Osteoporosis is a common metabolic bone disease, affecting a third of women and a fifth of men over age 65. In the US, annual health care costs associated with osteoporosis are estimated to be over $20 billion. Osteoporosis is associated with increased fracture risk, which has been demonstrated to predict mortality rates and nursing home admittance. Altered bone remodeling, defined by excessive bone resorption and/or impaired bone formation, is a major risk factor for osteoporotic fracture. In menopause-induced ovarian hormone deficiency, bone resorption exceeds formation. Menopause-induced bone resorption is associated with a reduced bone mineral density (BMD), and there is a relationship between low BMD and increased fracture risk. Given the importance of bone remodeling and fracture risk, there have been significant efforts to understand the ability of dietary components to slow bone loss.

Some observational studies report that fruit and vegetable consumption is associated with an increase in bone mineral content (BMC) and BMD. Dietary nitrates, present in high concentrations in leafy green vegetables, may serve as a dietary component that supports bone health. Vegetable intake accounts for ~80% of dietary nitrate consumption in the typical human diet. Dietary nitrates can be
reduced to nitric oxide (NO) via non-enzymatic reduction by lingual bacteria and a variety of mammalian reductases through the nitrate-nitrite-NO pathway. There is evidence suggesting that strategies to increase NO bioavailability using organic nitrates (i.e., nitroglycerin) as an NO donor may decrease bone turnover and loss in ovariectomized (OVX) animals, which are animals that have had their ovaries surgically removed to induce a postmenopausal-like state, defined by diminished ovarian production of female hormones, particularly estrogen and progesterone. Some follow up trials in postmenopausal women have demonstrated similar results. Since non-enzymatic reduction of dietary nitrate can account for up to 50% of NO production, this provides a biologically plausible link between dietary nitrate and bone health and suggests that dietary nitrate, as an alternate NO donor, may offer efficacious means of decreasing bone loss in postmenopausal women.

Here, using an in vivo approach, we examined the dose-response relationship between dietary nitrates and bone density, microarchitecture, and turnover in OVX rats. We show that dietary nitrates do not improve bone density, microarchitecture, or turnover. Further, dietary nitrate had no beneficial effect on mineral apposition rate, bone formation rate, or bone volume/tissue volume in OVX rats. Our novel findings demonstrate dietary nitrate does not slow ovx-induced bone loss. This knowledge clarified the relationship between a dietary compound found in large quantities in leafy greens and bone loss in an OVX model.

As an exploratory component of this study, we used 16S rRNA gene sequencing to profile the composition of fecal microbiota associated with sham surgical controls, OVX, and nitrate-treated groups to evaluate the ability of OVX or dietary nitrate to modulate the gut microbiome in OVX mammals. While dietary nitrate did not alter the gut microbiome in the context of OVX, we did observe gut microbiome changes in structure and composition associated with OVX status and identified specific phylotypes whose abundance stratify sham and OVX rats, including Proteobacteria, Firmicutes, and Bacteroidetes. These results add to the growing body of evidence that there is an association between the gut microbiome and sex steroid deficiency.
Our use of the OVX model also provided an important added opportunity to further our understanding of the tissue effects of sex steroid deficiency observed in menopause. While both progesterone and estrogen influence metabolism, most attention has been given to the effects of ovarian hormone deficiency. Complex interactions between estrogen and host metabolism suggest that tissues in addition to serum and urine must be examined to clarify the mechanistic underpinnings of ovarian hormone deficiency and associated disease risk. Insufficient data are available from tissue-based studies using NMR-based platforms in estrogen-deficient models. Metabolomic studies in OVX rats, a model for osteoporosis, and postmenopausal women have demonstrated major metabolic shifts in serum, plasma, and urine as a result of ovarian hormone deficiency. To our knowledge, metabolomics has not been used for investigating the metabolic phenotype observed in the postmenopausal colon. Metabolomic studies of ovarian hormone deficiency is limited to bone, adipose, skeletal muscle, serum, plasma, and urine. Further, while it is established that ovarian hormone deficiency causes metabolic dysregulation, and the gut is associated with metabolic diseases and bone metabolism, there is also limited information available on the effects of estrogen loss on both gut tissue and gut microbiota. In light of this and our observed gut microbiome differences in OVX rats, we evaluated the effects of OVX on the colon using a metabolomics approach. We found the OVX colon is characterized by elevated levels of antioxidants, such as taurine and hypotaurine, and osmolytes, such as glycerophospholine, glycine, and glutamate. These novel findings suggest alterations to the colonic transsulfuration and methylation pathways, and they are suggestive of hyperosmotic and oxidative stress in the OVX colon. Previous work has associated hyperosmotic and oxidative stress with inflammation and intestinal permeability. These processes may also be present in the OVX colon; however, we did not quantify either inflammation or intestinal permeability in the current body of work.

This dissertation takes advantage of a postmenopausal osteoporosis animal model and integrative techniques, such as osteologic, immunological, metabonomic, microbiomic and bioinformatic methods, to evaluate different metrics of
osteoporosis, including physiological determinants influencing the rate of bone loss and gut-associated changes in ovarian hormone deficiency. The central findings of this dissertation show that dietary nitrate does not slow bone loss in ovarian hormone deficiency-induced rat model of bone loss. Further, dietary nitrate does not appear to influence the gut microbiome in the context of OVX. This is important in furthering our understanding of the relationship between the consumption of this ubiquitous component of fruit and vegetables and bone health. We also demonstrate that OVX is associated with shifts in gut microbiome structure and composition. Finally, we show OVX influences the colon metabolic phenotype, inducing the upregulation of metabolites involved with hyperosmotic and oxidative stress. Since estrogen protects against oxidative stress in many other tissues, we hypothesize that ovarian hormone deficiency promotes oxidative and hyperosmotic stress in the colon, promoting intestinal permeability and elevating the inflammatory response. In response, the colonic transsulfuration and methylation pathways may adapt by upregulating osmolytes and antioxidants. However, the specific causative agents of oxidative stress cannot be derived from our current work. Further investigation of the role these metabolites and processes play in the development of inflammation and metabolic dysregulation will yield insights into the physiological effects of ovarian hormone deficiency in postmenopausal women, allowing for more informed diagnosis and treatment of susceptible individuals.
The Roles of Dietary Nitrate in Bone and the Gut in an Ovarian Hormone-deficient Rodent Model

by
Melissa N. Conley

A DISSERTATION

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APPROVED:

\underline{Major Professor, representing Nutrition}

\underline{Co-Director of the School of Biological and Population Health Sciences}

\underline{Dean of the Graduate School}

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Melissa N. Conley, Author
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I owe a debt of gratitude to the many remarkable people I have had the fortune of knowing both within and outside of graduate school, and without whom neither this dissertation nor the memorable experiences of graduate school would have been possible.

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Chapter 2: Melissa Conley, Thomas Sharpton, Urszula Iwaniec, and Norman Hord conceived and designed the experiments. Melissa Conley conducted or oversaw all experiments. Cooper Roberts and Ajay Machha prepared and analyzed samples for blood nitrate and nitrite analyses. Melissa Conley wrote the manuscript. Thomas Sharpton, Urszula Iwaniec, and Norman Hord edited the manuscript.

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Chapter 4: Melissa Conley wrote.
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Chapter 1

Introduction
Overview

Osteoporosis is a common metabolic bone disease, affecting one out every three women and one out of every five men over age 65 [1]. In the US, annual health care costs associated with osteoporosis are estimated to be over $20 billion. Osteoporosis is associated with increased fracture risk, which has been demonstrated to predict mortality rates and nursing home admittance. Altered bone remodeling, defined by excessive bone resorption and/or impaired bone formation, is a major risk factor for osteoporotic fracture. Menopause-induced ovarian hormone deficiency causes an increase in bone turnover [2]. Increased bone resorption prevailing over bone formation leads to reduced bone mass and quality [2]. This increased bone resorption is associated with a reduced bone mineral density (BMD), and there is a robust relationship between low BMD and increased fracture risk [3].

Estrogen plays a fundamental role in energy homeostasis. Estrogens influence energy balance by multiple mechanisms, such as controlling food intake and energy expenditure through the central nervous system, regulation of adipose tissue distribution, and dampening inflammatory processes [4]. Estrogen also plays a role in body fluid regulation [5–7]. Since estrogen is involved in the regulation of multiple tissues and metabolic pathways, clarifying the contribution of ovarian hormone deficiency to the pathophysiology of osteoporosis presents a critical challenge. Thus, it has been difficult to disentangle its specific contributions to osteoporosis. In order to address this issue, an improved understanding of the systemic and tissue-specific effects of ovarian hormone deficiency and the contribution of estrogens to osteoporosis is needed.
Animal models provide a controlled setting in which specific interactions within the host can be empirically explored. The most widely used models to study estrogen’s effects on the host have been rodents with ovariectomized-induced ovarian hormone deficiency, and estrogen receptor (ER) knockout models. These animal models of ovarian hormone deficiency demonstrate estrogen’s extensive actions across multiple tissues in health and disease. For instance, ovarian hormone deficiency is associated with dramatic shifts in lipid, amino acid, and glucose metabolism, and the promotion of metabolic dysfunction in liver, bone, and adipose tissue [8,9]. These studies have provided insight into mechanisms that contribute to the increased risks for osteoporosis and subsequent fractures, as well as increased risks for obesity, cardiovascular disease, and colorectal cancer in postmenopausal women [2,8,10,11]. The use of these models has also clarified the efficacy of specific effects of dietary compounds, such as fermented soybeans, dried plums, blueberries, as well as non-digestible oligosaccharides and certain probiotic bacteria, on the osteoporotic skeleton [12–17]. However, their application to the study of ovarian hormone deficiency on other host tissues is limited. Characterizing these relationships is useful given that estrogen affects are pleiotropic, that estrogen receptors are expressed throughout the body, and that recent evidence suggests estrogen plays a role in modulating inflammation and oxidative stress in multiple tissues, such as endothelium, kidney, eye, and liver [18].

Dietary factors like calcium and vitamin D are known to be important for bone health; however, these factors do not fully explain the associations between diet and osteoporosis risk [19]. A positive relationship between fruit and vegetable consumption and bone mineral content (BMC) and BMD has been reported in some observational studies of postmenopausal women [20–23]. Currently, we have an incomplete understanding of the mechanisms that
contribute to the beneficial effects of fruit and vegetables on bone in postmenopausal women. Thus, we addressed this dearth of information by utilizing the OVX model to determine whether dietary intervention with specific compounds could reduce bone loss. The use of this animal model provides an important added opportunity to further our understanding of the tissue effects of ovarian hormone deficiency. Our systemic understanding of ovarian hormone deficiency is limited to bone, adipose, skeletal muscle, serum, plasma, and urine. Further, while we understand that ovarian hormone deficiency causes metabolic dysregulation and that the gut is associated with metabolic diseases and bone metabolism, there is limited information on the effects of estrogen loss on the gut. This provides a rationale for investigation of the estrogen-deficient gut with a global, descriptive approach. The goal of this aspect of dissertation research was to establish a role for estrogen in metabolic pathways in colon tissues beyond what is currently known. A deeper understanding of the systemic adaptions to ovarian hormone deficiency will guide future research and ultimately allow for more informed diagnosis and treatment for susceptible individuals.

This dissertation takes advantage of the OVX rat model of estrogen-deficiency induced bone loss and an array of integrative techniques, such as osteologic, immunological, metabonomic, microbiomic and bioinformatic methods, to evaluate different metrics of osteoporosis, including physiological determinants influencing the rate of bone loss and gut-associated changes in this preclinical model. The overall goals of this dissertation are to further understand the complex interactions between ovarian hormone deficiency, osteoporosis, and potential dietary interventions to reduce risk of this condition. This was accomplished by first establishing the effects of dietary nitrate, a compound present in large quantities in leafy greens, on bone in the preclinical rat model of ovarian hormone deficiency-
induced bone loss. Secondly, we characterized gut microbiome-associated changes in rats rendered estrogen deficient via surgical ovariectomy and in response to dietary nitrate treatment. Finally, we investigated colonic metabolic profiles in the OVX model to assess changes to the colon in a rat model of ovarian hormone deficiency. The following aims were completed to accomplish these ends.

Aim 1. Evaluate the efficacy of dietary nitrate to slow bone loss in the OVX rat model of postmenopausal bone loss.
Aim 2. Characterize gut microbiome-associated changes in OVX and dietary nitrate treatment.
Aim 3. Characterize colonic metabolic phenotype of sham and OVX rats to identify a potential role of the colon in metabolic dysregulation observed in menopause.

The outcomes of these studies clarify the role of dietary nitrate in slowing bone loss in the OVX preclinical model, as well as provide novel information on the role of the gut microbiome and colon in the OVX preclinical model. The following sections clarify what is known about these topics and provide rationale for accomplishing these aims.

**Dietary components and bone metabolism**

There are many factors that can influence bone health, and understanding their respective roles allows for a more informed prescription for health. Of particular interest is the role of dietary components in slowing bone loss in ovarian hormone deficiency-induced bone loss. The ubiquitous advice to “eat your fruit and vegetables” is sensible; what remains unknown, however, are the recommended portions necessary to achieve optimal bone health. Epidemiologic evidence and clinical interventions have demonstrated
consistent beneficial effects of fruit and vegetable intake on indices of bone health across different age groups for both boys and girls [22], premenopausal women [21,23,24], postmenopausal women [25], and the elderly [20].

Observational research does not establish causality, but interest is growing in potential mechanisms involving dietary components, such as the alkalizing effect of fruit and vegetables on acid-base balance. Furthermore, the necessary role of bone in acid-base homeostasis, acting as a source of buffer in the preservation of the body’s pH and protection against acid-base disorders, has been known for over 30 years. Natural, pathologic, and experimental states of acidosis are associated with hypercalciuria and negative calcium balance, and negative effects of an acidic environment on BMD have been demonstrated [26,27]. It has been shown that administration of a base (i.e., potassium bicarbonate) results in a decrease in the urinary excretion of calcium and phosphorous, which was accompanied by decreased bone resorption and increased bone formation [28]. Fruit and vegetables are high in precursors of bicarbonate ions that function to buffer acids in the body. When bicarbonate ions are not available to maintain normal pH, the host is able to mobilize alkaline calcium salts from the bone to neutralize acids consumed from diet and produced by metabolism.

While the alkalizing effect of fruit and vegetable consumption has gained attention as a possible link to their positive influence on bone, it is important to highlight other plausible mechanisms, including phytoestrogens, vitamin K, and other potential dietary components acting alone or synergistically. Previous data have demonstrated that vegetables, salads, and herbs have an impact on bone resorption by an unknown mechanism not mediated by base excess [29]. A recent randomized, placebo-controlled
trial in 276 postmenopausal women supplementing their diet with base, defined by potassium citrate or 300 g/day of fruit and vegetables, did not increase BMD or reduce bone loss over two years [30]. This supports the suggestion that dietary factors other than base excess contribute to the benefit of fruit and vegetable intake on bone indices. The recent Dietary Approaches to Stop Hypertension (DASH)-Sodium trial demonstrated the impact of two dietary patterns on indices of bone metabolism. The DASH diet emphasizes fruit, vegetables, low-fat dairy products, and limited red meat intake. The control diet consisted of a typical Western, high-fat diet, low in dairy, fruit, and vegetables. The DASH diet, compared to the control diet, was found to significantly reduce bone turnover and urinary calcium excretion [31]. This is an important intervention study that shows a clear benefit of increasing fruit and vegetable intake from three to nine servings per day on markers of bone metabolism. The Aberdeen Prospective Osteoporosis Screening Study (APOSS) has demonstrated specific associations between nutrients commonly found in fruit and vegetables and bone mass and resorption markers. Women (45-49 years old) in the lowest quartile of potassium, magnesium, vitamin C, ß-carotene, and fiber intake were found to have significantly lower lumbar and femoral neck BMD compared to the highest quartile [24]. Furthermore, APOSS has displayed a longitudinal benefit of fruit and vegetables in bone loss during menopause transition [23]. Taken together, the evidence suggests a diet rich in fruit and vegetables can help reduce further bone loss, although the specific compounds responsible are not entirely known.

