UNIVERSITY OF CALGARY

A PROSPECTIVE POPULATION-BASED COHORT STUDY OF THE ASSOCIATION OF URINE MEASURES OF DIET DERIVED ACID EXCRETION WITH BONE LOSS AND FRACTURES IN ADULTS

by

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Abstract

The modern diet is purported to induce osteoporosis due to diet acid-ash, net acid excretion, altered acid-base balance and finally bone demineralization.

Objectives: The literature was systematically reviewed. Associations between urine measures of dietary acid load (pH, sodium, potassium, calcium, magnesium, phosphate, chloride, and sulfate, measured, and organic acids, calculated, in fasting morning urine samples (n = 795) were examined with the bone outcomes ((changes in bone mineral density (BMD) (femoral neck, lumbar spine, and total hip) over 5 years) and fractures over 7 years). Stability of the urine measures were assessed over 5-years.

Methods: A systematic review was undertaken based on Hill's criteria of causation. Fasting urine samples and DEXA BMD were measured at baseline and at 5-years among the Canadian Multicentre Osteoporosis cohort Study. Multiple linear (BMD) and logistic (fractures) regression analysis were undertaken controlling for potential confounders eg: age, gender, family history of osteoporosis, physical activity, smoking, calcium intake, vitamin D status, hormonal status, medications, renal function, urine creatinine, change of body mass index. Intraclass correlation coefficients (ICC) described the stability of the urine measures.

Results: The limitations of the literature included: primary outcome was urinary calcium, which may be confounded by absorption, and results of cohort studies are inconsistent. The internal consistency was dubious as a consequence of the conflicting roles of phosphate, sodium, and milk, and there is no mechanism described that would function to demineralize bone at physiological pH.

There was no association between urine pH and either the change of BMD or the incidence of fractures over 7 years. Among 11 measured associations between urine measures of dietary acid load and BMD at different sites and fragility fractures, only low urine potassium was associated with increased BMD in the lumbar spine over 5-years, percent explained variance =

4%. Urine potassium was not associated with change at the other bone sites. The ICCs for the urine measures of diet acid load were 0.08 to 0.64.

Conclusion: The finding of only one weak association between urine potassium and the change of spine BMD, and the systematic review, provided little support for the acid-ash hypothesis. It is unknown if this limited evidence was due to the absence of an association or the limitation of fasting urine samples to be assessed for diet acid load.

Dedication

I dedicate this thesis to my parents, Mary and Larry Root.

Table of Contents

Chapter One: Background

1.1 The Burden of Disease

Osteoporosis is defined as “a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture. Bone strength primarily reflects the integration of bone density and bone quality” (1). Bone strength is based on a combination of the degree of cross linked micro-architecture and bone mineral density (BMD). Together BMD and cross linked micro-architecture provide bone both strength and resistance to fracture (2). Bone strength is not readily measured for research or clinical purposes, so BMD is used routinely as a surrogate measure of bone strength and hence risk of osteoporosis (2).

Although osteoporosis is generally thought of as primarily affecting older adults, cross sectional studies have suggested that people in developed countries experience a gradual loss of bone mineral that begins as early as 25 to 30 years of age (3). For women, there is a period of accelerated bone loss for 10 to 15 years after menopause, during which approximately 25% of the skeletal mass may be lost (4-6). For men, bone loss is generally a slower and more constant process beginning in early adulthood (3, 7).

An individual with osteoporosis can experience a bone fracture within the course of routine daily activities. Fractures which occur without trauma, either spontaneously or due to a fall from standing height or less, are considered to be “non-traumatic” or “fragility” fractures (8). These fractures are considered to be a sign of decreased bone strength and therefore an indicator of osteoporosis.

