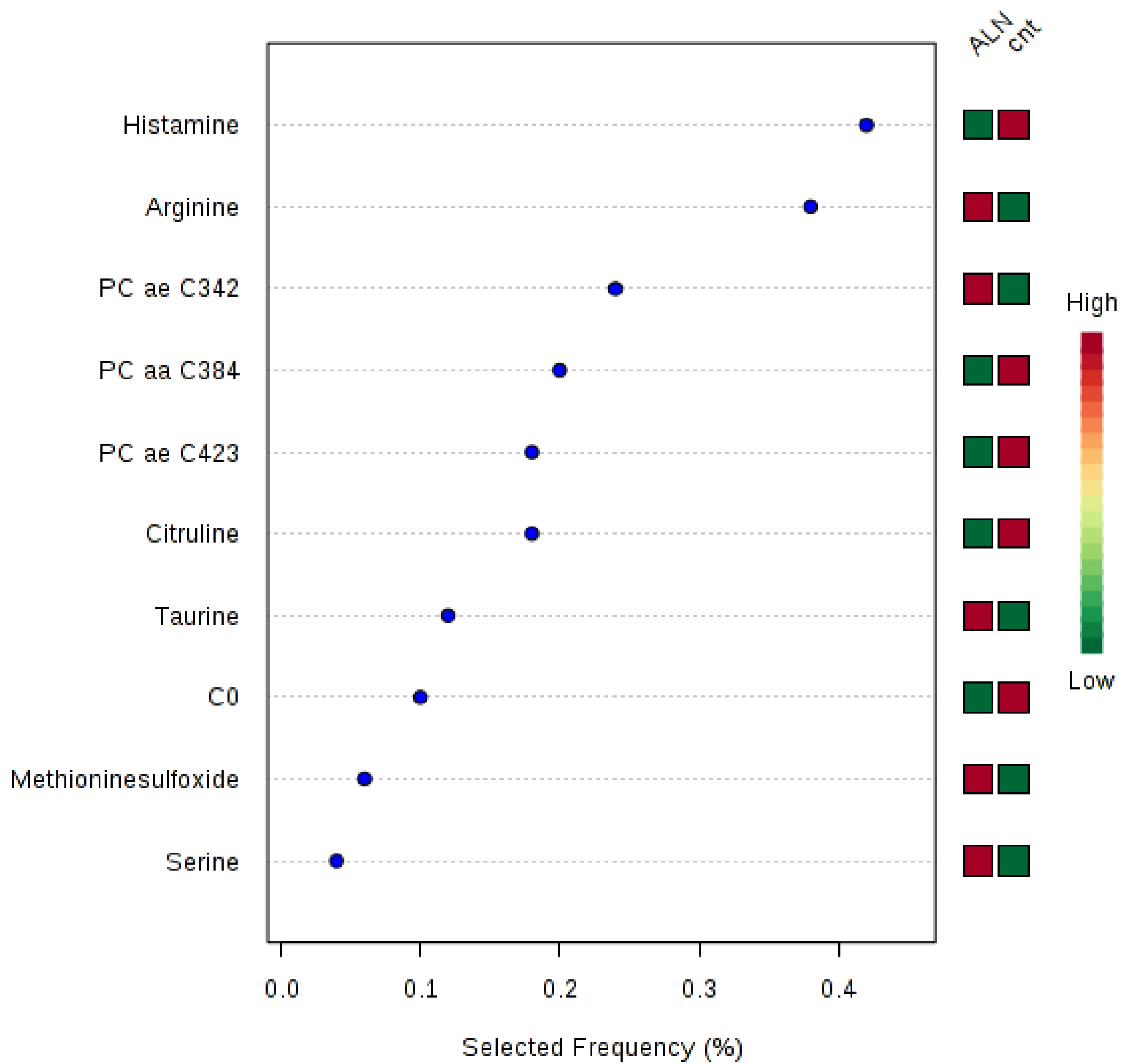


Figure 3.11.1: Variable importance in projection plot

3.4.2 Receiver operating characteristic curve of control versus Combo group

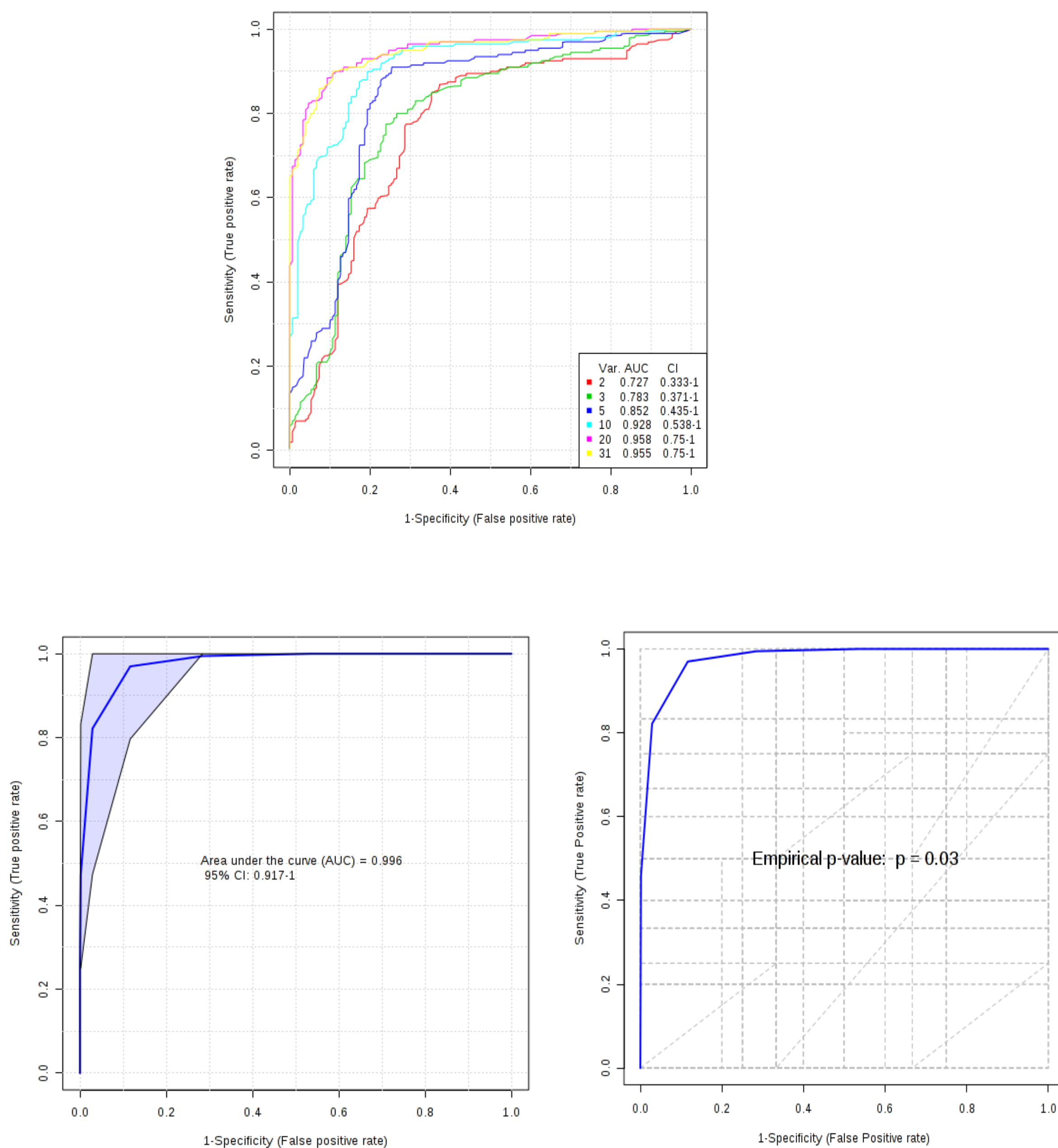


Figure 3.12: ROC curves of different models. (A) All Models: AUC: 0.955 (95% CI, 0.75–1); (B) 95% confidence for 10 metabolites 0.917, (C) permutation test P<0.05

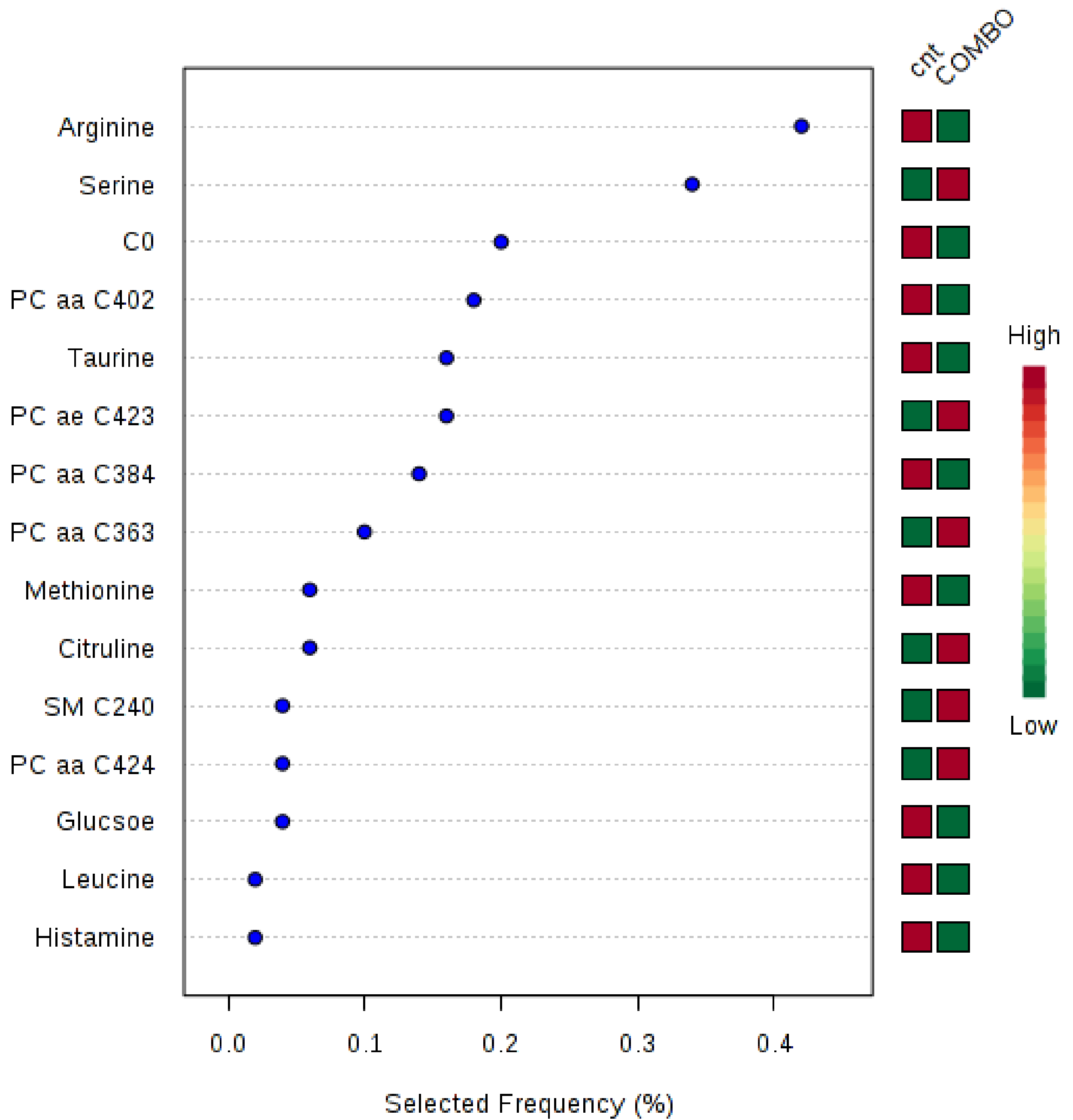


Figure 3.12.1: Variable importance in projection plot

3.5. Correlations between bone volume and plasma metabolites

A set of bivariate Pearson correlation tests was conducted to determine the strength and direction of the relationship between each of these predictors and the response variable (bone volume). This resulted in having five plasma metabolites (i.e., predictors) that can be used to

estimate the bone volume (i.e., response). These metabolites are arginine, proline, methionine sulfoxide, histamine, and trans-hydroxproline. A sample of all measures obtained from the three experimental groups combined (CNT 8 WKS, ALN 8 WKS, and baseline) was used. Arginine, proline, methionine, and trans-hydroxproline revealed a significant direct correlation with the bone volume at a 99% significance level with correlation values that ranged from 0.50 to 0.87. Histamine, on the other hand, showed an inverse relationship ($r = -0.4$) at a significant level of 0.95.

In order to understand how the response variable (bone volume) changes when any of the five plasma metabolites is varied while the other metabolites are held fixed, a multiple regression model was constructed and adopted. This model is also measuring the association between the response variable and each predictor. The analysis was performed using SPSS version 16.0 (SPSS is a registered trademark for SPSS Inc., Chicago IL).

Except for arginine, other metabolities showed significant association with the measured bone volume at $p<0.001$. For every unit increase in histamine, there is a 1.4 unit decrease in bone volume (0.95 CI = 1.91 to 0.81), holding all other predictors constant. For every unit increase in methionine sulfoxide, the bone volume is increased by about 4 fold (0.95 CI = 3.37 to 4.54), holding all other predictors constant. For every unit increase in trans-hydroxproline, there is a 0.75 unit increase in bone volume (0.95 CI = 0.87 to 0.62), holding all other predictors constant. As well, an increase in proline is contributing to a slight increase in bone volume.

Although it was significant in the bivariate analysis, arginine has shown to be insignificant in the multiple regression models. This may be due to the colinearity of arginine with the other predictors.