Fruit and vegetable intervention studies in humans present a unique challenge, as they are complicated by the presence of multiple dietary components. As a result, most intervention studies are accomplished using compounds isolated from fruit and vegetables. It is critical to investigate the
types of fruit and vegetables that have the most direct impact on the skeleton and what quantities are necessary for a maximum effect on bone metabolism. Dietary nitrates that are present in high concentrations in leafy, green vegetables are another component of vegetables and fruit that may serve such a function. Dietary nitrates can be reduced to nitric oxide (NO) via non-enzymatic reduction by lingual bacteria and a variety of mammalian reductases through the nitrate-nitrite-NO pathway [32,33]. NO is a free radical gas that plays extensive roles in physiological processes ranging from vascular homeostasis to host defense, cellular energetics, and nerve transmission [34]. There are two major sources of NO in the body: the endogenous L-arginine-NOS system and our diet (Figure 1). Aging is associated with decreased eNOS-dependent NO synthesis and endothelium-dependent vasodilation [35]. This suggests a role for NO donors, such as dietary nitrate or organic nitrates, to support NO production in age-related conditions, such as atherosclerosis and osteoporosis [36].

The relationship between NO and bone physiology is complex. Both osteoclasts (OCs) and osteoblasts (OBs) constitutively express the endothelial isoform of nitric oxide synthase (eNOS), implying a role for NO in bone metabolism. Exogenous nitrates, such as nitroglycerin, may influence bone cells indirectly as NO donors, inducing local vasodilation. The molecular targets for NO action in bone cells are poorly understood. In vitro work suggests that NO has biphasic effects on both OCs and OBs, demonstrating the ability to both stimulate and inhibit cell activity depending on the amount of NO present, as well as which NOS isoform is producing NO [37–39]. Nitrates may also have direct action on OCs and OBs. NO may influence OC activity, in part, via the receptor activator of NF-kappaB ligand (RANKL)/osteoprotegerin (OPG) pathway. High levels of NO stimulate OPG, which binds to RANKL and prevents the binding of RANKL to the receptor.
activator of NF-kappaB (RANK), decreasing OC activity [40]. eNOS-knockout animals demonstrate lower BMD, bone formation, and OB activity, with little to no effect on bone resorption, suggesting NO may be important for OB function [41,42]. Previous research shows that NO synthesis is induced in OB and osteocytes by mechanical strain and shear stress [43–46]. This dissertation addresses the effect of dietary nitrate, as an NO donor, on bone, as vegetables constitute ~80% of daily nitrate intake [47]. Notably, one study ascribed estrogenic activity to dietary nitrate in an in vitro transcription assay [48]. However, the strongest data associating nitrate intake with positive effects on bone health are from studies of organic nitrate administration in both OVX models and postmenopausal women.

Organic nitrates have been used as a therapeutic agent in treating angina pectoris and other cardiovascular disorders for over 100 years, while the oldest document account of using nitrate for angina dates back to the 8th century [49,50]. More recently, it was found that postmenopausal women using organic nitrates had improved BMD compared to non-nitrate users [51]. This led to the original hypothesis that age-associated decreases in NO production may be related to osteoporosis risk and that organic nitrates may slow bone loss. In vivo experiments demonstrate that the main effect of NO on bone metabolism occurs through OC inhibition. Studies in OVX rats have demonstrated there is equivalent efficacy of the NO donor nitroglycerin (NG) compared to a standard dose of estrogen in the prevention of bone loss [52–54]. Follow up human clinical trials have led to conflicting results. A pilot study evaluating the effects of nitroglycerine ointment compared to oral estrogen in oophorectomized women demonstrated that NG’s efficacy in preserving BMD was equivalent to a standard estrogen dose. Furthermore, NG significantly decreased NTx (bone resorption) and increased serum osteocalcin and serum bone specific alkaline phosphatase (BSAP) (bone
A randomized controlled trial conducted in postmenopausal women reported equivalent beneficial effects on lumbar BMD after 12 months of treatment with isosorbide mononitrate (ISMO) (20 mg/day) or alendronate (70 mg/week) [56]. A more recent randomized controlled trial comparing placebo and ISMO on markers of bone turnover in postmenopausal women found that compared with placebo, women randomized to ISMO at either 5 mg or 20 mg per day had a significant decrease in NTx (bone resorption) and increase in BSAP (bone formation) [57]. While pilot studies suggest a beneficial effect, the Phase III trial, NOVEL (Nitroglycerin as an Option: Value in Early Bone Loss) found no significant differences in lumbar spine BMD between the NTG and control group after two years of intervention. The authors suggest suboptimal dosing, non-compliance and side-effects were contributing factors to the null findings [55,56,58]. As a result, evaluation of the efficacy of alternative exogenous sources of NO, via dietary sources of nitrate, to slow bone loss is warranted.

The available evidence provides biological plausibility that dietary nitrate may act via the nitrate-nitrite-NO pathway to restore age-associated decreases in NO production that may be related to osteoporosis risk. Since dietary nitrate accounts for up to 50% of its NO production [59], this dietary compound may be a viable option for reducing bone loss. Thus, the first aim of this dissertation was to address the putative role of dietary nitrate in reducing bone loss in the ovariectomized rat model for postmenopausal osteoporosis by testing the novel hypothesis that dietary nitrates improve bone quality and bone mass by reducing bone turnover.

**Metabolomics approach to studying systems**

Recognizing the complex and interactive nature of the pathways underlying the function of organisms, researchers are increasingly using
system's biology approaches to advance the understanding of how multicellular biological systems operate in their environment. Traditional scientific methods evaluate particular genes, proteins, or substrates, while ‘-omics’ techniques examine the entire system in question, yielding a number of endpoints. The data are analyzed by looking for trends and patterns. This approach leads to a difference in how the scientific question is constructed. In the traditional approach, scientists use hypothesis-led strategies to examine one factor at a time, and subsequent experiments test that particular hypothesis. In systems biology, data are often collected without a pre-existing hypothesis. The trends and patterns seen post-analysis are used to generate a novel hypothesis that is then tested using suitable experimental methods. Occasionally, a single marker will provide an adequate measure of a disease endpoint. However, most diseases are polygenic in origin and conditionally linked to environmental influences (i.e., cardiovascular disease, osteoporosis). Accordingly, a complex fingerprint of molecular markers is often associated with a disease.

Emergence of the ‘-omics’ technologies was facilitated by advances in both high-throughput technologies, such as microarrays, allowing for a large number of biological endpoints to be measured simultaneously, concomitant with advances in computational power and mathematical modeling to interpret the massive datasets this approach produces. Systems-based analyses applying the ‘omics’ approach have mostly focused on other aspects of host biology, such as the genome, transcriptome, and proteome. Recently, evaluating metabolite profiles of the metabolome has received more attention. The metabolome refers to the complete set of low molecular weight metabolites in a given organism. As it represents the endpoint of biological processes, it may be most closely related to phenotype and function. While the genome is considered the ultimate potential of the
organism being investigated, exploring the information further downstream in the hierarchy can reveal what actually is happening. Identification of patterns of similarities in health or disease states, known as metabolic fingerprinting, provides a better understanding of underlying biochemical process without a priori knowledge of these processes in hopes of revealing potential therapeutic targets in disease.

The most commonly used analytical platforms for detection of these low molecular weight metabolites are mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR), the latter having the advantage that a separation step is not required and, therefore, less sample preparation is required. The principal techniques used in metabolomics for sample analysis are those of high frequency nuclear magnetic resonance spectroscopy ('H-NMR). NMR detects and characterizes thousands of endogenous molecules simultaneously. Mass spectrometry (MS), which is more sensitive than NMR but gives poorer pattern quantification, is also used. MS generally requires a separation step using either gas chromatography (GC), liquid chromatography (LC), or chemical derivization before the analysis stage. High performance liquid chromatography (HPLC) and optical spectroscopic techniques are other procedures with similar properties.

Metabolomic analyses have successfully clarified mechanistic relationships in disease, as revealed metabolic fingerprints associated with specific disease states, such as obesity and osteoporosis (cite). Metabolomic studies in ovariectomized (OVX) rats, a model for ovarian hormone deficiency induced osteoporosis, and postmenopausal women have demonstrated major metabolic shifts in serum, plasma, and adipose tissue in response to ovarian hormone deficiency. Menopause-associated
ovarian hormone deficiency has several well-established metabolic consequences. Menopause promotes atherosclerosis through alterations in lipid profiles, endothelial dysfunction, and oxidative stress [60,61]. Hormonal changes in menopause promote higher body fat and contribute to the increased CVD and metabolic risk [62,63]. In support, metabolomic studies reveal changes in lipid and amino acid metabolism associated with metabolic dysregulation.

Serum metabolomics studies reveal OVX rats have prominent changes in lipid metabolism associated with both obesity and inflammatory processes [64–67]. Specifically, changes in polyunsaturated fatty acids (PUFAs) (increased n-6 fatty acids and reduced n-3 fatty acids), cholesterol (increased), and low-density lipoproteins (LDL) are observed in OVX serum. PUFAs are precursors to potent lipid mediator signaling molecules and eicosanoids, and regulate inflammation [68]. Generally, n-6 PUFAs are pro-inflammatory, while n-3 PUFAs are anti-inflammatory [69,70]. Increased ratios of n-6 PUFA to n-3 PUFA are consistently associated with chronic, inflammatory diseases, such as obesity, cardiovascular disease (CVD), non-alcoholic fatty liver disease, and inflammatory bowel disease, for which postmenopausal women have an elevated risk [68,71–73]. This suggests that ovarian hormone deficiency-induced inflammation is, in part, driven by shifts in PUFA composition. Increased cholesterol and LDL are major risk factors for CVD, and postmenopausal women have increased cholesterol and LDL levels, as well as increased risk for CVD [62]. Further, visceral fat accumulation, particularly abdominal fat, increases in women after menopause. There has been one metabolomics study evaluating visceral fat composition in post-menopausal women that showed altered fatty acid metabolite profiles compared to pre-menopausal women [9]. However, it remains to be shown if these data are associated with the development of
metabolic syndrome that occurs in postmenopause. A large-scale study of over 26,000 persons evaluating the effects of menopause on serum metabolites demonstrated that menopause is associated with shifts in cholesterol and fatty acid concentrations, and a pattern of pro-atherogenic lipid changes [74]. These data support metabolic shifts in lipid metabolism during menopause that may contribute to the metabolic phenotype of obesity, as well as increased risk for CVD.

Ovarian hormone deficiency also alters amino acid metabolism. A consistent finding in OVX is increased serum levels of the branched-chain amino acids (BCAA’s) leucine, isoleucine, and valine [66,67]. The relationship between obesity and elevated BCAA’s has been observed for many years [75] and is attributed to lower levels of fatty acid oxidation and decreased energy expenditure. In OVX, higher urine concentrations of glycine and hippurate and lower levels of taurine, glutamate, and alanine are observed [64]. Antioxidants, such as taurine and glutathione, are upregulated to counter oxidative stress, as commonly seen in ovarian hormone deficiency [76,77]. This may explain the decreased urine levels of taurine and glutamate, the precursor to glutathione, in OVX. Altered amino acid metabolism in ovarian hormone deficiency has also been shown in humans [74]. The aforementioned large-scale study consistently showed altered amino acid metabolism, with postmenopausal women having higher glutamine, tyrosine, glycine, and isoleucine concentrations than premenopausal women. Both the BCAAs and the aromatic amino acids tyrosine and phenylalanine have been linked to insulin resistance and the development of type 2 diabetes in an obesity-dependent manner [78,79]. Further, postmenopausal women on hormone replacement therapy (HRT) had more similar metabolic profiles to premenopausal women, suggesting estrogen is a main driver in the metabolic changes observed [74]. The amino acids shifts observed in ovarian
hormone deficiency are consistent in both OVX models and humans. These observed changes are associated with the hallmarks of menopause, such as reduced energy expenditure, obesity, and oxidative stress. Thus, menopause may contribute to the risk for reduced energy expenditure, obesity, and oxidative stress by affecting amino acid levels, in addition to lipid changes.

These findings support the well established metabolic dysregulation observed in ovarian hormone deficiency, characterized by reduced energy expenditure, weight gain, dyslipidemia, oxidative stress, and inflammation. Available studies have mostly evaluated serum and plasma, likely due to cost, convenience, and efforts to assess global changes in ovarian hormone deficiency. However, estrogen plays an active role in dampening inflammatory responses in multiple tissues, and deficiency induces oxidative stress in many tissues, including endothelium, liver, eye, kidney, and heart [60,61,80–85]. Additionally, complex interactions between estrogen and host metabolism suggest that tissues in addition to serum, plasma, and urine must be examined to clarify the mechanistic underpinnings of ovarian hormone deficiency and associated disease risk. Insufficient data are available from tissue-based studies using NMR-based platforms in estrogen-deficient models. Our understanding of ovarian hormone deficiency is limited to bone, adipose, skeletal muscle, serum, plasma, and urine. While estrogen has been suggested to play a protective role in the colon, the physiological role of estrogen in the colon is unknown [86,87]. Further, while we understand that ovarian hormone deficiency causes metabolic dysregulation and that the gut is associated with metabolic diseases and bone metabolism, there is also limited information on the effects of estrogen loss on the gut [88–91]. A deeper understanding of the systemic adaptations to ovarian hormone deficiency will allow for more informed diagnosis and treatment for susceptible individuals. To our knowledge, metabolomics have not been
used for investigating the metabolic phenotype observed in the estrogen-deficient colon.

**Gut microbiome and bone metabolism**

One factor within the colon, the host gut microbiome, has been suggested to be an important determinant of human susceptibility to several age-related conditions, including metabolic syndrome, cancer, and osteoporosis. The gut microbiome constitutes trillions of bacteria that collectively contain more genes than the human genome [92]. The infant microbiome is largely determined by manner of birth and early environmental exposures [93,94]. The gut of the human infant is considered highly dynamic and unstable and exhibits a low diversity of microorganisms [95]. During early human development (i.e., infancy to three years of age), there is relatively rapid diversification of the gut microbiome [94]. Around three years of age, it stabilizes toward an adult-like structure [95]. During this time, the neonatal immune system quickly matures under the influence of the gut microbiome and environmental factors, such as diet, infections, and antibiotics. There are age-associated changes in the gut microbiome, however it is extremely variable between older persons [96,97].