Osteoporotic fractures are associated with pain, disability, diminished quality of life (due to pain and loss of function), increased need for institutionalization, and increased mortality (9-11). A recent Swedish study

demonstrated that most people (86%) experience pain with motion after osteoporosis-related fractures and 38% describe their pain as severe (12). The experience of pain from these fractures was associated with a reduction of participation in routine activities, especially those activities that took place outside of the house (13). Following a hip fracture, institutionalization was increased by 52% in women and 83% in men (14) and mortality was increased by 25% for 5-years (15).

The risk of osteoporotic fractures increases with decreased BMD, female gender, and increased age (16-18). In terms of age and gender, the ten-year probabilities of osteoporotic fractures among adults ranged from a low of 2.6% for men and 3.8% for women at age 45 up to 13% for men and 27% for women at age 85 (19). In terms of BMD and risk of fractures, the relative risk for fracture for women with low BMD (T-score of -2.0 or less) was 1.5 to 3.4 times compared to women with the normal BMD (T-score of 0) (20). With the loss of BMD that occurs with age, women at the age of 90 years have a 48% probability of a already having experienced a fracture (21).

1.2 Prevalence and Economic Impact of Osteoporosis in Canada

Osteoporosis is an expensive public health problem in Canada. The prevalence of osteoporosis in Canada is estimated to be 16% among women and 7% among men over the age of 50 years, and the prevalence increases with age, ultimately affecting 30% of women and 14% of men over the age of 80 years (3). If the prevalence of osteoporosis was reduced so that hip fractures were eliminated, in Canada we would anticipate saving $650 million dollars per year in 1996 dollars or an expected $2.4 billion per year by 2041 (22).

1.3 Nutrition recommendations for Osteoporosis

Current knowledge of physiology of bone indicates that the nutrients with the most established roles for the prevention of bone loss are calcium and vitamin D (23-27). Experts differ in opinion regarding the importance of some

other nutrients in the prevention of this disease. The Canadian Scientific Advisory Council advocates adequate protein intakes and limited intakes of caffeine and sodium along with adequate calcium and vitamin D intakes to support optimum bone health in their 2002 clinical practice guidelines. The Canadian group determined that there was insufficient evidence to make a statement regarding the role of phosphorus in osteoporosis. The American National Institutes of Health agrees with the Canadian advice regarding high intakes of sodium and caffeine, but in contrast, cautioned that high intakes of protein and phosphorus (amounts were not defined) can adversely affect calcium balance in individuals with low calcium intakes (28). Other than adequate calcium and vitamin D, the American Association of Clinical Endocrinologists does not specify any other nutrients of importance and only advocate good general nutrition in their 2003 statement (29). These discrepancies in recommendations from expert organizations speak to the limited evidence regarding the role nutrition plays in the prevention or progression of this disease.

Other experts identify other factors, such as potassium, may be important for the maintenance of bone health. One of the Institute of Medicine's books: Dietary Reference Intakes for Water, Potassium, Sodium, Chloride, and Sulfate states that the modern diet produces acid on metabolism, which "not only exchanges with bone sodium and potassium, but also titrates and is neutralized by basic salts of bone" possibly causing bone to demineralize (30). In summary, well-respected groups differ with regard to nutrients, other than calcium and vitamin D, are important to prevent the development of osteoporosis.

1.4 The acid-ash hypothesis of osteoporosis

The acid-ash hypothesis of osteoporosis suggests that modern diets a cause a loss of calcium in urine, and likely promote osteoporosis, through the metabolic production of acid that causes a demineralization of the skeleton to buffer the acid (31-34). According to the acid-ash hypothesis, osteoporosis develops as the skeletal pool of calcium is gradually diminished over time by this

calcium loss. There is evidence that some aspects of the diet cause acidic urine and elevated urinary calcium. However, it has not been established whether the acidic urine of the modern diet causes demineralization of the skeleton and osteoporosis. Consequently, the acid-ash hypothesis as a purported cause of osteoporosis could be flawed.