Our results suggest that the model calculated with multiple metabolites yields a higher correlation with bone density than any single metabolite. In fact, with a calculated correlation coefficient of 0.92, it can be inferred that 85% of the variance was being accounted for by the five independent variables.

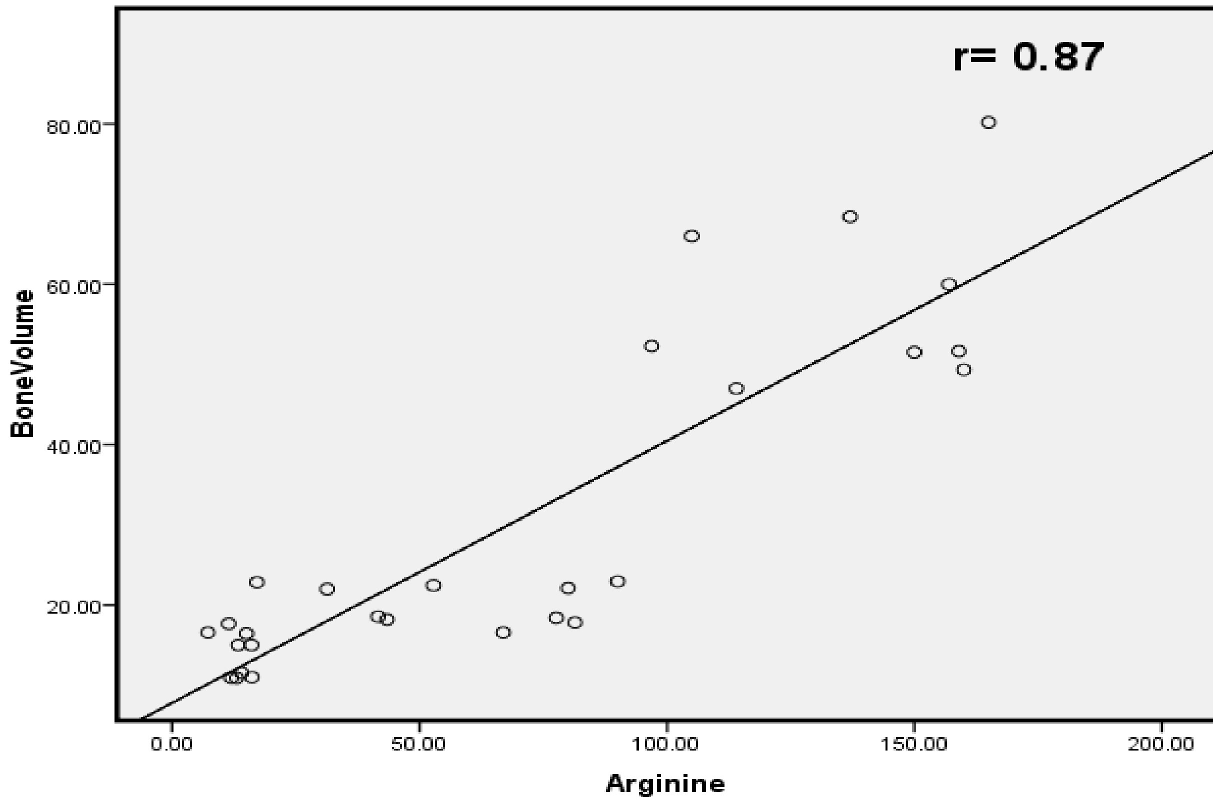


Figure 3.13: Scatterplot of trabecular bone volume and plasma arginine levels using three experimental groups combined (CNT 8 WKS, ALN 8 WKS, and baseline. The best fit line and corresponding Pearson correlation coefficient is also shown.

Correlations		BoneVolume	Arginine
Bone Volume	Pearson Correlation	1	0.872**
Arginine	Pearson Correlation	0.872**	1

**. Correlation is significant at the 0.01 level (2-tailed).

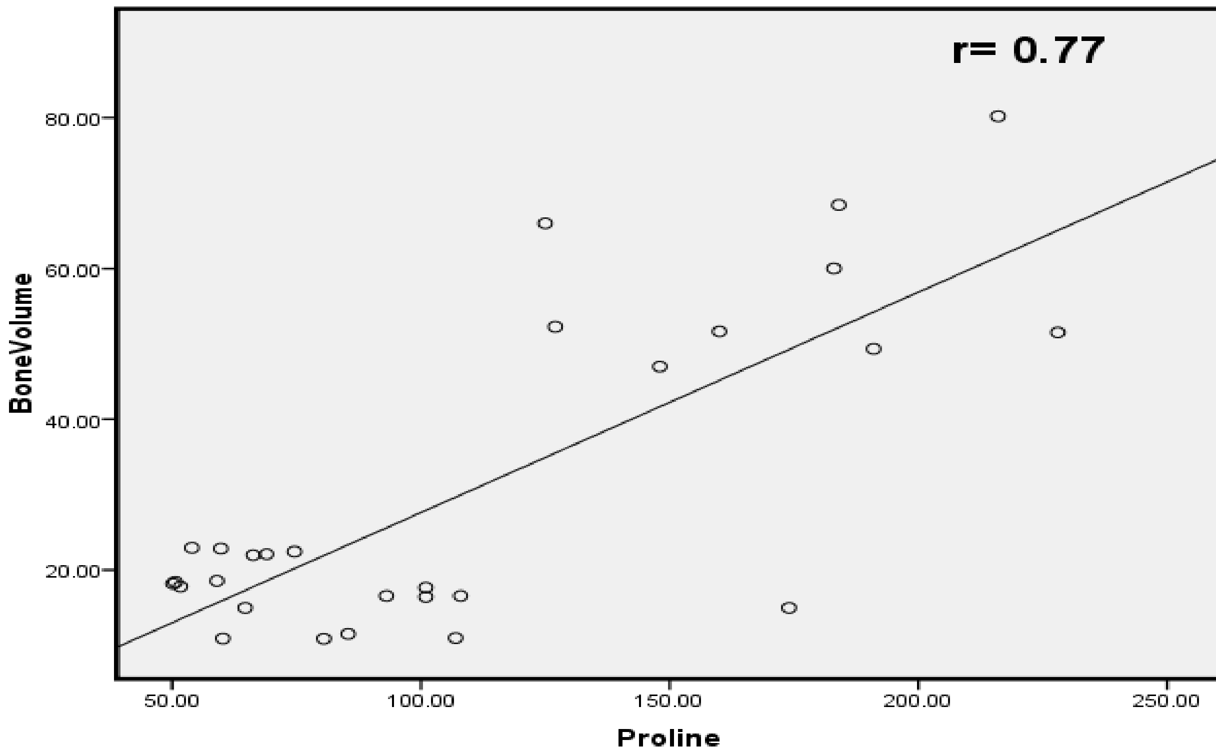


Figure 3.14: Scatterplot and trabecular bone volume and plasma proline levels using three experimental groups combined (CNT 8 WKS, ALN 8 WKS, and baseline. The best fit line and corresponding Pearson correlation coefficient is also shown.