The gut microbiota form a complex and dynamic ecosystem that constantly interacts with host metabolism, facilitating the absorption of complex carbohydrates and influencing amino acid homeostasis, as well as protecting from invading pathogens. The gut microbiome composition is modifiable by environmental factors, such as diet, antibiotics, and pollutants. Changes in gut microbiota and the metabolites they produce can be either beneficial or harmful to the host and have been shown to influence gut permeability, the immune system, and disease progression. During
symbiosis, gut microbiota protect the host from invading bacteria and other pathogens by supporting immune and epithelial processes, also known as colonization resistance. Perturbations to the gut microbiome, called dysbiosis, caused by pathogens, diet, or antibiotics can induce inflammation, increased intestinal permeability, and tissue damage that may contribute to the development of disease. Changes in the gut microbiome may also contribute to inflammation through the mediation of nutrient metabolism and immunity [98,99]. This may include shifts in bacterial metabolites and in the production of inflammatory mediators influencing local and systemic processes such as gut permeability and immune function. This may, in turn, increase the host’s susceptibility to inflammation and related chronic diseases [100]. The gut microbiome presents a massive source of potential antigens for the host’s immune system to manage. Given the immune system’s ability to sense commensal microbiota and trigger various immune-related responses, it is plausible that gut microbiome changes influence immune-related disorders [101,102]. Dietary changes, antibiotics, and pathogens can also shift the composition of the gut microbiome and thus disturb the balance in metabolic and immune regulatory networks that normally control intestinal inflammation [103–105]. Gnotobiotic animals have provided insight into the effects of the gut microbiome on the immune system and other tissues. Germ-free (GF) animals have immature mucosal immune systems and poorly developed gut-associated lymphoid tissue (GALT). GF mice also have a reduced number of CD4+ cells in the spleen, as well as smaller and fewer germinal centers within the spleen. This suggests the gut microbiome is able to shape systemic immunity [102].

Bone remodeling is a coordinated process by OBs (bone formation) and OCs (bone resorption). Beyond structural support, bone serves as a reservoir for mesenchymal and hematopoietic progenitor cells. OBs are
derived from pluripotent mesenchymal stromal cells, while OCs are derived from hematopoietic stem cells that also produce immune cells. As such, OC respond and produce many of the cytokines that regulate monocytes and macrophage activities. It is now known that many of the major pro-inflammatory cytokines, such as TNF, IL-6 and IL-1, regulate OC differentiation and activity and subsequent bone resorption. Thus, the role of inflammation in bone health is a major area of research and examples of inflammation and bone loss have been well established. For instance, in autoimmune diseases like rheumatoid arthritis, bone resorption by OCs is driven by pro-inflammatory cytokines produced by activated T cells [106]. Menopause-induced ovarian hormone deficiency causes increased OC formation and extended survival [2]. This is due to many factors, including loss of the immunosuppressive effects of estrogen, resulting in increased production of cytokines promoting osteoclastogenesis, and direct effects of estrogen on OCs [107,108]. Several studies show low-grade inflammation affects bone turnover and plays a role in bone conditions like osteoporosis. Moderately elevated serum levels of high-sensitivity C-reactive protein (hsCRP), as a marker of low-grade systemic inflammation, are associated with low BMD, higher bone resorption, bone loss, and fracture risk [109–112]. Inhibition of inflammatory cytokines tumor necrosis factor alpha (TNFα) and interleukin 1 (IL-1) consistently causes a decrease in bone resorption markers in postmenopausal women [113]. Further, mice depleted of T cells in vivo by treatment with anti-CD4 and anti-CD8 antibodies are protected against OVX-induced bone loss [114]. The authors demonstrate higher levels of TNF-producing T cells in the bone marrow of OVX mice, further implicating a role of T cells and T cell-produced cytokines in bone turnover [115]. In sum, bone remodeling imbalances are associated with immune status and may lead to bone loss and osteoporosis.
The immune system’s interactions in the regulation of bone remodeling, as well as with the gut microbiome suggest that the gut microbiome may affect bone metabolism by influencing immune status. There is some evidence that the gut microbiome influences bone metabolism in rodents. This relationship has largely been explored in three different contexts: GF conditions, after treatment of antibiotics, and after ingestion of pre- and probiotics. It was recently shown that GF mice have both increased cancellous and cortical bone mass compared to conventionally raised mice [116]. The trabecular bone volume/tissue volume (BV/TV, %) increased by 39% in the distal femur of GF mice compared to conventionally raised. There was also a decreased number of OCs present in GF bone, and GF bone marrow had a decreased incidence of CD4+ T cells and OC precursor cells. Contrastingly, there were no changes in bone formation observed in GF mice. This suggests that the observed increase in bone mass in GF mice is caused by both diminished bone resorption and osteoclastogenesis. GF mice also have reduced expression in bone of pro-inflammatory cytokines involved in osteolysis, such as IL-6 and TNF-alpha, suggesting that decreased osteoclastogenesis is caused through immune-mediated mechanisms [116]. Of interest, colonization of mice born in a GF environment with a conventional mouse microbiome at three weeks of age normalize both trabecular and cortical bone mass, as well as CD4+ T cells and OC precursors in bone marrow.

A recent study investigated the role of the gut microbiome in bone loss caused by sex-steroid deficiency. Both GF and conventional mice were treated with Leuprolide, a gonadotropin-releasing hormone agonist that inhibits sex-steroid production and is used to mimic OVX. Leuprolide treatment caused more trabecular and cortical bone loss in conventional mice compared to GF, establishing that the gut microbiome plays a role in
bone loss observed in sex-steroid deficiency [114]. Further, Leuprolide treatment increased serum levels of the bone resorption marker CTx in conventional mice but not in GF mice. This indicates that GF mice are protected from increased bone resorption that occurs in sex-steroid deficient mice. Leuprolide treatment also resulted in elevated levels of TNF-alpha+, CD4+, TNF-alpha+, and CD8+ T cells in conventional bone marrow but not in GF mice. This suggests that the gut microbiome is necessary to shape the immune system during sex-steroid deficiency. Taken together, it appears the gut microbiome plays a role in inducing bone loss and increasing bone turnover in sex-steroid deficiency by providing antigens required for bone marrow T cell expansion and increased TNF-alpha production. Thus, it has been speculated that the gut microbiome composition may influence the magnitude of bone loss in postmenopausal women. This may be caused by an increased inflammatory status due to altered gut microbiome composition, resulting in greater bone loss in postmenopausal women when they lose the immunosuppressive effects of estrogen. It has been proposed that the most probable mechanism by which gut microbiota affect bone mass involves systemic and bone marrow immune status, which subsequently regulates osteoclastogenesis [117].

Further supporting a role of the gut microbiome in bone mass regulation, a study demonstrated that antibiotic treatment at weaning increases bone mass in mice after three weeks of treatment [118]. A follow-up study showed that low-dose penicillin provided from birth or weaning resulted in increased BMD in adult female mice [104]. Tetracycline treatment has also been reported to prevent OVX-induced bone loss, and this was partially due to diminished bone resorption, as similarly described in sex-steroid deficient GF mice [119]. These data show that antibiotics have the
capability of influencing the gut microbiome and bone mass and support the notion that the gut microbiome plays a role in bone metabolism.

**Prebiotics, probiotics and bone mass**

The common use of antibiotics has raised interest in using treatments like prebiotics and probiotics to improve health status by supporting the indigenous bacteria potentially altered as a result of antibiotic treatment. Probiotics are defined as live, commensal microorganisms, such as bacteria, that when administered in adequate amounts can confer a health benefit on the host, including improved intestinal function and maintenance of the integrity of the intestinal lining [120]. Probiotics have also been suggested to positively affect immune responses in the gastrointestinal tract. Several strains of probiotics are currently being examined for use as a treatment of inflammatory gastrointestinal diseases [121]. Prebiotics are non-digestible food components, such as dietary fiber and oligosaccharides, that promote the growth and activity of gut microbiota [122]. Prebiotics have been suggested to confer beneficial effects through multiple mechanisms, including increased solubility and absorption of minerals, and enhanced anti-inflammatory pathways [123]. A study evaluating the effects of probiotics on bone mass in chickens showed that treatment with a diet supplemented with probiotic strains for six weeks increased tibial bone mass [124]. In light of this work and the previous studies showing the involvement of the gut microbiome in modulating bone loss in sex-steroid deficient female mice, it was suggested that probiotic treatment might protect OVX mice from bone loss [119,125]. Indeed, follow-up studies have demonstrated probiotics protect OVX mice from OVX-induced bone loss, elevated bone resorption, and reduced expression of the pro-inflammatory cytokines TNF-alpha and IL-1beta in bone [126]. Probiotic treatment also appears to suppress OVX-induced increase in bone marrow CD4+ T cells, further supporting the
modulation of immune status of bone by gut microbiota [17]. In sum, it appears that probiotic treatment has positive effects on bone by supporting immune status, which subsequently diminishes bone resorption.

The ability of prebiotics to modulate the gut microbiome is well documented. The effect of prebiotic interventions on bone mass and the composition of the gut microbiome has been examined in both rats and humans. In rats, galactooligosaccharide (GOS) treatment changed the composition of the gut microbiome and increased bone mass [127]. In adolescent girls, GOS treatment for three weeks improved calcium absorption and resulted in elevated proportions of bifidobacteria [128]. Further, a prebiotic mixture of short- and long-chain inulin-type fructans provided as a daily supplement to young adolescents for one year improved bone mineralization and resulted in a greater increase in BMD compared to controls [129]. This observation may be due to improved calcium absorption.

Collectively, this evidence suggests that the gut microbiome plays a regulatory role in bone mass and that the inhibitory effect of the gut microbiome on bone mass is mediated through immune system-related events. Studies demonstrating that antibiotics, prebiotics, and probiotics all play a role in bone metabolism further support a role for the gut microbiome in bone metabolism. The gut microbiome has been suggested as a novel therapeutic target for osteoporosis, as well as a potential biomarker for fracture risk prediction. However, limited data are available regarding alterations in the gut microbiome in OVX. Additional research is needed to determine the effects of menopause on the gut microbiome, as well as potential therapies that modulate the gut microbiome and bone mass and associated fracture risk in osteoporosis.
This evidence led us to evaluate the gut microbiome of our OVX rats, as well as alterations of the gut microbiome by a dietary intervention with dietary nitrate. We found that there was no effect of dietary nitrate treatment on the gut microbiome in our study, but we did observe differences in the gut microbiomes between sham and OVX rats. This motivated us to further explore this relationship by looking at potential determinants of the effects of ovarian hormone deficiency within the colon, where a large number of indigenous bacteria live. Thus, the second and third aims of this dissertation were to i) evaluate potential gut microbiome changes associated with OVX and dietary nitrate treatment, and ii) evaluate the effects of OVX on colon metabolism.

**Summary**

Osteoporosis is a common metabolic bone disease, affecting one out every three women and one out of every five men over age 65 in the United States [1]. This is a major public health burden, as annual health care costs associated with osteoporosis are estimated to be over $20 billion. Menopause is the primary cause of osteoporosis in women. Therefore, there have been considerable efforts to develop strategies, such as dietary interventions, to slow the rate of bone loss in postmenopausal women. A positive relationship between fruit and vegetable consumption and bone mineral content (BMC) and BMD has been reported in some observational studies of postmenopausal women [20–23]. However, we currently have an incomplete understanding of the mechanisms that contribute to the beneficial effects of fruit and vegetables on bone in postmenopausal women. Animal models are valuable to evaluate the effects of dietary components on the skeleton in ovarian hormone deficiency. Previous work demonstrates the efficacy of the NO donor, organic nitrates, to slow bone loss in the OVX rat model. The ability of another NO donor, dietary nitrate, to benefit the
osteoporotic skeleton is unknown. Thus, we addressed this dearth of information by utilizing the OVX rat model to determine whether dietary intervention with nitrate could reduce bone loss. The use of this animal model provides an important opportunity to further our understanding of the tissue effects of ovarian hormone deficiency. While ovarian hormone deficiency has been shown to have deleterious effects on many tissues and organs, the potential effects on the gut remain to be fully defined. A deeper understanding of the systemic adaption to ovarian hormone deficiency will allow for more informed diagnosis and treatment for susceptible individuals.

The overall goals of this dissertation were to further understand the complex relationship between ovarian hormone deficiency and osteoporosis and to determine if dietary interventions have the potential to reduce the risk for osteoporosis. This dissertation takes advantage of an OVX rat model of estrogen-deficiency induced bone loss and an array of integrative techniques to evaluate different metrics of osteoporosis, including physiological determinants influencing the rate of bone loss and gut-associated changes in this preclinical model. In this dissertation, we clarified the effects of dietary nitrate, a biologically active compound present in large quantities in leafy greens, on the skeleton in the OVX rat model. This work suggests that dietary nitrate supplementation does not have a direct, beneficial role on the osteoporotic skeleton. In addition, this dissertation demonstrates a relationship between OVX status and the gut microbiome composition. These observations add to the increasing evidence that suggests a link between the gut microbiome and sex steroid deficiency. Our work also addresses the shifts in colon metabolism observed in OVX. These results improve our understanding of the effects of ovarian hormone deficiency on the colon, and in conjunction with the observed gut microbiome compositional shifts, have generated many exciting hypotheses about
presently unidentified mechanisms that may be contributing to the metabolic
dysregulation observed in OVX.

**Figure 1.1** Enzymatic and non-enzymatic production of nitric oxide. Nitric oxide can be produced enzymatically through the classic pathway involving NO synthases (NOS) that convert L-arginine and molecular oxygen to NO and L-citrulline under normoxic condition, and under hypoxic conditions from nitrite by the nitrate reductase activity of proteins, such as deoxyhemoglobin (DeoxyHb), deoxymyoglobin (DeoxyMb), xanthine oxidoreductase (XOR), and carbonic anhydrase (CA).
Chapter 2

Increasing dietary nitrate has no effect on cancellous bone loss or gut microbiome in ovariectomized rats

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Molecular Nutrition and Food Research
Abstract

Scope
Some studies suggest that diets rich in fruit and vegetables reduce bone loss in older adults, although the specific compounds responsible are not known. The objectives of the study were to investigate the capability of dietary nitrate, a component in leafy green vegetables, to reduce bone turnover and loss in an ovariectomized rat model.

Methods and results
Forty, six-month-old female Sprague Dawley rats [30 ovariectomized (OVX) and 10 sham-operated (sham) at 5.5 month of age] were randomized by weight into 1 of 3 treatment groups (n = 10 per group): (1) vehicle (water) control, (2) low-dose nitrate (LDN, 0.1 mmol nitrate/kg bw/day), or (3) high-dose nitrate (HDN, 1.0 mmol nitrate/kg bw/day) for three weeks. The sham controls received vehicle. Serum bone turnover markers, bone mass, bone mineral density, histomorphometric parameters, bone quality parameters, and feces were examined. OVX resulted in increased bone turnover, cancellous bone loss, and changes in gut microbiome composition and structure. Three weeks of LDN or HDN supplementation had no effect on the skeleton or gut microbiome in OVX rats.

Conclusions
These data indicate dietary nitrate does not slow bone turnover and loss in an OVX rat model, and gut microbiome composition is associated with OVX.
Introduction

Osteoporosis is a common metabolic bone disease, affecting a third of women and a fifth of men over age 65 [1]. In the US, annual health care costs associated with osteoporosis are estimated to be over $20 billion. Menopause-induced ovarian hormone deficiency causes an increase in bone turnover [2]. Increased bone resorption relative to bone formation contributes to reduced bone mass and quality and bone mineral density (BMD), and there is a relationship between low BMD and increased fracture risk in postmenopausal women [2,3].