Correlations		BoneVolume	Prolinr
BoneVolume	Pearson Correlation	1	0.770**
Prolinr	Pearson Correlation	0.770**	1

**. Correlation is significant at the 0.01 level (2-tailed).

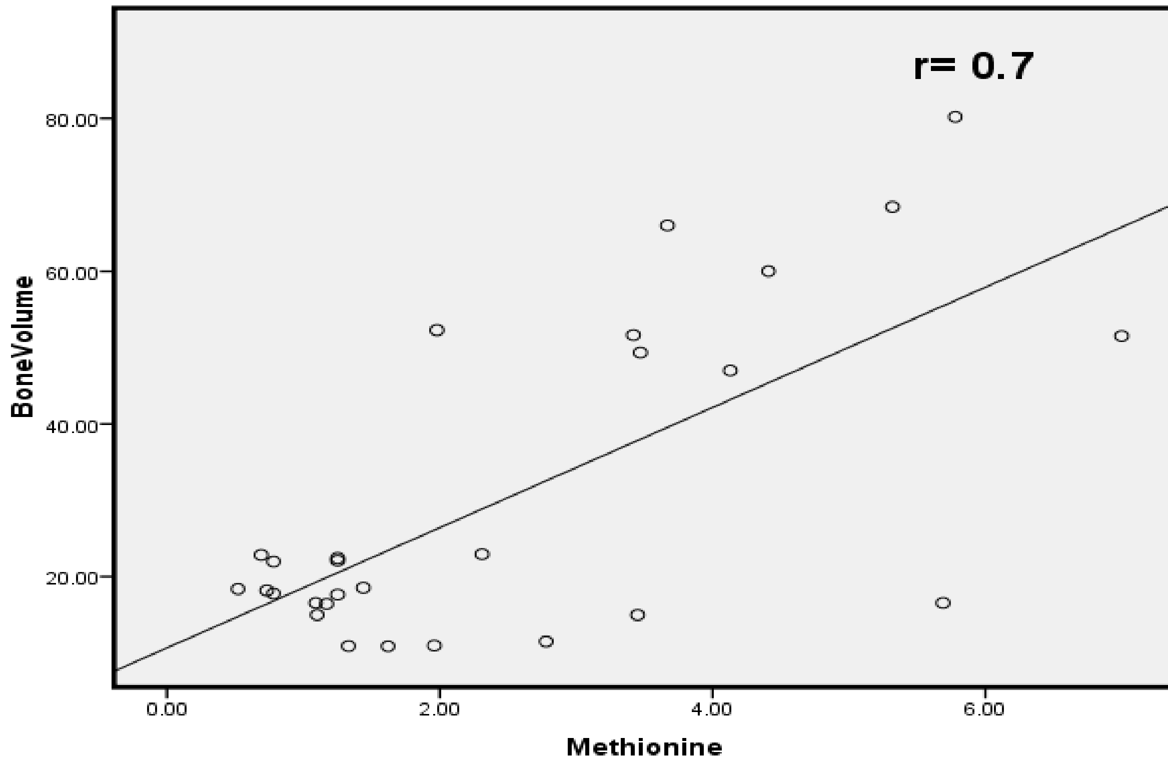


Figure 3.15: Scatterplot and trabecular bone volume and plasma methionine levels using three experimental groups combined (CNT 8 WKS, ALN 8 WKS, and baseline. The best fit line and corresponding Pearson correlation coefficient is also shown.

Correlations		BoneVolume	Methionine
BoneVolume	Pearson Correlation	1	0.691**
Methionine	Pearson Correlation	0.691**	1

**. Correlation is significant at the 0.01 level (2-tailed).

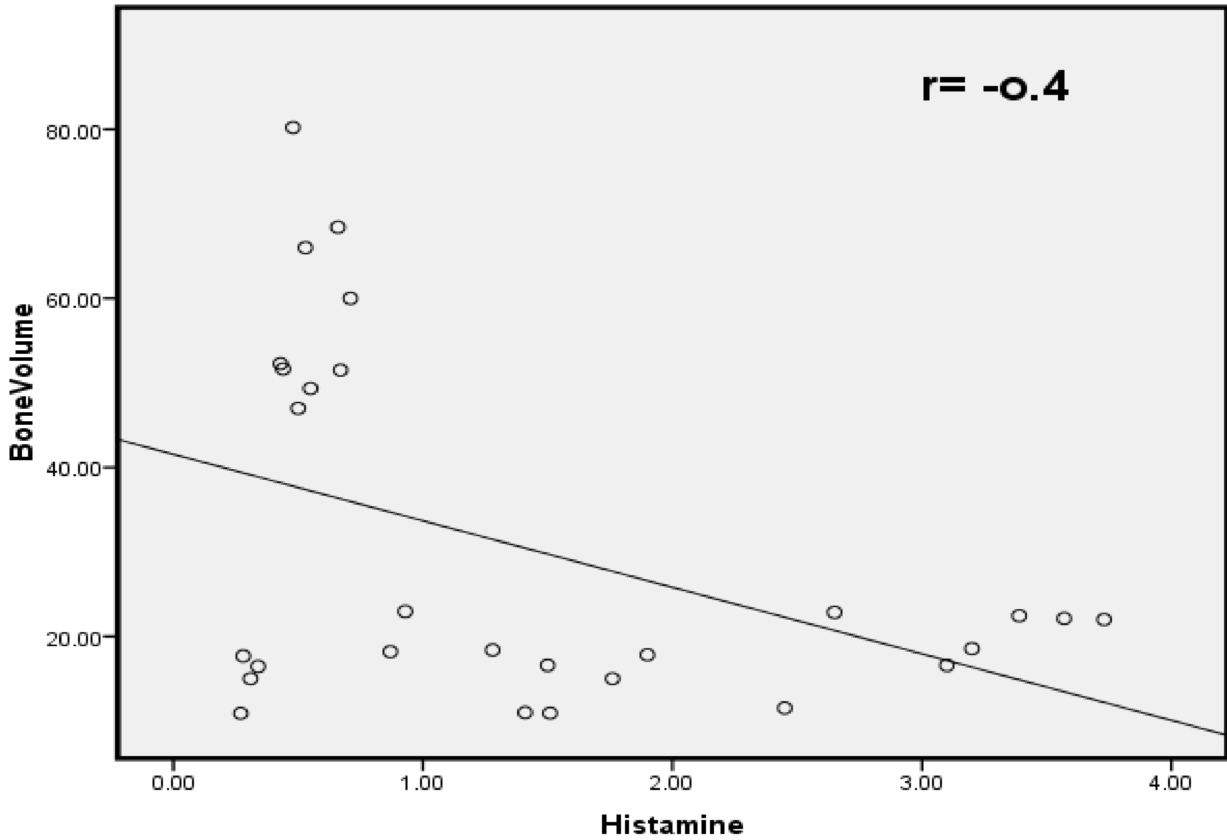


Figure 3.16: Scatterplot and trabecular bone volume and plasma histamine levels using three experimental groups combined (CNT 8 WKS, ALN 8 WKS, and baseline. The best fit line and corresponding Pearson correlation coefficient is also shown.