Some observational studies report that fruit and vegetable consumption is associated with an increase in bone mineral content (BMC) and BMD [4–8]. Dietary nitrates, present in high concentrations in leafy green vegetables, may serve as a dietary component that supports bone health. Vegetable intake accounts for ~80% of dietary nitrate consumption in human diets [9]; dietary nitrates can be reduced to nitric oxide (NO) via non-enzymatic reduction by lingual bacteria and a variety of mammalian reductases through the nitrate-nitrite-NO pathway [10,11]. NO is a free radical gas involved in physiological processes ranging from vascular homeostasis to host defense, cellular energetics, and nerve transmission [12]. There are two major sources of NO in the body: the endogenous L-arginine-NOS system and our diet. Under normal conditions, these contribute approximately equally to NO homeostasis [13]. Aging is associated with decreased eNOS-dependent NO synthesis and endothelium-dependent vasodilation [14]. This suggests a role for NO donors, such as
dietary nitrate or organic nitrates, to support NO production in age-related conditions [15].

Organic nitrates (e.g., nitroglycerin, NGT) have been used as a therapeutic agent in treating angina pectoris and other cardiovascular disorders for over 100 years [16]. Postmenopausal women using organic nitrates had higher BMD compared to non-nitrate users [17]. This led to the hypothesis that age-associated decreases in NO production may be related to osteoporosis risk and organic nitrates may slow bone loss. Studies in an ovariectomized (OVX) animal model of bone loss demonstrated increased NO bioavailability using organic nitrate (i.e., nitroglycerin) as an NO donor and increased BMD and decreased bone turnover [18–20]. Follow-up human clinical trials have led to conflicting results. While pilot studies suggest a beneficial effect, the Phase III trial NOVEL (Nitroglycerin as an Option: Value in Early Bone Loss) found no significant differences in lumbar spine BMD between the NTG and control group after two years of intervention. The authors suggest suboptimal dosing, non-compliance, and side-effects were contributing factors to the null findings [21–23]. As a result, evaluation of the efficacy of alternative exogenous sources of NO, via dietary sources of nitrate, to slow bone loss is warranted.

The available evidence provides biological plausibility that dietary nitrate may act via the nitrate-nitrite-NO pathway to restore age-associated decreases in NO production that may be related to osteoporosis risk. Since dietary nitrate accounts for up to 50% of its NO production [24], this dietary compound may be a viable option for reducing bone loss. Thus, the first aim of this study was to define the relationship between dietary nitrate, bone density, turnover, and microarchitecture in the ovariectomized rat model for postmenopausal osteoporosis.

The gut microbiome has been demonstrated to be an important component of human susceptibility to several age-related conditions,
including metabolic syndrome, cancer, and osteoporosis. There is a growing body of literature that posits a relationship between bone mass and the gut microbiome [25–27]. Given that changes in microbiome composition can associate with chronic disease and that specific microbiota can interact with the immune system [28–32], it has been hypothesized that gut microbiome composition may influence the magnitude of bone loss in postmenopausal women [25]. Sex steroid deficiency results in a chronic inflammatory state that, in part, contributes to osteoporosis [33]. The microbiome’s effects on bone mass may be mediated via alteration of the immune system and regulation of osteoclastogenesis, which results in greater bone loss in postmenopause when women lose the immunosuppressive effects of estrogen [25,34,35]. OVX rats can provide empirical insight into whether the gut microbiome influences bone loss in postmenopausal women and whether specific dietary components can interact with the gut microbiome to impact bone health. To date, there are limited data regarding alterations in the gut microbiome in OVX and its relationship to bone loss. While diet can rapidly modify the gut microbiome, there is a dearth of experimental data about the effects of specific dietary components on gut microbiome composition [36]. Therefore, the second aim of this study was to characterize both the effects of OVX and dietary nitrate on gut microbiome composition.

Materials and Methods

Experimental design

A total of 40 six-month-old female Sprague Dawley rats [30 ovariectomized (OVX) and 10 sham-operated (sham) at 5.5 month of age] were purchased from Charles River Laboratory (Hollister, California). Three days after arrival at Oregon State University OVX rats were randomized by weight into 1 of 3 treatment groups (n = 10 per group): (1) vehicle (water)
control, (2) low-dose nitrate (LDN, 0.1 mmol nitrate/kg bw/day), or (3) high-dose nitrate (HDN, 1.0 mmol nitrate/kg bw/day). The sham controls received vehicle. At study initiation, sodium nitrate was added to water at 0.14 or 1.4 grams per liter to achieve 0.1 or 1.0 mmol nitrate intake per kilogram body weight per day. Food (TD.2018 chow, Teklad Lab Animal Diets, 118/5.4 nmol of nitrate/nitrite per g) was provided ad libitum to all animals. The rats were housed individually and maintained on a 12-hour light:12-hour dark cycle for the duration of study (3 weeks). The Institutional Animal Care and Use Committee at Oregon State University approved the experimental protocol under ACUP 4532. Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Food and water consumption and body weight were measured twice a week. These data were used for adjusting nitrate levels in the water throughout the study. Water was replaced every other day for the duration of experiment. The fluorochrome calcein (20 mg/kg, Sigma-Aldrich, St. Louis, MO), was injected subcutaneously 9 and 2 days before sacrifice to label mineralizing bone. Fecal samples were collected at the end of the 3-week study and stored at -80°C. For tissue collection all rats were fasted overnight, then anesthetized with 2–3% isoflurane delivered in oxygen, and death was induced by exsanguination from the heart. Serum and blood were collected and stored at −80°C for measurement of serum global markers of bone turnover and blood nitrate and nitrite levels. Uteri and abdominal white adipose tissue (WAT) were excised and weighed. Tibiae were removed and stored in 70% ethanol for analysis using dual-energy X-ray absorptiometry (DXA), microcomputed tomography (μCT), and histomorphometry.

Quantification of blood nitrate and nitrite levels

*Blood collection and pretreatment*
Whole blood was obtained by cardiac puncture and pretreated for nitrite and nitrate analysis, as described previously [37]. Briefly, heparinized whole blood was mixed with nitrite-preserving solution [K3Fe(CN)6, N-ethylmaleimide, water, Nonidet P-40] and kept frozen at −80°C until analysis. For nitrate analysis, heparinized whole blood was mixed with deionized water at a 1∶9 ratio between the blood and water and kept frozen at −80°C until analysis.

Nitrate and nitrite analysis

Nitrate and nitrite content was analyzed using a standard gas phase chemiluminescence method (NO Analyzer, model 280i, GE Analytical Instruments, Boulder, CO) with helium as carrier gas, as described elsewhere [4]. Briefly, for nitrate analysis, samples were injected into purge vessel of the NO analyzer containing 7 ml of heated (95°C) vanadium chloride (0.8 g of vanadium chloride dissolved in 100 mL of 1M hydrochloric acid) solution. For nitrite analysis, samples were deproteinized with methanol, centrifuged for 3 min at 15,000 rpm, and then supernatants were injected into the purge vessel of the NO analyzer containing 7 ml of tri-iodide (1 g potassium iodide, 0.65 g iodine, 20 ml water and 70 ml glacial acetic acid) solution. The concentration of nitrate and nitrite in analyzed samples was deduced from standard concentration vs. peak area curves constructed with sodium nitrate (10 µM) and sodium nitrite (1 µM), respectively. A correction for the nitrite concentration in nitrite-preserving solution and methanol, nitrate concentration in deionized water, and molar mass of sodium was made when calculating levels of nitrate and nitrite ions in the samples analyzed.

Average daily nitrate intake from food was derived from average daily food intake and quantification of dietary nitrate present in TD.2018 diet, as described in the methods section. Average daily nitrate intake from water was derived from average daily water intake and sodium nitrate
concentrations in water provided. Total average daily nitrate intake was determined by totaling average daily nitrate intake from food and water.

**Serum markers of bone turnover**

Serum osteocalcin was measured using a rat Gla-osteocalcin High Sensitive EIA kit obtained from Clontech Takara. Serum C-terminal telopeptide (CTx) was measured using a rat CTx ELISA kit from Life Sciences Advanced Technologies (St. Petersburg, FL).

**Dual-energy X-ray absorptiometry**

Tibial bone mineral content (BMC; mg) and area (cm²) were measured ex vivo using dual-energy x-ray absorptiometry (DXA; Piximus; Lunar Corp., Madison, WI). Bone mineral density (BMD) was calculated as BMC per area (mg/cm²).

**Microcomputed tomography**

Nondestructive three-dimensional evaluation of bone microarchitecture was completed using microcomputed tomography (μCT). Tibiae were scanned at a voxel size of 16 x 16 x 16 µm (55 kVp x-ray voltage, 145 µA intensity, and 200 ms integration time) using a Scanco μCT40 scanner (Scanco Medical AG, Basserdorf, Switzerland). Filtering parameters sigma and support were set to 0.8 and 1, respectively. Threshold for analysis was determined empirically and set at 245 (range, 0-1,000) for both cancellous bone and cortical bone. Cancellous bone was assessed in 94 slices (1,504 µm) (1,504 µm distal to the growth plate) and 33 ± 1 slices (528 ± 16µm) in the proximal tibia metaphysis and proximal tibia epiphysis, respectively. (Figure 1: VOIs). Cortical bone was assessed at 62 slices (992
µm) distal to the midshaft. Midshaft was defined as midpoint between the top and bottom of each tibia. Direct cancellous bone measurements included cancellous bone volume fraction (bone volume/tissue volume, BV/TV; volume of tissue occupied by cancellous bone, %), trabecular number (number of trabecular intercepts, mm⁻¹), trabecular thickness (mean thickness of individual trabeculae, um), and trabecular separation (mean distance between trabeculae, um). Direct cortical measurements included cross-sectional volume (volume of cortical bone and bone marrow, mm³), cortical volume (mm³), marrow volume (mm³), and cortical thickness (µm).

**Histomorphometry**

Histological methods applied here have been previously described [38]. Briefly, proximal tibiae were dehydrated in a graded series of ethanol and xylene and embedded un-decalcified in modified methyl methacrylate. Longitudinal sections (4 µm thick) were cut with a vertical bed microtome (Leica/Jung 2165) and affixed to slides pre-coated with a 1% gelatin solution. One section was mounted unstained for measurement of fluorochrome labels. One section was stained for tartrate resistant acid phosphatase and counterstained with toluidine blue (Sigma-Aldrich, St. Louis) for measurement of cells.

Fluorochrome-based measurements of bone formation included: i) mineralizing perimeter (mineralizing perimeter/bone perimeter: cancellous bone perimeter covered with double plus half single label normalized to bone perimeter, %), ii) mineral apposition rate (the distance between two fluorochrome markers that comprise a double label divided by the 7 day inter-label interval, µm/day), and iii) bone formation rate (bone formation rate/bone perimeter: calculated by multiplying mineralizing perimeter by mineral apposition rate normalized to bone perimeter, µm²/µm/year).

Osteoclast perimeter was determined as the percentage of cancellous bone
perimeter covered by multinucleated (two or more nuclei) cells with acid phosphatase positive (red-stained) cytoplasm (osteoclast perimeter/bone perimeter, %). The sampling site for the proximal tibia metaphysis was located 1 mm distal to the growth plate and extended approximately 1.2 mm in the marrow excluding cortical bone. Histomorphometric data were collected using the OsteoMeasure System (OsteoMetrics, Inc., Atlanta, GA, USA) and are reported using standard two-dimensional nomenclature (Dempster, 2012).

**Fecal DNA isolation and 16S amplicon sequencing**

Fecal DNA was isolated using QIAamp DNA stool mini-kits (Qiagen, Valencia, CA) per manufacturer’s instructions. 16S rRNA PCR amplification was conducted according to established methods [1]. Briefly, each sample’s extracted DNA was subjected to PCR reactions to amplify the V4 region of the 16S locus using PCR primers (515F and 806R) that include Illumina adapters and sample-specific barcodes. PCR amplicons from individual rat samples were cleaned using the Qiagen QIAquick PCR cleanup kit (Germantown, MD) and pooled. An aliquot of the pooled 16S library was sequenced on an Illumina MiSeq (v3 chemistry) at the Center for Genome Research and Biocomputing core facility (Oregon State University, OR). This generated ~596 thousand 300 bp single end reads (median reads per sample = 15,025).

**Experimental design of effects of dietary nitrate supplementation on bone in growing rat model**

We did not observe a beneficial effect of nitrate on the skeleton of ovx rats. However, it is possible that a normal diet provided sufficient levels of nitrate. We therefore evaluated the effects of nitrate deficiency and supplementation on expression of genes related to bone formation and
resorption. Growing rats were studied based on the expectation that the growing skeleton would be especially sensitive to nitrate levels. We found no significant changes in gene expression in response to either dietary nitrate or nitrite treatment (data not shown). A total of 24 female 6-week-old Sprague Dawley rats were obtained from Charles River Laboratory (Hollister, CA). Three days after arrival, the animals were randomized by weight into a control (Group 1) and 3 treatment groups (Groups 2 through 4) (n = 6 per group). Rats in the control group were provided with TD.2018 chow (Teklad Lab Animal Diets (Madison, WI), 118/1.84 nmol of nitrate/nitrite per g), and distilled, deionized water. Rats in groups 2, 3, and 4 were fed with a purified low-nitrate TD.99366 chow (Teklad Lab Animal Diets, 16.5/1.5 nmol of nitrate/nitrite per g). Group 3 received sodium nitrate in distilled, deionized water to deliver an approximate dose of 0.7 mmol/kg BW/day. Group 4 received sodium nitrite in distilled, deionized water to deliver an approximate dose of 50 μmmol/kg BW/day. Food and water were provided ad libitum to all animals. The rats were housed individually and maintained on a 12-hour light: 12-hour dark cycle for the duration of study (one week). The Institutional Animal Care and Use Committee at Oregon State University approved the experimental protocol. Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Food and water consumption and body weight were measured and water replaced every other day. for the duration of experiment. After one week of treatments, animals were fasted overnight and then sacrificed; animals were anesthetized with 2–3% isofluorane delivered in oxygen, and death was induced by exsanguination from the heart. Distal femurs were collected and snap frozen in liquid nitrogen for RNA analyses.

**Rat Osteoporosis RT² Profiler PCR Array**
RNA was extracted from distal femur using Trizol (Life Technologies) according to the manufacturer’s protocol. RNA was quantified via spectrophotometry using a nanodrop-1000. 1 µg of RNA was reverse transcribed in a 20ul reaction volume using a Qiagen kit. qRT-PCR was performed using the 7900HT fast machine from Applied Biosystems, according to the manufacturer’s protocol. Gene expression profiling of 84 genes in the distal femurs was performed using The Rat Osteoporosis RT² Profiler PCR Array (PARN-170ZE-4) (SuperArray, USA). Results were normalized to housekeeping genes in the array, B2m, Hprt1, and Ldha. Multiple tests were controlled for using Benajimini-Hochberg with a false discovery rate of 5%. Nitrate-deficient controls were defined as the control group that both nitrate and nitrite treatment groups were compared to. Statistics were performed with the RT2 Profiler™ PCR Array Data Analysis online software (http://www.sabiosciences.com/pcrarraydataanalysis.php#Excel)

**Bioinformatic and statistical analysis**

Mean responses of individual variables were compared between sham, OVX vehicle, LDN, and HDN groups using separate one-way analyses of variance (ANOVA). A modified F test was used when the assumption of equal variance was violated, with Welch’s two-sample t-test used for pairwise comparisons [40]. The Kruskal-Wallis nonparametric test was used when only the normality assumption was violated, in which case the Wilcoxon-Mann-Whitney test was used for pairwise comparisons. The Benjamini and Hochberg method for maintaining the false discovery rate at 5% was used to adjust for multiple comparisons [41]. Differences were considered significant at p <0.05. All data are expressed as mean ± SE. Bioinformatic analyses were performed as previously described [2]. Samples were rarefied to 12,000 reads, and alpha- (i.e., richness) and beta-diversity
(i.e., weighted and unweighted UniFrac distances) were subsequently quantified using the core_diversity_analyses.py script in QIIME. Statistical analyses were conducted in R. The coin package was used to implement robust statistical tests and identify differences in the gut microbiome communities of sham and OVX rats (i.e., Wilcoxon tests). For OTU level analyses, OTUs were filtered based on presence in three or more samples. False discovery rates were quantified using Bonferroni corrections for phylum level tests, and the q-value software package was used for remaining comparisons at the taxonomic level. Data analysis was performed using R version 2.12

Results

Dietary nitrate intakes

Average daily nitrate intakes for each group are shown in Table 1. There were no differences in average daily nitrate intake from food between sham, OVX vehicle, LDN, or HDN groups. Total daily nitrate intakes were not significantly different between OVX and sham, while LDN and HDN groups had significantly higher total nitrate intake compared to OVX vehicle.

Effects of dietary nitrate on blood nitrate and nitrite levels

The effects of OVX and dietary nitrate supplementation on blood nitrate and blood nitrite levels are summarized in Figure 2. There were no significant differences in blood nitrate levels between OVX vehicle and sham controls (Figure 2A). The LDN and HDN groups had significant increases in blood nitrate compared to OVX vehicle. There were also no significant differences in blood nitrite levels between OVX vehicle and shams (Figure 2B). There were no significant differences in blood nitrite levels in LDN group compared to vehicle, while the HDN group had significantly higher blood nitrite levels compared to OVX vehicle and LDN groups.
Effects of dietary nitrate on food and water intake, body weight, uterine weight, and abdominal white adipose tissue weight

Average daily food and water intake, body weights, uterine weights, and abdominal white adipose tissue (WAT) weights for each group are shown in Table 1. All OVX groups had significantly increased body weight and decreased uterine weight compared to sham controls with no differences observed between OVX, LDN, and HDN groups. There were no significant differences in food intake between OVX and sham, or OVX vehicle, LDN, and HDN groups. There were no observed differences in WAT weights between groups.

Effects of ovariectomy on bone

The effects of OVX on bone mass and architecture are summarized in Table 2. Significant differences in tibial bone area, BMC, or BMD were not detected between OVX and sham controls (Table 2). In the proximal tibia metaphysis, OVX resulted in significantly lower BV/TV, trabecular number, and trabecular separation and no differences in trabecular thickness. In the proximal tibia epiphysis, OVX resulted in significantly lower BV/TV and no significant differences in OVX on trabecular number trabecular separation, or trabecular thickness compared to sham. Significant differences in cortical endpoints (cross-sectional area, cortical area, marrow area, or cortical thickness) were not detected between OVX and sham.

Histological analyses of proximal tibia metaphysis revealed the OVX vehicle group had significantly higher mineral apposition rate, mineralizing perimeter, bone formation rate, and osteoclast perimeter compared to sham controls (Figure 3A-D). OVX vehicle had significantly higher osteocalcin levels compared to sham controls (Figure 3E). No significant differences in CTx levels between OVX vehicle and sham were observed (Figure 3F)
Effects of dietary nitrate supplementation on bone in ovariectomized rats

The effects of dietary nitrate supplementation on bone are summarized in Table 2. Significant differences in tibial BMC, bone area, or BMD were not detected between OVX rats treated with vehicle, LDN, or HDN. Significant differences in cancellous bone (BV/TV, trabecular number, trabecular thickness, and trabecular separation) were not observed among treatment groups. Similarly, significant differences in cortical endpoints (cross-sectional volume, cortical volume, marrow volume, or cortical thickness) were not detected among OVX rats treated with vehicle, LDN, or HDN. Histological analyses of proximal tibia metaphysis revealed there were no significant differences in mineral apposition rate, mineralizing perimeter, or bone formation rate between OVX vehicle, LDN, or HDN groups (Figure 3A-D). Significant differences in cortical endpoints (cross-sectional area, cortical area, marrow area, or cortical thickness) were not detected between OVX, LDN, and HDN groups.

Effects of dietary nitrate supplementation on serum biochemical markers of bone remodeling

The effects of OVX and dietary nitrate supplementation on serum biochemical markers of bone resorption (CTx) and bone formation (osteocalcin) across treatment groups are shown in Figures 3E and 3F. There were no significant differences in osteocalcin or CTx levels between OVX vehicle group, LDN, or HDN.

Effects of ovariectomy and dietary nitrate supplementation on fecal microbiota composition
In order to identify possible gut microbiota signatures with OVX or dietary nitrate, we compared the gut microbiota composition of sham rats to OVX rats treated with vehicle, LDN, or HDN. We first assessed the intersample diversity between the sham and OVX rats. We used UniFrac, which normalizes intersample taxonomic differences by the phylogenetic diversity of the microbial lineages observed in the samples (i.e., samples containing more phylogenetically similar taxa produce a relatively lower distance). A principal coordinates analysis (PCoA) of fecal samples based on their weighted UniFrac distances reveals that samples weakly but significantly cluster by OVX status, as supported by a permutational multivariate analysis of variance (R^2 = 0.17, p = 0.017) (Figure 4). This finding suggests the sham and OVX rats exhibit microbial communities with different evolutionary histories. These results are qualitatively consistent with those obtained using unweighted UniFrac and indicate that there are distinct phylogenetic differences in the gut microbiome between sham and OVX rats. We did not observe any clustering between OVX vehicle, LDN, or HDN groups based on UniFrac distances.

We then explored the structure of these communities at various taxonomic levels to understand potential taxonomic signatures that may stratify samples based on OVX status. Using 16S rRNA ribosomal genes as a marker, sequences were clustered into operational taxonomic units (OTUs) using a threshold of 97% sequence similarity from the GreenGenes database [42] using the QIIME open-reference OTU-picking protocol. OTUs were then taxonomically annotated using UCLUST. As expected, given prior characterizations of the rodent gut microbiome, all rats were dominated by the phyla Bacteroidetes and Firmicutes [43,44]. We found marginally significant increases in Proteobacteria abundance in the OVX rats (Bonferroni adjusted p = 0.052), whereas Firmicutes were more abundant in the sham rats (Bonferroni adjusted p = 0.015). We also observed a trend of increased
abundance of Bacteroidetes in OVX animals. At the family level, Bacteroidaceae, Erysipelotrichaceae, and Alcaligenaceae were lower in OVX (q < 0.20). The genera Bacteroides and Sutterella were also lower in the OVX rats (q < 0.20). Consistent with our analysis at the phylogenetic level, we did not observe any significant differences in gut microbiome communities between OVX vehicle, LDN, or HDN groups at the taxonomic level.

Discussion

The effects of dietary nitrate on bone were evaluated in a rat model of OVX-induced bone loss. Three weeks of dietary nitrate supplementation in water at 0.1 mmol nitrate/kg bw/day or 1.0 mmol nitrate/kg bw/day had no effect on tibial cancellous or cortical bone mass and architecture or histomorphometric indices of bone formation or resorption in OVX rats. Furthermore, increased dietary nitrate had no significant effect on serum biochemical markers of bone turnover. Blood concentrations due to nitrate supplementation were comparable to other rodent studies using similar doses [13], indicating that lack of an effect was not due to insufficient nitrate supplementation.

We are not aware of prior animal studies investigating dietary nitrate effects on bone mass and turnover. In vitro evidence on dietary nitrates and bone is also scarce. We are aware of one study ascribing estrogenic activity to the nitrite anion in an in vitro transcription assay [45]. The relationship between NO and bone physiology is complex. Osteoclasts and osteoblasts both constitutively express the endothelial isoform of nitric oxide synthase (eNOS), implying a role for NO in bone metabolism. Exogenous nitrates, such as nitroglycerin, may influence bone cells indirectly as NO donors, inducing local vasodilation. The molecular targets for NO action in bone cells are poorly understood. In vitro work suggests that NO has biphasic effects on both osteoclasts and osteoblasts, demonstrating the ability to stimulate and
inhibit cell activity depending on the amount of NO present, as well as which NOS isoform is producing NO [46–48]. Nitrates may also have direct action on osteoclasts and osteoblasts. NO may influence osteoclast activity, in part, via the receptor activator of NF-kappaB ligand (RANKL)/osteoprotegerin (OPG) pathway. High levels of NO stimulate OPG, which binds to RANKL and prevents the binding of RANKL to the receptor activator of NF-kappaB (RANK), decreasing osteoclast activity [49]. eNOS global knockout animals demonstrate lower BMD, bone formation, and osteoblast activity; with little to no effect on bone resorption, suggesting NO may be important for osteoblast function [50,51]. Previous research shows NO synthesis is induced in osteoblasts and osteocytes by mechanical strain and shear stress [52–55].

Aging is associated with decreased eNOS-dependent NO synthesis and endothelium-dependent vasodilation [14]. Reduced cofactor availability for eNOS can decrease NO production [15]. Previous reports show that OVX rats have significantly lower plasma NO metabolite [NOx; plasma nitrate+nitrite] levels compared to sham controls [56]. In postmenopausal women receiving hormone replacement therapy (HRT), plasma NOx levels are positively correlated with estrogen status [57]. Thus, age-associated decreases in NO production may contribute to osteoporosis risk. In the current study, we did not observe a significant decrease in either blood nitrate or nitrite levels in OVX controls compared to sham.

Studies in OVX rats show organic nitrates slow bone loss by reducing bone turnover [18–20]. Human data investigating organic nitrate effects on bone are generally concordant with the animal data, but there are conflicting results. A randomized controlled trial to study the effects of nitroglycerin ointment compared to oral estrogen in oophorectimized women demonstrated that nitroglycerin’s efficacy in preserving BMD was equivalent
to a standard estrogen dose [21]. Furthermore, nitroglycerin significantly decreased NTx (bone resorption) and increased serum osteocalcin and serum bone-specific alkaline phosphatase (bone formation) [21]. As a follow-up study, a randomized controlled trial conducted in postmenopausal women reported equivalent effects of isosorbide mononitrate (20 mg/day) or alendronate (70 mg/week) on lumbar BMD after 12 months of treatment [22]. This suggests organic nitrate is able to increase BMD and slow bone turnover in humans. However, the Phase III clinical trial NOVEL, evaluating effects of NTG on lumbar spine BMD in postmenopausal women, found no significant differences between the NTG and control group after two years of intervention [23]. The research group reports that poor compliance and suboptimal dose (22.5 mg NTG/day) likely explain the null findings. Further, most patients were taking <15 mg active NTG per day, far below the proposed therapeutic window for bone. Given the inconsistent results, side effects, and non-compliance, it is not clear if organic nitrates are a suitable treatment for osteoporosis.

We did not observe significant effects of dietary nitrate on bone density, turnover, or microarchitecture in the OVX rat. Several reasons might explain why dietary nitrate does not affect the ovx rat skeleton in a manner similar to organic nitrates. Although both organic and inorganic nitrates mediate their principal effects through NO, there are many notable differences. Inorganic nitrates are small, water-soluble ions present in the diet and produced endogenously by oxidation of NO, while organic nitrates are synthetic and structurally more complex [58]. These structural differences, together with pharmacokinetic differences, may contribute to the observed differences in animal studies.
Dietary nitrates rely on lingual reduction of salivary nitrate via enterosalivary circulation [59], while organic nitrates do not use this pathway. Biotransformation of organic nitrates is not fully understood and can vary widely with the class of organic nitrate [60]. Organic nitrates typically undergo liver first-pass metabolism; however, the exact mechanism of denitrification (release of NO) in the vasculature or different tissue sites remains a matter of debate [61,62]. Also, the pharmacodynamics of each compound must be considered. Organic nitrates have potent acute effects, while inorganic nitrate’s effects are more subtle and dependent on certain conditions [i.e., pH, oxygen tension] [58]. Thus, differential metabolism may explain why dietary nitrate does not affect the ovarian hormone-deficient skeleton in an analogous manner to organic nitrates. In humans, the form of nitrate supplemented may alter efficacy. For instance, vegetable juices were more efficacious in blood pressure lowering in humans than sodium nitrate [63]. In the current study, we supplemented sodium nitrate in water. While we demonstrate that this has no effect on bone, it remains unknown if nitrate-rich foods have a beneficial effect on the osteoporotic skeleton.

We observed differences in the community structure of the microbiome between sham and OVX rats. We also identified specific taxa that stratify OVX and sham animals. Here, we observed increased Firmicutes abundance in OVX compared to sham (p = 0.015), as well a non-significant decrease in Bacteroidetes (p = 0.12). A similar observation was made in OVX rats bred to have low aerobic capacity [64]. Indeed, there is an accumulating body of literature that suggests a role for the gut microbiome in the regulation of bone mass. For instance, germ-free (GF) mice have significantly increased bone mass compared to conventionally raised mice [65]. Further, OVX GF mice are protected from both cancellous and cortical bone loss [25]. The microbiome’s effects on bone mass may be mediated via alteration of the immune system and regulation of osteoclastogenesis [25]. This evidence
suggests a putative role of the gut microbiome in the bone loss observed in ovarian hormone deficiency. It is unclear from our study design if these changes in the composition of the microbiome are related to the etiology of bone loss and other observed phenotypes in the OVX rat, such as weight gain. Future investigations are required to determine whether there is a causal role of the gut microbiome in bone loss in OVX [25, 64, 66] and to explore specific mechanisms by which the gut microbiome may effect bone metabolism in ovarian hormone deficiency. While salivary nitrate reduction to nitrite by nitrate reductases in lingual bacteria is one of the earliest examples of commensalism [13], relatively little is known about the interaction between dietary nitrate and the gut microbiome. In the current study, we did not observe significant differences in gut microbiome communities between OVX vehicle, LDN, or HDN groups.

In sum, we evaluated the potential of dietary nitrate, a compound present in large quantities in leafy green vegetables, to slow bone loss in the pre-clinical model for osteoporosis. In the context of OVX, dietary nitrate supplementation for three weeks has no effect on the skeleton, including bone density, turnover, or architecture. We used a variety of methods to evaluate this relationship, including μCT, histology, DXA, and serum markers of bone turnover. In addition, our investigation of the effect of dietary nitrate and nitrite on bone growth also revealed no effect of these dietary constituents on the expression of genes related to bone growth. We also demonstrated that OVX alters gut microbiome composition and structure. However, dietary nitrate feeding had no effect on the gut microbiome in an OVX rat model of bone loss. Collectively, these data do not support an effect of increasing dietary nitrate on reducing bone loss in an OVX rat model.