Correlations		BoneVolume	Histamine
BoneVolume	Pearson Correlation	1	-0.432*
Histamine	Pearson Correlation	-0.432*	1

*. Correlation is significant at the 0.05 level (2-tailed).

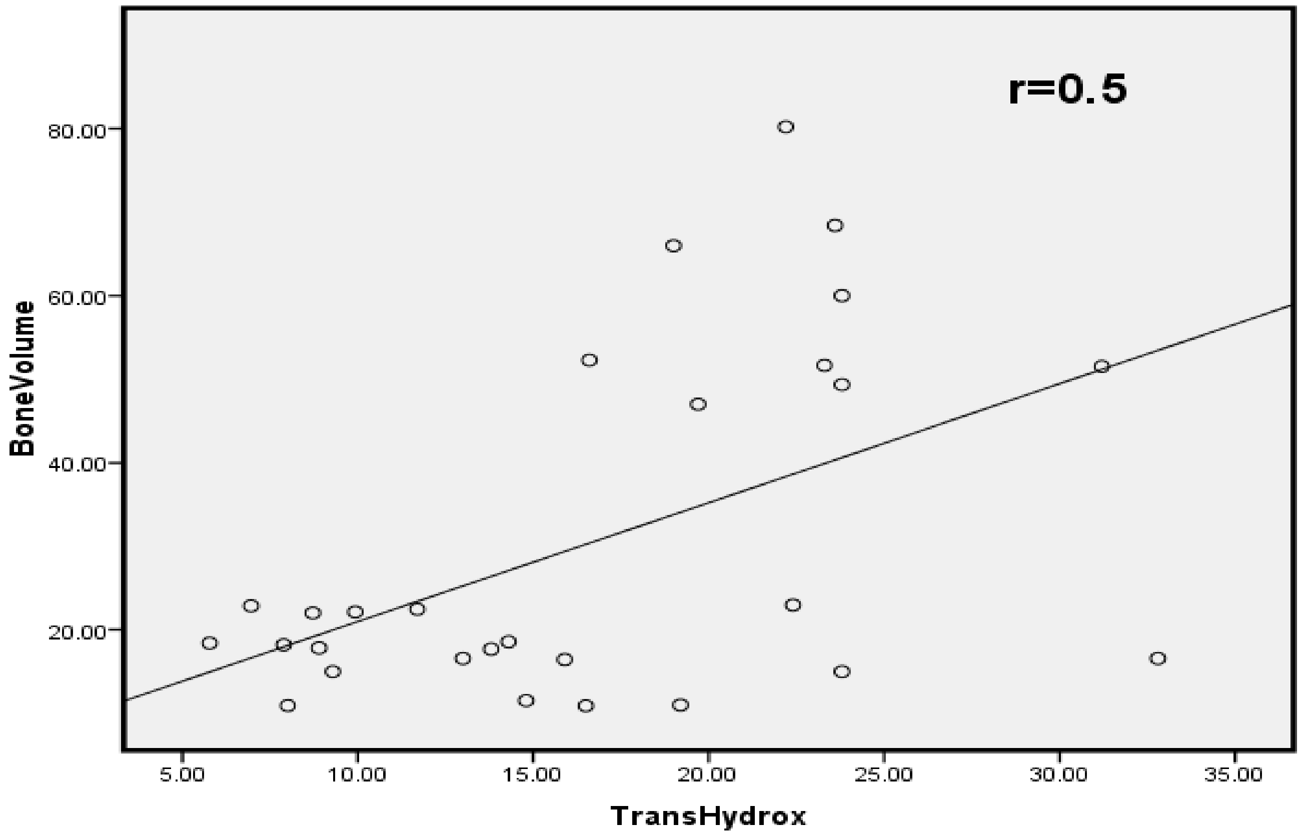


Figure 3.17: Scatterplot and trabecular bone volume and plasma transhydroxyproline levels using three experimental groups combined (CNT 8 WKS, ALN 8 WKS, and baseline) The best fit line and corresponding Pearson correlation coefficient is also shown.

Correlations		BoneVolume	TransHydrox
Bone Volume	Pearson Correlation	1	0.501**
Trans Hydrox	Pearson Correlation	0.501**	1

**. Correlation is significant at the 0.01 level (2-tailed).

Table 3.1: Results of correlation analysis of the relationship between plasma metabolites and bone volume.

Correlations		Bone volume	Arginine	Proline	Histamine	Methionine	Hydroxproline
Bone Volun	Pearson Correlatior	1	0.872**	0.770**	-0.432*	0.691**	0.501**
Arginine	Pearson Correlatior	0.872**	1	0.689**	-0.335	0.587**	0.454*
Proline	Pearson Correlatior	0.770**	0.689**	1	-0.464*	0.857**	0.779**
Histamine	Pearson Correlatior	-0.432*	-0.335	-0.464*	1	-0.402*	-0.424*
Methionine	Pearson Correlatior	0.691**	0.587**	0.857**	0.402*	1	0.892**
Hydroxyprolin	Pearson Correlatior	0.501**	0.454*	0.779**	0.424*	0.892**	1

**. Correlation is significant at the 0.01 level (2-tailed

*. Correlation is significant at the 0.05 level (2-tailed)

Table3.2: Multiple regression analysis for association between plasma metabolites (independent variables) and bone volume (dependent variable)

Parameter	B	Std. Error	95% Wald Confidence Interval		
			Lower	Upper	**Sig.**
Arginine	0.006	0.0111	0.028	0.016	0.579
Proline	0.042	0.0097	0.023	0.061	<0.001
Histamine	-1.360	0.2793	-1.908	-0.813	< 0.001
Methionine	3.953	0.2978	3.370	4.537	<0.001
Hydroxproline	0.747	0.0631	0.870	0.623	<0.001