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Conflict of Interest
The authors have declared no conflicts of interest.
Figure 2.1. Regions of interest analyzed in the tibia using microCT.
Figure 2.2 a) OVX had no effect on blood nitrate levels and increasing dietary nitrate resulted in increased blood nitrate levels and b) OVX had no effect on blood nitrite levels compared to sham, while significantly increased blood nitrite levels were observed in the OVX-HDN treatment group. OVX, ovariectomized; LDN, low-dose nitrate 0.1 mmol sodium nitrate kg/day; and HDN, high-dose nitrate 1 mmol sodium nitrate kg/day. Data represent means ±SE (n=10 rats/group). Differences were considered significant at Benjamini-Hochberg adjusted p < 0.05.
Figure 2.3. a) Dietary nitrate had no effect on a) mineral apposition rate (MAR), b) mineralizing perimeter (mineralizing bone/bone perimeter, M.Pm/B.Pm), c) bone formation rate (bone formation rate / bone perimeter, BFR/B.Pm), or d) osteoclast perimeter (osteoclast perimeter / bone perimeter, Oc.Pm/B.Pm) in the proximal tibia metaphysis of ovariectomized Sprague Dawley rats as determined by histology; Dietary nitrate had no effect on serum biochemical markers of e) bone formation (Osteocalcin), and f) bone resorption (CTx). OVX, ovariectomized; LDN, low-dose nitrate 0.1 mmol sodium nitrate kg/ day; and HDN, high-dose nitrate 1 mmol sodium nitrate kg/ day. Data represent means ±SE (n=10 rats/group). Differences were considered significant at Benjamini-Hochberg adjusted p < 0.05.
Figure 2.4. Principal coordinates analysis using weighted UniFrac distance on 16S sequences from fecal microbiota of sham (blue) and OVX (red) rats showing there are distinct phylogenetic differences in the gut microbiome between sham and OVX rats (Adonis, $R^2 = 0.17$, $p = 0.017$). Points represent individual rats. Ellipses represent 95% confidence intervals.
Table 1. Average body weight, food and water intake, daily nitrate intake, uterine weights, and abdominal WAT across each group.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Sham-Control (n=10)</th>
<th>Ovariectomized Control (n=10)</th>
<th>Low NO3 (n=10)</th>
<th>High NO3 (n=10)</th>
<th>FDR-adjusted p-value comparing the four groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>314.3 ± 5.9</td>
<td>370.1 ± 10.38^a</td>
<td>368.6 ± 9.94^a</td>
<td>365.8 ± 9.97^a</td>
<td>0.004</td>
</tr>
<tr>
<td>Average daily food intake (g/d)</td>
<td>16.24 ± 0.43</td>
<td>18.97 ± 0.71</td>
<td>18.77 ± 0.65</td>
<td>17.91 ± 0.45</td>
<td>0.110</td>
</tr>
<tr>
<td>Average daily water intake (mL/g)</td>
<td>22.48 ± 1.77</td>
<td>17.16 ± 0.95</td>
<td>18.73 ± 1.36</td>
<td>21.29 ± 1.15</td>
<td>0.398</td>
</tr>
<tr>
<td>Average daily nitrate from food (uM/d)</td>
<td>2.35 ± 0.06</td>
<td>2.75 ± 0.1</td>
<td>2.72 ± 0.09</td>
<td>2.5 ± 0.06</td>
<td>0.110</td>
</tr>
<tr>
<td>Average daily nitrate from water (uM/d)</td>
<td>- ± -</td>
<td>- ± -</td>
<td>30.85 ± 2.24</td>
<td>350.67 ± 18.91</td>
<td>N/A</td>
</tr>
<tr>
<td>Average total daily nitrate intake (uM/d)</td>
<td>2.35 ± 0.06</td>
<td>2.75 ± 0.1</td>
<td>33.57 ± 2.29^b</td>
<td>353.3 ± 18.91^b</td>
<td>0.003</td>
</tr>
<tr>
<td>Uterine weight (g)</td>
<td>0.728 ± 0.060</td>
<td>0.157 ± 0.01^a</td>
<td>0.155 ± 0.01^a</td>
<td>0.161 ± 0.01^a</td>
<td>0.003</td>
</tr>
<tr>
<td>Abdominal white adipose tissue weight (g)</td>
<td>9.37 ± 0.54</td>
<td>13.22 ± 1.06</td>
<td>14.15 ± 1.44</td>
<td>13.39 ± 0.51</td>
<td>0.096</td>
</tr>
</tbody>
</table>

Data are mean ± SE

^a different than sham, p < 0.05

^b different than ovx control, p < 0.05

*The Benjamini-Hochberg method for maintaining the family-wise error rate at 10% was used to adjust for multiple comparisons

Food intake, water intake, and body weights were collected twice per week for duration of the study.

These data were used to calculate averages.

Average total daily nitrate intake quantified by totaling the average daily nitrate from food and water.
Table 2. Dietary nitrate had no effects on BMC and BMD in total tibia, on cancellous bone in the proximal tibial metaphysis or proximal tibial epiphysis and on cortical bone in the tibial diaphysis in ovariectomized Sprague Dawley rats.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Sham-Operated</th>
<th>Ovariectomized</th>
<th>FDR-adjusted p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=10)</td>
<td>Control (n=10)</td>
<td>Low NO3 (n=10)</td>
</tr>
<tr>
<td><strong>Total Tibia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone Area (cm²)</td>
<td>2.40 ± 0.05</td>
<td>2.62 ± 0.06</td>
<td>2.55 ± 0.03</td>
</tr>
<tr>
<td>BMC (g)</td>
<td>0.364 ± 0.009</td>
<td>0.373 ± 0.015</td>
<td>0.361 ± 0.008</td>
</tr>
<tr>
<td>BMD (g/cm³)</td>
<td>0.152 ± 0.005</td>
<td>0.142 ± 0.003</td>
<td>0.142 ± 0.002</td>
</tr>
<tr>
<td><strong>microComputed Tomography</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal Tibia Epiphysis (cancellous bone)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone Volume/Tissue Volume (%)</td>
<td>43.52 ± 1.47</td>
<td>37.14 ± 1.12</td>
<td>38.42 ± 1.12</td>
</tr>
<tr>
<td>Trabecular Number (1/mm)</td>
<td>98 ± 3</td>
<td>95 ± 2</td>
<td>95 ± 2</td>
</tr>
<tr>
<td>Trabecular Spacing (µm)</td>
<td>182 ± 6</td>
<td>212 ± 6</td>
<td>202 ± 5</td>
</tr>
<tr>
<td>Proximal Tibia Metaphysis (cancellous bone)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone Volume/Tissue Volume (%)</td>
<td>28.95 ± 3.22</td>
<td>14.14 ± 1.09</td>
<td>14.77 ± 1.72</td>
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<tr>
<td>Trabecular Number (1/mm)</td>
<td>5.13 ± 0.19</td>
<td>3.93 ± 0.22</td>
<td>3.97 ± 0.15</td>
</tr>
<tr>
<td>Trabecular Thickness (µm)</td>
<td>98 ± 3</td>
<td>95 ± 2</td>
<td>95 ± 2</td>
</tr>
<tr>
<td>Trabecular Spacing (µm)</td>
<td>98 ± 6</td>
<td>122 ± 6</td>
<td>120 ± 5</td>
</tr>
<tr>
<td>Midshaft Tibia (cortical bone)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cross-Sectional Volume (mm³)</td>
<td>5.96 ± 0.19</td>
<td>6.43 ± 0.26</td>
<td>6.05 ± 0.15</td>
</tr>
<tr>
<td>Cortical Volume (mm³)</td>
<td>4.56 ± 0.14</td>
<td>4.93 ± 0.17</td>
<td>4.73 ± 0.12</td>
</tr>
<tr>
<td>Marrow Volume (mm³)</td>
<td>1.40 ± 0.07</td>
<td>1.50 ± 0.10</td>
<td>1.32 ± 0.04</td>
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<tr>
<td>Cortical Thickness (µm)</td>
<td>654 ± 13</td>
<td>676 ± 14</td>
<td>677 ± 14</td>
</tr>
<tr>
<td>(I_{\text{polar}}) (mm⁴)</td>
<td>5.96 ± 0.37</td>
<td>7.06 ± 0.55</td>
<td>6.20 ± 0.29</td>
</tr>
</tbody>
</table>

Data are mean ± SE

\( ^a \) different than sham, \( p < 0.05 \)

\( ^b \) different than ovx control, \( p < 0.05 \)

*The Benjamini-Hochberg method for maintaining the family-wise error rate at 10% was used to adjust for multiple comparisons.

BMC, bone mineral content; BMD, bone mineral density

Rats were ovariectomized at 6 months of age and treated with one of two doses of nitrate for three weeks.
Chapter 3

Effects of ovarian hormone deficiency on colonic metabolic phenotype

Melissa N. Conley, Norman Hord, Thomas J. Sharpton, Sandrine P. Claus

Formatted for submission to PLOS ONE
Abstract

Menopause is an estrogen-deficient state that is associated with increased bone loss, weight gain, changes in body composition, alterations in lipid profiles, and oxidative stress. As a result, postmenopausal women are at high risk for osteoporosis and subsequent fractures, and are at increased risk of obesity, cardiovascular disease, and colorectal cancer. While ample data supports evidence of metabolic shifts associated with menopause that lead to metabolic dysfunction in liver, bone, and adipose tissue, there is a dearth of information regarding these metabolic shifts in the colon. Previously, we observed changes in the structure of gut microbiome as a result of ovariectomy (OVX) in rats; Given the described interaction between bugs and colonic tissue, we anticipated there would be concordant changes in the colon metabolic profile. As such, we employed a high-resolution proton nuclear magnetic resonance spectroscopy ($^1$H-NMR) to describe the colonic metabolic phenotype, or metabotype, of the OVX rat model to determine the biochemical consequences of ovarian hormone deficiency on the colon. Here, we show that, relative to sham controls, OVX rat colons have higher levels of glycerophosphocholine, hypotaurine, taurine, glycine, glutamate, glutamine, glycine, tyrosine, phenylalanine, alanine, and branched chain amino acids. These observations indicate alterations of the methylation and transsulfuration pathways that may be indicative of hyperosmotic and oxidative stress in OVX rat colon. Our results demonstrate the metabolic effects of ovarian hormone deficiency may extend to the colon.
Introduction

Menopause is defined as the permanent termination of menstruation due to diminished ovarian production of female hormones, particularly estrogen and progesterone[1]. The postmenopausal deficiency in circulating ovarian hormones is accompanied by major metabolic shifts, such as increased bone loss, weight gain, changes in body composition, alterations in lipid profiles, and oxidative stress [2,3]. As a result, postmenopausal women have a high risk for osteoporosis and subsequent fractures, as well as increased risks for obesity, cardiovascular disease, and colorectal cancer [4,5,2,6].

Animal models of ovarian hormone deficiency, such as ovariectomy (OVX), demonstrate ovarian hormones’ extensive actions across multiple tissues in health and disease. For instance, ovarian hormone deficiency is associated with dramatic shifts in lipid, amino acid, and glucose metabolism, and the promotion of metabolic dysfunction in liver, bone, and adipose tissue [2,7,8]. Since ovarian hormones are involved in multiple pathways, clarifying the contribution of ovarian hormone deficiency to the pathophysiology of these chronic diseases presents a critical challenge. In order to address this issue, an improved understanding of the systemic and tissue-specific effects of ovarian hormone deficiency and the contribution of ovarian hormones to energy balance is needed. While metabolic dysregulation is associated with colonic metabolic shifts, the putative role of ovarian hormone loss needs to be clarified (cite).

Colonic metabolic processes include reabsorption of water and nutrients, and motility. The effects of ovarian hormone loss on these gut metabolic processes are unknown. From a clinical perspective, postmenopause is associated with gastrointestinal symptoms, including abdominal pain, altered bowel movements, bloating, and flatulence
Clarifying the metabolic state of the colon in an ovarian hormone-deficient model may explain the metabolic determinants that are associated with these observations. We have previously reported that OVX rats exhibit differences in gut microbiome structure relative to sham surgical controls (Conley et al., submitted manuscript). The gut microbiome has been demonstrated to affect energy metabolism through facilitating the absorption of complex carbohydrates (fiber breakdown) through fermentative processes, influencing the homeostasis of amino acids, and modifying bile acids [9,10]. Prior work has clarified the important role of microbiome-produced metabolites in mammalian physiology and their responses to host-derived metabolism. Given these described interactions, and our described effects of OVX on the microbiome, we hypothesize that the colonic metabolic profile is also different in OVX relative to sham controls.

Metabonomic approaches combining spectroscopic profiling techniques with pattern recognition analysis have proved useful in the assessment of metabolic alterations in various disease states. This approach has revealed metabolic fingerprints associated with a number of diseases, such as obesity, diabetes, and inflammatory bowel disease [15–20]. Complex interactions between estrogen, host metabolism, and gut microbial metabolism suggest that tissues in addition to serum and urine must be examined to clarify the mechanistic underpinnings of ovarian hormone deficiency and associated disease risk. Insufficient data are available from tissue-based studies using NMR-based platforms in estrogen-deficient models. Metabonomic studies in OVX rats, a model for osteoporosis, and postmenopausal women have demonstrated major metabolic shifts in serum, plasma, and urine as a result of ovarian hormone deficiency [21–24]. To our knowledge, metabonomics have not been used for investigating the metabolic phenotype observed in the postmenopausal colon.
In this study, we employed a high-resolution proton nuclear magnetic resonance spectroscopy ($^1$H-NMR) approach to investigate the colonic metabolic phenotype, or metabotype[25], of the OVX rat model to determine the biochemical consequences of ovarian hormone deficiency on the colon.

Materials and Methods

Animals, experimental design, and sample collection

A total of 20 six-month-old female Sprague Dawley rats (10 ovariectomized (OVX) and 10 sham-operated (sham) at 5.5 month of age) were purchased from Charles River Laboratory (Hollister, CA), and used in the experiment. Food (TD.2018 chow, Teklad Lab Animal Diets) was provided ad libitum to all animals. The rats were housed individually and maintained on a 12 h light: 12 h dark cycle for the duration of study (three weeks). The experimental protocol was approved by the Institutional Animal Care and Use Committee at Oregon State University, and the animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Food and water consumption and body weight were measured twice a week. All rats were fasted overnight, then anesthetized with 2–3% isoflurane delivered in oxygen, and death was induced by exsanguination from the heart. Blood and serum were collected and stored at −80°C. Whole colons were excised, placed in foil, and frozen in liquid nitrogen. Uteri and abdominal white adipose tissue (WAT) were excised and weighed.

$^1$H-NMR spectroscopy

Whole colon was measured from top to bottom and a one centimeter section from the midpoint was cut for $^1$H-NMR spectroscopic analyses. Colon samples were homogenized and extracted in methanol/water (1:1), as
previously described [26]. The supernatant containing the aqueous phase was collected, concentrated through evaporation in a vacuum concentrator at 45°C for three hours, and dissolved in 600 µL of phosphate buffer in D$_2$O with 0.1% 3-(Trimethylsilyl) [2,2,3,3-$^{2}$H$_4$] propionic acid (TSP). 550 µL were used for analysis by NMR spectroscopy. All $^1$H NMR spectra were acquired on a Bruker Avance 700 MHz Spectrometer (Bruker Analytische GmbH, Rheinstetten, Germany) operating at 700.19 MHz and equipped with a 5 mm CryoProbe® from the same manufacturer. All spectra were acquired using a standard 1-dimensional pulse sequence (noesypr1d) with water suppression applied during recycle delay of 5 s, a mixing time of 100 ms and a 90° pulse set at 14.33 µs. Total acquisition time was 3.34 s. For each spectrum, a total of 128 scans were accumulated into 32K data points with a spectral width of 9803.9 Hz. 2D NMR spectra (COSY) were performed on selected samples using the same equipment to facilitate metabolite identification. The FIDs were multiplied by an exponential function corresponding to 0.3 Hz line broadening. All spectra were manually phased and calibrated to the chemical shift of TSP (δ0.00) in MestReNova (Mestrelab Research S. L., Spain, version 10.0.1). Baseline was automatically corrected using the Whittaker smoother algorithm implemented in the same software. Metabolites were assigned using data from the literature and the Chenomx and Biological Magnetic Resonance Bank (BMRB) databases.

**Statistical analyses**

Mean responses of individual variables were compared between sham and OVX with Welch's two-sample t-test used for pairwise comparisons [27]. The nonparametric Wilcoxon-Mann-Whitney test was used for pairwise comparisons when the normality assumption was violated. The Benjamini and Hochberg method for maintaining the false discovery rate at 5% was used to adjust for multiple comparisons [28]. Data analysis was performed
using R version 2.12. Differences were considered significant at \( P < 0.05 \). All data are expressed as mean ± SE.

**Multivariate data analysis**

All NMR spectra were digitalized and imported to Matlab (version R2015, Mathworks) for statistical analysis. The spectra range 0.5 to 9 ppm was used for analysis. Initially, Principal Component Analysis (PCA) was performed on mean-centered and unit variance scaled data to visualize the general structure of the dataset. Differences between sham and OVX in \(^1\)H-NMR were investigated by orthogonal partial least-square discriminant analysis (OPLS-DA) models, where the data matrix was regressed against a dummy matrix of ones and zeroes indicating sham or OVX [29]. The models were evaluated by assessment of the cross-validated scores from the model based on seven-fold cross-validation (data were mean-centered and scaled to unit variance before modeling).

To aid interpretation, the OPLS coefficients were plotted into a spectral domain using the back-scaling method [30]. Using this method, the weights of each variable are back-scaled to their initial metric of the data and then the shape of NMR spectra and the sign of the coefficients are preserved. However, the weights of the variables can still be compared using a color code corresponding to the square of the actual OPLS coefficients. By construction, the OPLS coefficients are directly proportional to the correlation coefficients between the discriminant axis and the NMR data. For this reason, the square of the coefficients can be represented in terms of correlation after applying the same corrective factor to all coefficients, allowing by this way an estimation of the amount of variance of each NMR variable involved in the discrimination.
Results

Effects of ovariectomy on food intake, body weight, uterine weight, and abdominal white adipose tissue weight

Average weekly food intake, body weights, uterine weights, and abdominal white adipose tissue (WAT) weights for both groups are summarized in Figure 1. OVX group had significantly increased food intake, body weight, and WAT weight compared to sham controls. This is consistent with the key role played by estrogens on appetite regulation and energy metabolism [31]. OVX group also had significantly reduced uterine weights compared to shams.

Colon $^1$H-NMR spectroscopy profiles

Estrogen affects metabolism in multiple tissues, such as bone, liver, and adipose. The physiological role of estrogen in the colon remain to be elucidated. Thus, we evaluated colon $^1$H-NMR spectroscopy profiles of sham controls and OVX rats to provide insight into potential roles of ovarian hormones on colon metabolism. Spectra from colonic tissue extracts contain prominent signals from metabolites representing numerous metabolic pathways. Typical spectra obtained from a sham and OVX rat are displayed in Figure 4A and 4B, and Table I shows the NMR assignment and corresponding resonance multiplicity.

Score plots from principal component analysis (PCA) suggest distinct metabolic differences in the colon between sham and OVX rats, captured by the first two principal components representing a total variance of 57% (Figure 2). Each point represents an individual rat colon metabolome. This indicates that within-group variation is less than between-group variation, implying distinct metabolic differences within the colon between sham and
OVX rats. Our results indicate that OVX induced marked metabolic changes in the rat colon.

In order to evaluate which metabolic changes were characteristic of OVX, we constructed orthogonal projection to latent structure discriminant analysis (OPLS-DA) models derived from the NMR-based metabolic profiles of the colons of sham and OVX rats, as described in Materials and Methods. Pairwise comparison models of colonic metabolic profiles were used to compare sham and OVX groups. The OPLS-DA regression model is displayed in Figure 3. We observed colonic metabolic adaption as a result of OVX, as indicated by the OPLS-DA scores, $Q^2_Y = 0.52$, $R^2_Y = 0.81$, and $R^2_X = 0.35$.

The colon $^1$H-NMR profiles were characterized by high levels of glutamate and lactate. The colonic profiles also displayed patterns similar to those observed in human colon biopsies [32] (Figure 4A and B). Aqueous extract profiles of colon tissue from OVX rats were markedly different from those from sham rats (Figure 4C). The metabolite profile of the OVX rat colon was mainly characterized by higher levels of branched chain amino acids, glycerophosphocholine, hypotaurine, taurine, alanine, glutamate/glutamine, glycine, tyrosine, and phenylalanine compared to sham rats. The statistical model built from all colon spectra displayed five outliers, two from the sham group and three from the OVX group (data not shown). These highly diluted samples were removed from the subsequent analysis and the model was recalculated with eight individuals in the sham group against seven individuals in the OVX group.
Discussion

Ovarian hormone deficiency causes metabolic dysregulation. Consequently, postmenopausal women have increased risks for obesity, cardiovascular disease, and osteoporosis. Emerging evidence suggests the colon is a highly metabolic organ, which is largely due to metabolic processes of colonic gut bacteria [33]. We previously observed changes in the gut microbiome in an OVX-induced model of ovarian hormone deficiency (Conley, submitted manuscript). Adverse physiological changes to the colon are associated with chronic diseases that present increased risk in postmenopause, such as obesity, cardiovascular disease, and metabolic syndrome [34–37]. It is thus critical to understand how metabolic dysregulation observed in OVX affects the metabolic state of the colon. However, the metabolic state of the colon in ovarian hormone deficiency has not been described. In part due to the complicated nature of understanding colon metabolism given it is a function of host physiological and gut microbiomic processes. Here, we gained new insights into the impact of ovarian hormone deficiency on colon metabolism.

We characterized the colon metabotypes of sham and OVX rats using $^1$H-NMR spectroscopy and found that sham and OVX rats have distinct colon metabolic fingerprints (Figure 4). Our results agree with OVX metabonomics studies of serum and plasma that associated ovarian hormone deficiency with shifts in metabolites involved in amino acid metabolism [21,23,24,38]. We also report alterations of the methylation and transsulfuration pathways, indicative of hyperosmotic and oxidative stress in OVX rat colon, likely associated with increased gut permeability, as previously shown [39]. Compared to sham controls, OVX rat colons have higher levels of glycerophosphocholine, hypotaurine, taurine, glycine, glutamate, glutamine, glycine, tyrosine, phenylalanine, alanine, and branched chain amino acids.
This shows the metabolic effects of ovarian hormone deficiency extend beyond major metabolic organs to the colon.

**Alteration of colonic methylation pathway in OVX rats**

Figure 5 describes the methylation pathway and the transsulfuration pathway for taurine and glutathione (GSH) synthesis. Metabolites in the methyl pathway transfer a methyl group from a methyl donor (i.e., betaine) by synthesizing S-adenosylmethionine (SAM), which donates a methyl group to a methyl acceptor in many biochemical reactions. SAM loses a methyl group to form S-adenosylhomocysteine (SAH), and SAH is hydrolyzed to release homocysteine. Homocysteine is either remethylated to enter the methylation pathway again via methionine or conjugated to serine to form cystathionine, the cysteine precursor. Cysteine has two fates: (i) under normal conditions, most cysteine is used in glutathione synthesis; (ii) when cysteine levels rise, the cysteine dioxygenase (CDO) is upregulated to detoxify cysteine into hypotaurine. Both glutathione and hypotaurine are known to be potent hepatic antioxidants and act as scavengers of reactive oxygen species (ROS) generated by oxygenases and lipid β-oxidation [40–42].

We observe changes of the methylation pathway in the OVX rat colon. Specifically, higher concentrations of glycine and glycerophosphocholine (GPC) are observed in colon profile of OVX rats compared to controls. Increased glycine production from choline is the likely source of methyl that contributes to increased GPC levels. Elevated homocysteine levels observed in ovarian hormone deficiency may also be contributing to increased conversion of choline to glycine, and GPC production [43]. Upregulation of this pathway may deplete choline status in ovarian hormone deficiency. This suggests that plasma homocysteine levels are influenced by choline status. Further, choline supplementation reduces plasma homocysteine levels [44].
Estrogen regulates phosphatidylethanolamine N-methyltransferase (PEMT) activity, the enzyme responsible for conversion of phosphatidylethanolamine (PE) to phosphatidylcholine (PC), and expressed in multiple tissues, including the colon [45]. Therefore, it has been suggested that postmenopausal women may have higher dietary requirements for choline, as they have a higher risk of becoming choline deficient, compared to pre-menopausal women, due to limited PC production [46,47]. Our data suggest that increased conversion of choline to glycine may also contribute to the increased choline demand in ovarian hormone deficiency. These alterations to the methylation pathway in the OVX rat colon appear linked to subsequent upregulation of the transsulfuration pathway, suggesting adaptation to oxidative stress.

**Alteration of colonic transsulfuration pathway in OVX rats**

High concentrations of hypotaurine and taurine are seen in the OVX rat colon, indicating colonic oxidative stress. This is consistent with previous literature that ovarian hormone deficiency increases oxidative stress in multiple tissues, such as endothelium, bone, heart, kidney, eye, and brain, and in many cases estrogen treatment reduces oxidative stress [48–54]. As described above, the transsulfuration pathway converts cysteine to hypotaurine and glutathione. Hypotaurine is an osmotic regulator and potent antioxidant that scavenges the reactive hydroxyl radical [41,42]. Thus, hypotaurine is produced in large quantities under oxidative stress conditions, as shown by recent investigations demonstrating a hepatoprotective role of hypotaurine against oxidative injury [55,56]. High hepatic concentrations of hypotaurine’s end product, taurine, are seen in ovarian hormone deficiency [57]. Cysteine sulfinic acid decarboxylase (CSAD) and cysteine dioxygenase (CDO) are two rate-limiting enzymes in the de novo synthesis of taurine from hypotaurine [57]. Both CSAD and CDO hepatic expression and serum taurine
are elevated in OVX rats, and estrogen treatment decreases their expression [57]. The other major end product of cysteine, GSH, is unaltered in OVX rat colon. While lowered GSH levels are observed in ovarian hormone deficiency and CDO overexpression can decrease GSH levels, the elevated levels of glutamate in the OVX rat colon may have supported GSH production, thereby compensating for GSH lost in oxidative stress and resulting in no overall change in glutathione levels [49,58]. It is also possible the high levels of hypotaurine compensate for glutathione. The stable glutathione levels and high levels of glycine, an essential amino acid for GSH synthesis, suggest a perturbed gamma-glutamyl cycle and further argue for altered cysteine metabolism in the OVX rat colon [59].

Taken together, the increases in hypotaurine and taurine, concomitant with the increased levels of glycine and GPC, in OVX rats can be interpreted as a signature of a colonic compensatory mechanism to neutralize oxidative stress. This alteration to the methylation and transsulfuration pathways is likely an adaptive mechanism in hyperosmotic stress, as the upregulated metabolites are osmolytes.

**Hyperosmotic stress in the OVX colon**

An imbalance between extracellular and intracellular fluid osmolarity affecting osmotic pressure is the underlying cause of osmotic stress [60]. Increased extracellular osmolarity caused, for example, by sodium chloride (NaCl), induces hyperosmotic stress and has many damaging effects on cells by promoting water efflux from the cell, triggering cell shrinkage, and increasing oxidative stress [61]. The ability to regulate and preserve distinct solutes in the intracellular and extracellular microenvironments is crucial to maintain cellular homeostasis. The loss of intracellular water due to hyperosmotic stress adversely affects protein structure and function,
including enzyme activity [42]. Cells have several adaptive response mechanisms to counter hyperosmotic stress and restore osmotic equilibrium, including the induction of genes involved in the synthesis and transport of osmolytes [62]. This strategy is referred to as osmoadaptation and involves accumulation of osmolytes, allowing organisms to adapt to significant fluctuations in external osmolarity without compromising cell activity. Osmolytes preserve cell volume by preventing water efflux from the cell, stabilizing protein structures to preserve enzyme function, and restoring osmotic balance [42,63]. As a response to hyperosmotic stress, the upregulation of osmolytes also counters increased generation of reactive oxygen species (ROS) and oxidative stress [42,64]. Bacteria are also known to respond to hyperosmotic stress with upregulation of osmolytes to preserve cell volume, growth, and function [65].

Most investigations have focused on the effects of hyperosmotic stress in the kidneys, which routinely face wide fluctuations in osmolarity due to the nature of their physiological function. Recent data indicate many non-renal tissues encounter and respond to hyperosmotic stress. Hyperosmolarity has been shown to stimulate the release of pro-inflammatory cytokines from both immune and epithelial cells. The putative role of hyperosmolarity in influencing intestinal epithelial production of pro-inflammatory cytokines is supported by the hyperosmolarity observed in inflammatory bowel diseases. As such, increased cytokine production may be a functional consequence of impaired osmoadaptation of colonic cells. It is becoming increasingly clear that local and systemic hyperosmolarity is present in a variety of inflammatory disorders. Some disorders involve systemic hyperosmolarity (e.g., dehydration, diabetes mellitus, inflammatory bowel disease), and in certain inflammatory disorders, the skin, intestine, and cornea are sites of local hyperosmolarity.
We observe elevated levels of primary osmolytes, such as taurine, glycine, and glycerophosphocholine (GPC), in the OVX rat colon compared to sham, indicating hyperosmotic stress. These osmolytes are by-products of both bacterial metabolism and of the conversion of choline to glycine in the methylation pathway [66,67]. These observations likely represent metabolic shifts in both the host and in resident bacteria during hyperosmotic stress. High glycine levels are seen in osmotically stressed epithelial cells; this is further supported by the demonstrated increased conversion of choline to glycine in high salinity [68]. Similarly, bacteria respond to hyperosmotic stress with elevated glycine production [66,69]. Thus, both changes to host methylation pathways and bacterial production of glycine may contribute to our observations. GPC also plays a protective role in maintaining osmotic pressure. In the kidney, GPC synthesis is upregulated to defend against high-salinity conditions [70]. Conversion of GPC to choline occurs through both phospholipase activity and bacterial glycerol diesterase, GDP-D5 [71–73]. Bacteria also respond to hyperosmotic stress with increased GPC production through inhibition of GDP-D5 [73]. Altered GDP-D5 activity, as a result of hyperosmotic stress, may also explain the accumulation of GPC we observe in the OVX rat colon. Other osmolytes elevated in the OVX rat colon also play a protective role against oxidative stress. For instance, osmotic and oxidative stress result in the increased production of glutamine, an amino acid that plays a crucial role in protecting against oxidative stress-induced apoptosis and in numerous other defense mechanisms [74]. Bacteria also increase glutamine and glutamate synthesis during hyperosmotic stress [66,75]. Our observation of increased glutamate further supports a bacterial response to hyperosmotic stress, as upregulated glutamate and potassium (K+) are the primary response in bacteria after exposure to hyperosmolarity [76,77]. Taurine is the predominant osmolyte in
mammals and is upregulated in oxidative stress, along with its precursor, the antioxidant hypotaurine \(^59,65\); the elevated levels of these metabolites in the OVX rat colon offer additional support to the role of hyperosmotic stress and oxidative stress in ovarian hormone deficiency. It is difficult to disentangle the relative contributions of osmoadaptation from host and bacterial metabolism in the current study; however, in light of prior work and our observations, it is likely they both contribute to observed changes in the OVX rat colon.