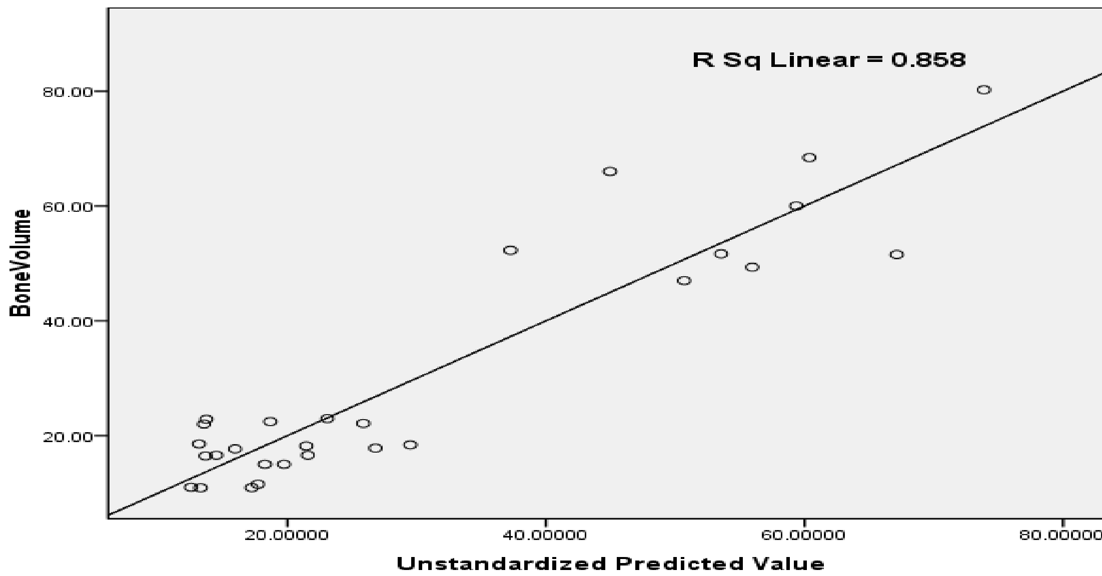


Figure 3.18: Scatter plot of multiple regression analysis for association between arginine; proline, hydroxyproline, methionine sulfoxide; and histamine metabolites and trabecular bone volume.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	**0.926**	**0.858**	**0.825**	8.69925

a. Predictors:(Constant), Trans-Hydroxproline, Histamine, Arginine, Proline, Methionine

b. Dependent Variable: Bone Volume

CHAPTER 4: DISCUSSION

4.1. The effect of bisphosphonates and vitamin D on the body weight change:

In our study, the ovariectomized control group was found to have significantly greater body weight than for all other treated animals ($p<0.05$) over the course of the experimental period. This result is in agreement with previous (101)studies. (102,103)

In our experiment, the sudden, unexpected decrease in the body weight of the ovariectomized vitamin D group relative to the control group at week 2 was clearly a consequence of an excessively high cumulative dose of vitamin D. The vitamin D treatment was discontinued as soon as the adverse events began to show themselves. The primary intention of including vitamin D with our experiment was to investigate if the active form of vitamin D would alter the metabolomic profile of bisphosphonate-burdened skeletal bone. In this study, our use of vitamin D at the previously suggested dose of 400 µg/kg/wk led to similar or even more intensive changes in the osseous tissue as found via micro CT studies (results not shown). Feskanich et al. demonstrated the correlation between the occurrence of bone fractures and vitamin D intake in women in this investigation (104)

As reported in previous studies, diminished level of estrogens induced by ovariectomization leads to an increase in body mass, which supports our experimental findings. In those studies, ovariectomized rats were shown to have significantly higher body weights compared to sham-operated rats due to fat deposition caused by estrogen deficiency. Additionally, an earlier study(105) suggested that increased body weight likely provides an additional stimulus for bone neoformation, thus serving as partial protection against the osteopenia that occurs in long bones from supporting a greater total body weight.

As seen in the results section, there was significantly increased body weight in the ovariectomized rats treated with both ALN and vitamin D (OVX-Combo) when compared to ovariectomized rats treated with vitamin D alone. This increase in body weight suggested a protective effect of ALN in halting hypercalcemia in the presence of high-dose vitamin D. As expected, administering alendronate to female OVX rats throughout the experiment resulted in significantly improved trabecular bone volume of the osseous tissue over that of untreated OVX rats. The results confirm the protective effect of alendronate on the bony tissue in estrogen-deficient animals and humans. The combination treatment of ALN and vitamin D showed an additional increase in trabecular bone volume over ALN monotherapy alone. This observation may warrant further investigation as to the potential for cumulative synergistic effects with combination therapy, as recently reported for humans. [110] This may be indicative of a more prominent influence of alendronate on the bone and prove that concurrent administration of alendronate and vitamin D leads to therapeutically favorable outcomes.

4.2. The Micro-Computed Tomography Analysis

Numerous parameters can be used to describe trabecular bone microarchitecture. According to Bouxsein et al. at least four parameters (BV/TV, Tb.Th, Tb.Sp, and Tb.N) should be used to evaluate trabecular bone microarchitecture. For this reason, these four parameters were selected for evaluation of both types of bones in the present study. (16)

In this study, we aimed to confirm the curative effect of the combination of bisphosphonates and vitamin D or its derivatives as therapeutic agents for osteoporosis using a rat model. We did not to attempt to elucidate the mechanism of action of the combined treatment (106)

Nevertheless, some studies hypothesized that restoring bone loss in OVX rats treated by bisphosphonate and vitamin D may due to stimulating bone formation directly, via enhancing calcium absorption from the gastrointestinal tract, while alendronate may indirectly act on osteoblast cells to decrease bone turnover and bone resorption. It may be that alendronate blocks the catabolic action of bone dissolution, but not the anabolic activity, of 1α (OH) D3 in the combined treatment. (68,107,108)

Interestingly, not all combination treatments lead to positive outcomes. It has been reported that combined therapy between tiludronate (a non-nitrogenated bisphosphonate) and PTH may reduce bone mass in elderly females, and bone formation was blunted when bone resorption was inhibited by tiludronate. (109) Moreover, although several previous studies demonstrated that a single treatment with 1-α (OH) vitamin D3 significantly increased bone mass in OVX rats(110) Unfortunately our study was not able to show significant anabolic actions of vitamin D (other than an increase in combo therapy bone volume) due to the adverse drug reactions that we encountered in our study. In conclusion, the combination therapy with alendronate and vitamin D exhibited superiority to ALN alone or vitamin D alone regarding improving bone volume, trabecular number, trabecular thickness and a decrease in trabecular separation.

4.3. Direct injection-mass spectrometry and metabolomics

In the present work, a decrease in glucose levels was found in all three treated groups of rats. For the OVX rats, an increase in glucose levels might be expected, according to Chau et al. (111,112)

who claimed that osteoporosis-like conditions may occur with diabetes disease comorbidity in postmenopausal women. However, variable findings on glucose levels in osteoporosis have been reported in the literature. For example, while some studies report an increase in the glucose levels

in OVX rats, (113,114) other studies report no effect of antiresorptive therapy on glucose levels, (93) while still other studies reported a high correlation between osteoporosis on glucose levels by impacting the osteoblast cells. (115) Those findings are somewhat contradictory and would require additional validation, as the results may depend on the strain of the examined animals.

Arginine emerged as one of the interesting metabolites in our study. It was found to be significantly increased in ALN, and ALN+Vitamin D treated groups when compared with the OVX control group. Arginine is a semi-essential amino acid that is derived both from endogenous and dietary sources and considered as a critical building block to repair damaged tissue and bone(116). Arginine is required for the production of nitric oxide (117). The latter is an atypical messenger molecule biosynthesized from arginine and molecular oxygen by nitric oxide synthase. Nitric oxide has been reported to play an essential role as a potent inhibitor of osteoclastic bone resorption(118). Another study has also reported that arginine could potentially increase bone formation over bone resorption, and consequently, increase bone mass(119).