Our observations of elevated oxidative stress and hyperosmotic stress also suggest alterations of intestinal permeability and inflammation. Oxidative stress and inflammatory mediators increase paracellular permeability in epithelial cells, but it is unclear how they are regulated \([78]\). Cytokines, such as gamma-interferon, interleukins, and TNF-alpha, appear to promote intestinal permeability in inflamed tissue by altering tight junction space \([79,80]\). Many conditions are characterized by local osmotic stress and correlate with the secretion of pro-inflammatory cytokines. It was recently demonstrated that hyperosmolarity contributes to both colonic inflammation and the exacerbation of intestinal inflammatory disease via upregulation of the epithelial cytokine response \([81]\). In colorectal epithelial cells, the induction of pro-inflammatory cytokine secretion through hyperosmolarity is mediated by the methylation of protein phosphatase 2A (PP2Ac). The methylation of PP2Ac then translocates the transcription factor NF-κB which regulates the synthesis of multiple cytokines \([82]\). Hyperosmotic stress can also promote intestinal permeability and inflammation in the colon by the induction of cytokines TNF-alpha and lymphotixin-betain in osmotically stressed T cells through a process mediated by the osmoregulator, nuclear factor of activated T cells 5 (NFAT5) \([83]\). NFAT5 is activated in osmotic stress in many tissues and has been implicated in the response to local hyperosmolarity associated with local inflammation \([84]\). Thus, there are likely multiple mechanisms involved in the hyperosmotic stress response that contribute to inflammation and intestinal permeability.

Disturbances of intestinal integrity, such as increased intestinal permeability and inflammation, have been reported in ovarian hormone deficiency \([85,86]\). Colon permeability is highly sensitive to circulating estrogens, which reinforce the intestinal epithelial barrier through ER-β-
dependent upregulation of tight junction proteins, reducing paracellular space [87]. Taken together with the observations described above, elevated levels of antioxidants and osmolytes in the OVX rat colon may be an adaptive response to hyperosmotic stress, associated oxidative stress, and increased intestinal permeability. Impaired barrier function may have pathological consequences by promoting inflammatory processes. For example, increased permeability in the colon permits more antigen access, which can initiate or perpetuate inflammation and is often clinically referred to as leaky gut syndrome [88,89]. Leaky gut syndrome has been implicated in diseases associated with inflammation, such as obesity, diabetes, and osteoporosis, but supportive data are lacking. Leaky gut syndrome is observed in inflammatory gastrointestinal diseases. For instance, the colons of Crohn’s and ulcerative colitis patients demonstrate increased osmolarity and permeability compared to healthy controls [88,90–92]. While it is established that intestinal permeability can influence local inflammation, the effects on systemic inflammation are less clear. However, one study demonstrated that hyperosmolarity upregulates colonic inflammation and plasma inflammatory marker serum amyloid A (SAA), suggesting local hyperosmolarity can modulate systemic inflammation [81]. Further experimentation is warranted to distinguish between local and systemic effects of inflammation as a result of colon permeability in ovarian hormone deficiency.

There are many reasons that the colon may experience hyperosmotic stress in ovarian hormone deficiency. While intestinal permeability likely plays a role, the cause may be a direct or indirect consequence of ovarian hormone deficiency. Visceral fat accumulation and obesity also promote systemic inflammatory processes that may ultimately drive increased gut inflammation and permeability, as well as hyperosmolarity [93,94]. Increased food intake in the ovarian hormone-deficient rats, as others and we have
observed, may also influence hyperosmotic stress. As hyperosmolarity is a postprandial response, it is possible that increased food intake contributes to an exaggerated hyperosmotic stress. However, it is difficult to determine the driving factor(s) from the current study. Further experimentation is needed to clarify the cause(s) of hyperosmotic stress in the colon.

**Conclusions**

We show that ovarian hormone deficiency influences the colon metabolic phenotype, inducing the upregulation of metabolites involved with hyperosmotic and oxidative stress. Since estrogen protects against oxidative stress in many other tissues, we hypothesize that ovarian hormone deficiency promotes oxidative and hyperosmotic stress in the colon, promoting intestinal permeability and elevating the inflammatory response. In response, the colonic transsulfuration and methylation pathways adapt by upregulating osmolytes and antioxidants. However, the specific causative agents of oxidative stress cannot be derived from our current work. Future work should evaluate the temporal nature of the development of the observed metabolic phenotype, as well as clarifying the contribution of specific cell types within the colon, and gut microbiomic metabolism, in the observed hyperosmotic and oxidative stress in OVX. Further investigation of the role these metabolites and processes play in the development of inflammation and metabolic dysregulation will yield insights into the physiological effects in postmenopausal women.

**Acknowledgements**

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Conflict of Interest

The authors have declared no conflicts of interest.
Figure 3.1. OVX results in increased food intake, body weight, and abdominal white adipose tissue, and reduced uterine weights; * indicates statistical significance.
Figure 3.2. PCA score plots from colon metabolomes of sham (blue) and OVX (red) rats suggest distinct metabolic differences in the colon between sham and OVX rats, captured by the first two principal components representing a total variance of 57%. Each point represents an individual rat colon metabolome.
Figure 3.3 OPLS-DA scores derived from the model calculated from 700-MHz $^1$H-NMR spectra of colon from sham (black) and ovx (red); (1 predictive component plus 1 orthogonal component; $Q^2_Y=0.52$, $R^2_Y=0.81$, $R^2_X=0.35$). T axis represents raw OPLS scores and Tcy axis represents cross-validated T scores.
Figure 3.4. $^1$H-NMR spectra of colonic aqueous extracts of sham (A) and OVX (B) rats. (C) Plot of OPLS-DA coefficients related to the discrimination between $^1$H-NMR spectra of colon from sham (top) and OVX (bottom). For panel C, metabolites are color coded according to their correlation coefficient, red indicating a very strong positive correlation ($r^2 > 0.7$). The direction of the metabolite indicates the group with which it is positively...
associated as labeled on the diagram. For identification of the peak numbers, refer to codes in Table I.

**Figure 3.5:** Methylation pathway and its relationship with hypotaurine, taurine, glycine, and glycerophosphocholine. Adapted from Castro et al. 2013. Key: BHMT, betaine homocysteine S-methyltransferase; CDO, cysteine dioxygenase; CK, choline kinase; CL, cystathionine-γ-lyase; CPT, choline phosphate transferase; CS, cystathionine-β-synthase; CT, CTP:phosphocholine cytidylyltransferase; DG, diacylglycerol; GCL, γ-glutamylcysteine ligase; MAT, methionine adenosyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine N-methyltransferase; SAH, S-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase; SAM, S-adenosylmethionine; HTD, Hypotaurine dehydrogenase; THF, tetrahydrofolate. Colored arrows represent our observations (red) and those gleaned from the literature to be associated with ovarian hormone deficiency (grey) and hyperosmotic stress (purple).
<table>
<thead>
<tr>
<th>Code</th>
<th>Metabolite</th>
<th>$\delta^1$H (multiplicity) group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>glutamate</td>
<td>2.08 (m), 2.34(m), 3.75 (m)</td>
</tr>
<tr>
<td>2</td>
<td>glutamine</td>
<td>2.15 (m), 2.46 (m), 3.77 (m)</td>
</tr>
<tr>
<td>3</td>
<td>glycerophosphocholine</td>
<td>3.23 (s), 4.32 (m)</td>
</tr>
<tr>
<td>4</td>
<td>glycine</td>
<td>3.56 (s)</td>
</tr>
<tr>
<td>5</td>
<td>hypotaurine</td>
<td>2.64 (t), 3.37 (t)</td>
</tr>
<tr>
<td>6</td>
<td>isoleucine</td>
<td>0.95 (t), 1.01 (d), 1.26 (m), 1.48 (m), 1.98 (m), 3.68 (d)</td>
</tr>
<tr>
<td>7</td>
<td>lactate</td>
<td>1.33 (d), 4.12 (q)</td>
</tr>
<tr>
<td>8</td>
<td>leucine</td>
<td>0.96 (d), 1.71 (m), 3.73 (t)</td>
</tr>
<tr>
<td>9</td>
<td>phenylalanine</td>
<td>3.13, 3.28, 4.0, 7.33 (m), 7.39 (t), 7.43 (m)</td>
</tr>
<tr>
<td>10</td>
<td>taurine</td>
<td>3.27 (t), 3.43 (t)</td>
</tr>
<tr>
<td>11</td>
<td>tyrosine</td>
<td>3.06, 3.16, 3.94, 6.87 (d), 7.18 (d)</td>
</tr>
<tr>
<td>12</td>
<td>uracil</td>
<td>5.78 (d), 7.52 (d)</td>
</tr>
<tr>
<td>13</td>
<td>valine</td>
<td>0.99 (d), 1.05 (d), 2.28 (m), 3.62 (d)</td>
</tr>
</tbody>
</table>

The numbering/nomenclature of compounds follows the IUPAC system.
Key: s, singlet; d, doublet; t, triplet; m, multiplet;
Chapter 4

General Conclusions
The overall goals of this dissertation were to further understand the complex interactions between ovarian hormone deficiency, osteoporosis and potential dietary interventions to reduce risk of this condition. This dissertation takes advantage of an ovariectomized (OVX) rat model of estrogen-deficiency induced bone loss and an array of integrative techniques, including osteologic, immunological, metabonomic, microbiomic and bioinformatic methods to evaluate different phenotypes of the osteoporotic state. These approaches have allowed for the elucidation of physiological determinants influencing the rate of bone loss and gut-associated changes observed in this preclinical model. In this dissertation, we clarified the effects of dietary nitrate, a biologically active compound present in large quantities in leafy greens, on the skeleton in a rat model of ovarian hormone deficiency-induced bone loss. This work suggests that dietary nitrate supplementation does not have a direct, beneficial role on the osteoporotic skeleton. In addition, this dissertation demonstrates an association between OVX status and the gut microbiome composition. These observations add to the increasing support that suggests a link between the gut microbiome and bone health. Finally, this dissertation demonstrated that there exist shifts in colon metabolism in OVX. These results improve our understanding of the effects of ovarian hormone deficiency on the colon, and in conjunction with the observed gut microbiome compositional shifts, have generated many exciting hypotheses about yet to be identified mechanisms that may be contributing to metabolic dysregulation observed in OVX.

In Chapter 2, we evaluated the ability of dietary nitrate to slow bone loss in an OVX model. Previous investigations suggest that an NO donor, organic nitrates, can slow bone loss in this model. We demonstrated that dietary nitrate, which serves as a substrate for endogenous production of NO and other nitrogen oxide (NOx) species, does not beneficially affect bone
density, microarchitecture, or turnover in OVX. We used a variety of proxies to evaluate this relationship, including DXA, μCT, histology, and serum markers of bone turnover. Several reasons might explain why dietary nitrate does not affect the osteoporotic rat skeleton in a similar manner as organic nitrates. Although both organic and inorganic nitrates mediate their principal effects through NO, there are many notable differences. Inorganic nitrates are small, water-soluble ions present in the diet and produced endogenously by oxidation of NO, while organic nitrates are synthetic, structurally more complex, and require metabolic activation for bioactivity. These structural differences, together with pharmacokinetic differences, may contribute to the observed differences in animal studies. Dietary nitrates rely on lingual reduction of salivary nitrate, via enterosalivary circulation, while organic nitrates do not use this pathway. Biotransformation of organic nitrates are not fully understood and can widely vary with the class of organic nitrate. Organic nitrates typically undergo liver first-pass metabolism, however the exact mechanism of denitration (release of NO) in the vasculature or different tissue sites remains a matter of debate. The pharmacodynamics of organic and inorganic nitrates are also different. Organic nitrates have potent acute effects while inorganic nitrate’s effects are more subtle and dependent on certain conditions [i.e. pH, oxygen tension]. Thus, differential metabolism may explain why dietary nitrate does not affect the estrogen-deficient skeleton in an analogous manner to organic nitrates.

As a follow up investigation, we also examined the ability of dietary nitrate to influence bone metabolism in a growing rat model. Previous studies have found fruit and vegetable intake is also associated with bone health in earlier life stages, including adolescence. Consistent with our first investigation, we also observed no positive effects of dietary nitrate on bone metabolism in a growing model. This suggests dietary nitrate does not play a
direct role in the relationship between fruit and vegetable intake and bone health in the earlier stages of the life cycle. Taken together, these results do no support a direct role for dietary nitrate in bone metabolism.

As an exploratory component of the first study, we used 16S rRNA gene sequencing to profile the composition of fecal microbiota associated with sham, OVX, and nitrate treated groups to evaluate the ability of OVX or dietary nitrate to alter the gut microbiome structure composition. We found no significant changes in gut microbiome structure or composition in response to dietary nitrate treatment. These novel findings clarify the relationship of a commonly consumed dietary component and the gut microbiome. However, our analysis identified an association between gut microbiome structure and OVX status, and revealed specific groups of taxa whose abundance stratify sham and OVX rats, including Proteobacteria, Firmicutes, and Bacteroidetes. Our data demonstrates that gut microbiome composition is associated with OVX status. At this time, there is little known concerning the relationship between ovarian hormone deficiency and the gut microbiome, but these findings warrant further investigation of taxa associated with OVX status and the role of gut microbiome in health status of postmenopausal women. Exploring these host-microbe interactions will be an important area of future research, particularly expanding our understanding of the role the gut microbiome may play, as a cause or consequence, in the major metabolic shifts observed in postmenopausal women.

In Chapter 3, we employed a high-resolution 1H-NMR spectroscopic approach to investigate the colonic metabolic phenotype of the OVX rat model to determine the biochemical consequences of ovarian hormone deficiency on the colon. We found OVX rats had a distinct colonic metabolic
fingerprints compared to sham. Compared to sham controls, OVX rat colons have higher levels of glycerophosphocholine, hypotaurine, taurine, glycine, glutamate, glutamine, glycine, tyrosine, phenylalanine, alanine, and branched chain amino acids. Our results agree with OVX metabolomics studies of serum and plasma that associated ovarian hormone deficiency with shifts in metabolites involved in amino acid metabolism. We also report upregulation of metabolites in the methylation and transsulfuration pathways, indicative of hyperosmotic and oxidative stress in OVX rat colon. This is consistent with prior work that demonstrates ovarian hormone deficiency increases oxidative stress in multiple tissues, such as endothelium, bone, heart, kidney, eye, and brain. Here, we demonstrated similar observations in the estrogen-deficient colon. These results show the metabolic effects of ovarian hormone deficiency extend beyond the skeleton to include the colon. Taken together, this work suggests ovarian hormone deficiency influences the colon and the associated gut microbiome and supports further exploration of the role of the gut in postmenopausal women and associated metabolic dysfunction.

The conclusions from this dissertation suggest the field could benefit from further analyses of the effects of OVX on both the gut microbiome and the colon, to explore if OVX is influencing both the gut either locally, systemically, or both. Additionally, the identification of host-microbe interactions, in the context of ovarian hormone deficiency, will be useful in understanding the putative role of the gut microbiome in the development of metabolic dysfunction observed post-menopause. The current data suggests that the estrogen deficient colon experiences hyperosmotic and oxidative stress, both indicative of increased intestinal inflammation and permeability. Our data are consistent with the hypothesis that some of these observed metabolic changes may be derived from bacterial metabolism. However, the precise role that the gut microbiome plays in determining the gut metabolic
phenotype is unknown. A deeper understanding of gut-associated changes in ovarian hormone deficiency will ultimately contribute to more informed diagnosis and treatment strategies for susceptible individuals.
Bibliography


[57] Jamal SA, Cummings SR, Hawker GA. Isosorbide mononitrate increases bone formation and decreases bone resorption in postmenopausal