Our data revealed that arginine increased significantly after exposure to the ALN and ALN+Vitamin D treatments, whereas it decreased considerably in the OVX control group. However that reduction in arginine levels was not statistically significant. One possible explanation for these effects of vitamin D may relate to increasing nephrogenous cyclic AMP production, as previously reported (120). Additionally, there is prior evidence demonstrating that arginine is capable of stimulating IGF-1 production and collagen synthesis in osteoblast-like cells, which explains, in part, its direct impact on bone formation (121). Therefore, as a general rule, the greater the amount of arginine, the greater the level of bone formation. This might explain the lower quantities of arginine

measured in the plasma of ovariectomized rats. Evidence suggests that amino acids such arginine may benefit bone health. However, arginine may exert a detrimental effect on bone density when calcium is low (122)

The results of this study show that arginine significantly increased in the ALN and combo groups, compared with the control group. These results were consistent with previous studies that have been shown to affect femur bone mineral content in growing female rats. (123)In addition, we took the

opportunity to examine the relation between arginine and bone volume where the arginine markedly increased when bone volume increased in treated rats. These data suggest that arginine potentially could be used as an acceptable and safe natural supplement for treating osteoporosis.

Another exciting cluster of metabolites, as revealed by our MS-based metabolmic studies, was a group of phsophatidylcholine compounds (i.e., PC aa, and PC ae, where aa = diacyl and ae = acyl-alkyl). Glycerophospholipids occupied a large part of the distinctive metabolite differences between the treated group and the controls. They were typically increased in the OVX control group at 8 weeks.

Many studies have showed PCs as essential biomarkers of anti-osteoporosis(124), bone health and bone formation. (75,125) Furthermore, an elevated level of PCs is an indication of an overwhelmed antioxidant defense mechanism due to the generation of reactive oxygen species(126). Numerous studies showed a direct relationship between levels of glycerophospholipids and osteoporosis. Fukumoto et al. inferred that glycosphingolipids play an important role in the RANKL-induced osteoclastogenesis via lipid rafts(127). Another study interested by Moayyeri indicates that alterations in phospholipid composition and concentrations are associated with the development of OP. (128) OP has also been demonstrated to affect glycerophospholipids in the regulation of the signaling steps leading to neutrophil

activation(129). Several of the PCs were down-regulated after treatment with alendronate or the combination treatment. This suggests that the regulation of bone metabolism by alendronate or alendronate+vitamin D was associated with glycerophospholipid metabolism.

In this study, phosphatidylcholine diacyl (PC aa) C40: 2 ($P<0.01$), phosphatidylcholine acyl-alkyl PC ae C36: 2 ($P<0.011$), PC ae C34: 2 ($P<0.01$) were detected as the most significantly altered PC metabolites between control and treated rat groups.

Taken together, the literature supports the notion that glycerophospholipids can influence both osteoclasts and osteoblasts in bone. Our findings agree with these results, as we observed a clear change in glycerophospholipids metabolism. This may support the notion that glycerophospholipids play a vital regulatory role in the pathology process in osteoporosis and osteonecrosis(130).

Taurine (2-aminoethane sulphonic acid) is a sulfur-containing amino acid. Taurine is a sulfonic acid derived from cysteine but it does not contain the usual carboxylic acid groups and does not incorporate into the structure of proteins. Our results also showed taurine was higher in both alendronate-treated groups, despite not reaching statistical significance. In alignment with that, increased levels of taurine have been proven to have anti-osteopenic effect in low Ca diet-induced osteopenia in rats. Taurine was one of the metabolites that rose in the plasma of both ALN, ALN + vitamin D, and vitamin D treatment groups. Evidence from the literature indicates that increased taurine increases bone mineral density in rats(131).

Taurine is known to increase cell proliferation and generate an increase in [Mg2+] accompanied by ERK 1/2 activation in human osteoblast cells (132). This observation might explain the high concentration of taurine in our treated groups. Therefore, it is reasonable to propose that taurine may play an important role in bone metabolism. Interestingly, taurine has been found to enhance bone mass via a different mechanism. According to Koide et al., taurine was able to inhibit experimental bone resorption and osteoclast formation(131)via stimulatory actions on alkaline phosphatase activity and collagen synthesis(132). Consequently, it is not surprising to see taurine at a high level after treatment with alendronate, and as such, our result was consistent with that from the literature.

Other key findings from the plasma metabolomic studies were decreased levels of proline and hydroxyproline in the treated groups, which have been documented as potential biomarkers for osteoporosis and osteonecrosis. We measured increased proline, as well hydroxyproline (an oxidation product of proline) in OVX control rats, which can be expected seeing as the main organic protein component of bone tissue (namely collagen) is known to be significantly reduced in osteoporosis.

The collagen in bone is an abundant reservoir for proline, and it has been noted that matrix metalloproteinases degrading collagen in bone are activated by nutrient stress to make proline available, and the metabolism of proline generates electrons to produce ROS which in turn initiates a variety of downstream effects, including a blockade of the cell cycle, and apoptosis. (133,134)

Previous studies have shown that proline and hydroxyproline are involved in the osteonecrosis process. It has been proposed that, if there were a disruption in the metabolism of collagen or an increase in degradation of this protein, these changes might be reflected by increased levels of excretion of this amino acid in plasma or urine. (130,135)

Although our treatment significantly reduced the level of these amino acids, to what extent is still unknown. Accordingly, our results agree with previous findings that identified proline

and hydroxyproline as potentially interesting candidate biomarkers of osteonecrosis and osteoporosis. (130,136)

Methionine sulfoxide is one of the more interesting metabolites detected in the plasma of treatment groups that was significantly different compared to the control group. Methionine sulfoxide is an oxidation product of methionine.. Methionine is an amino acid that showed improvement in bone density and significantly decreased the degree of osteoclast development. (137) Reduction of methionine sulfoxide back to methionine is catalyzed by the enzyme methionine sulfoxide reductase. (138) It has been documented that oxidation of methionine in essential proteins could be a lethal alteration if there were no mechanism to repair the damage to these proteins. Therefore, the repair of this damage appears to be necessary for cells to survive in the presence of ROS. Consequently, methionine sulfoxide reductase plays a vital role in protecting cells against oxidative damage. (139)

DI-MS analysis of the plasma of the treated groups of rats showed decreased levels of methionine sulfoxide in the blood when compared with the control group. Reduced amounts of methionine sulfoxide in the plasma samples of the treated group with high levels of control could be related to protective effect by decreasing in producing of ROS in the cell. Taken together it is tempting to speculate that oxidative damage, which has been implicated in osteoporosis diseases, can be improved by anti osteoporotic drugs.

Another metabolite that was altered by our bisphosphonate treatment was serine. Our results showed a significant change in serine compared to the control group. Serine is required to activate the obligatory glycine binding site as a co-agonist. (140) Serine is also an essential component for maintaining normal osteoclast metabolism. That explains the higher concentration of serine in the control OVX groups. Moreover, decreased concentrations of serine in the treated groups may contribute to the measured treatment effect. Therefore, serine

metabolism caused by treatment may be responsible for inducing osteoclast apoptosis, as reported in previous studies. (137,141,142)

A decreased level of amino acid metabolites is indicative of the inhibitory effect of bisphosphonate on serine metabolism during osteoclast differentiation. Previous research has reported that amino acids could increase the activity of alkaline phosphatase, a key enzyme in osteoclast differentiation. (143)

In summary, our results revealed the potential role of BP and vitamin D treatment in the metabolic response to amino acids, which was consistent with previous findings that amino acids were related to the development of osteoporosis. (88,144)

4.4.Association between plasma metabolites and bone volume

This study was conducted to explore the associations between the impact of different metabolites on the bone volume of OVX rats. Our results indicate that there is a correlation between 5 metabolites (arginine, proline, trans-hydroxyproline, histamine and methionine in terms of the association between bone volumes with plasma metabolites

Proline is abundant in collagen and collagen fibers and it is believed to confer tensile strength to bone. Hydroxyproline is released from bone as collagen degrades. (145) The proline and hydroxyproline concentrations were significantly increased in the OVX control group in this study. Previous studies on the association between bone density and serum proline and hydroxyproline showed several inconsistencies. Some metabolomics studies have reported hydroxyproline concentrations decreased in the osteoporosis group compared with healthy group using GC-MS. (146,147) In contrast, other studies reported increased plasma hydroxyproline levels in ovariectomized rats. (88,148) This latter result is consistent with our

observation and supports the idea that proline and hydroxyproline may be potential biomarkers of osteoporosis.

Notably, one of the significantly positive correlated metabolites is methionine. Methionine is known to down regulate TLR4/MyD88/NF κB signaling in osteoclast (137,149)precursors to reduce bone loss during osteoporosis. (137) Diana Cabrera et al. (150)reported that low plasma levels of methionine are associated with low bone density, although these results are not statistically significant. However, this observation is consistent with our results. (150)

Arginine is a semi essential amino acid that is derived both from endogenous and dietary sources. Arginine is metabolized through several pathways of which protein synthesis and conversion to urea and ornithine by the enzyme arginase are the main ones. In the liver, catabolism of arginine through the hepatic urea cycle is isolated from the metabolism of arginine within the cytosolic free amino acid pool(151)

In our studies, we verified that arginine has the most potential to be used as an osteoporosis biomarker due to its significant correlations with bone volume, and high contribution in distinguishing the treated groups from the control groups calculated by the receiver operating characteristic curve (ROC). The arginine may be useful for osteoporosis prediction and diagnosis. As shown a more significant correlation with bone volume, which was complementary to results obtained from metabolomics data.

In rat plasma, arginine has been shown to affect femur bone mineral content in growing female rats and OVX rats; therefore, our results of this study are also consistent with the previous studies. (123,152,153)

Up to now, there has been no relationship reported between a bone volume and changes in histamine levels, however, it has been reported in one study with knockout mice (in whom the transformation of histidine into histamine was blocked) that the activity of osteoclasts was suppressed. These researchers claimed that histamine could be a cause of osteoporosis. (154)

Previous works were showing inhibition of the effect of histamine on trabecular bones. Histamine inhibited the uptake of proline and thymidine, which suggests that histamine could inhibit new bone synthesis in rat bones. (155)Moreover, the synthetic processes of thymidine and proline incorporation by bone in the presence of histamine might suggest an effect on the osteoblast (156). Histamine is also involved in the promotion of the inflammatory response of bone cells. Following activation, mast cells will synthesize and release prostaglandin D2 and leukotrienes which promote inflammation and are chemotactic for neutrophils and eosinophils.

Osteoclasts may also be recruited. It is assumed that mast cell degranulation will cause tissue damage and promote an uncoupling of bone resorption and formation as the rate of bone turnover is not increased. (155). This suggestion explained how histamine was significantly higher in control rats at week 8.

Not unexpectedly, the level of histamine was significantly higher in OVX rats treated with alendronate. The mechanism of alendronate induced inflammatory is uncertain. However, there is some evidence that alendronate may induce of inflammatory reaction by prolonged induction of the histamine-forming enzyme in rats. (157). This suggests that alendronate may affect the functions of mast cell type in certain situations. Furthermore, a mutual augmentation of inflammatory reactions is seen with OVX treated with alendronate compared to the control group. A very interesting finding was that Combo group (vitamin D +alendronate) could reduce histamine level in OVX rats. However, In terms of this protective effect, it is not clear

how vitamin D inhibits the inflammatory actions of alendronate. Thus, studying the pharmacological kinetics may help clarify the mechanisms underlying the protective effect of vitamin D.

Our findings suggest that plasma proline; trans-hydroxyproline, arginine methionine, and histamine levels are potential biomarkers of osteoporosis and could be used as indirect, quick and potentially quantitative measures of bone loss.

CHAPTER 5: CONCLUSION

Metabolomics has paved the way for an upsurge in new and unexpected biomedical discoveries, ranging from the metabolic causes of cancer to the dietary causes of atherosclerosis. The impact of metabolomics can also be seen in the field of osteoporosis research. This metabolomics study focused on the identification of key metabolites involved in osteoporosis conditions. The animal models used in this experimental study were osteoporotic rats, which among other models, are conventionally used for osteoporosis research.

Results of this study reinforced the significant role that bisphosphonates can play to prevent bone density loss and other bone related diseases. It was found out that bisphosphonate is a potent anti-osteoporosis therapy effectively that alters the regulation of amino acid and lipid metabolism involved in the crucial processes such as bone resorption and bone formation. Marked changes in the plasma metabolic profile preceded changes in known osteoporosis biomarkers. As suggested by DI-LC-MS/MS profiling, a number of endogenous metabolites changed significantly. This work may pave the way for further metabolic investigations of the pathology of osteoporosis, the metabolic mechanisms of osteoporosis, and even the potential to identify novel targets for anti-osteoporosis drug research.

The findings of this study indicate that the utility of metabolomics profiling as a medical research tool holds great promise for elucidating both biological activity and toxicity of bisphosphonates. While the use of a limited (180 metabolite) targeted metabolomic approach employed in this study limited the discovery of potentially more useful biomarkers or pathological mechanisms, it is possible that other metabolomic techniques such as NMR and GC-MS/MS techniques could be used to gain a more comprehensive understanding of the subject.

Likewise the small number of animals limited the statistical significance of the reported findings. It is highly recommended that larger-scale metabolomic studies be undertaken to investigate more completely the complex pathological mechanisms of bisphosphonates as far as osteoporotic research is concerned.

These larger studies would also help clarify and validate the proposed biomarkers and the proposed metabolic mechanisms suggested from this work.

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Appendix

In this thesis, we conducted all statistical analysis excluding vitamin D group due to the reasons mentioned before in the thesis.