The acid-ash hypothesis consists of a sequential series of cause and effect assumptions:

- Through absorption and metabolism, the foods consumed are broken down and consequently, mineral salts are introduced into the body. The kidney actively reabsorbs most salts and eliminates some excess salts into urine. The pH of systemic body fluids is buffered and maintained within a narrow range by respiratory excretion of CO_2 and renal excretion of bicarbonate, respectively.

- The salt composition and pH of urine depends primarily on the diet of an individual. When the diet is rich in foods that generate phosphate, chloride, sulfate and organic acids, the ionized form of these acids, along with accompanying hydrogen ions, are excreted in the urine in lieu of bicarbonate, rendering the urine more acidic (35). This excretion of mineral anions and hydrogen ions is referred to in the literature as the renal “acid load” (36-39). The acid load from the diet has been calculated from the difference in dietary intake of phosphate, chloride, and sulfate less the intakes of potassium, sodium, calcium and magnesium (40).

- Chronic consumption of diets that generate larger acid loads and produce acidic urine are assumed to promote urinary calcium loss and bone mineral resorption that is clinically manifested as osteoporosis (30, 41-45).

- In contrast, chronic consumption of diets high in fruit and vegetables, which include large amounts of potassium with organic-conjugate-acids, are associated with urine bicarbonate excretion, high urine pH, and reduced loss of calcium in the urine.

In spite of the lack of causal evidence, respected researchers, authors of medical textbooks and numerous review articles, as well as writers for lay audiences and complementary medicine have regarded the acid-ash hypothesis as an important factor for bone health and some advocate alternate diets and dietary supplements to reduce the risk of osteoporosis on the basis of this hypothesis (30, 41-46). However, the source of the additional calcium noted in the urine in the literature may be due to improved calcium absorption and there is limited direct evidence to demonstrate that the additional urine calcium comes from the bone. Consequently, the acid-ash hypothesis needs to be examined to determine its importance in terms of the progression of osteoporosis.

The terminology used to refer to the acid-ash hypothesis varies from one research group to another. Early food composition studies burned away the organic components of foods and analyzed the remaining inorganic components in the ash (47, 48). Researchers a century ago considered the food's "acid" content as equivalent to the sum of the anions (phosphate, chloride and/or sulfate) and the "alkaline" content was equivalent to the sum of the cations (calcium, magnesium, sodium, and/or potassium) (47, 48). In 1918 Sawyer et al suggested that bone might be the source of the increased urinary calcium excretion observed when subjects were fed an acid load (49). In 1968, Gonick referred to this concept as "acid-ash" and this term has been used more recently by others (50, 51). In current literature, Tucker used the term "acid-base hypothesis" (52), Cloutier referred to the "acid-ash hypothesis" (53), and Remer referred to the "dietary ash hypothesis" (31). The term "net acid excretion" has been used frequently (35, 54-56). Remer, New, Sebastian and their associates refer to "acid-base balance" and the "dietary acid load" (34, 56, 57). Perhaps the most descriptive phrase would be the "diet-acid-mediated-calcuria hypothesis', but for brevity's sake, this paper will use the term "acid-ash hypothesis".

Morris and Sebastian of the University of California have patented the "Treatment of osteoporosis using potassium bicarbonate" in the United States (58).

Chapter Two: Overview of hypothesized risk factors for Osteoporosis

Osteoporosis is sometimes described as a disease that is “silent” until a fracture occurs. The identification of risk factors is important to aid identification of those at risk so that measures can be taken to prevent or slow the progression of the disease and to decrease the occurrence of fractures. Additionally, in epidemiological research, it is important to consider risk factors for a disease in the study of a relationship between an exposure and the disease since these additional or extraneous variables might distort the understanding of the relationship under study.

When the estimated effect of the exposure, such as the diet acid load, depends on the level of another extraneous factor, the effect of the risk factor of interest is said to be “modified” by the second factor (59). When the extraneous factors sometimes “confuse” the assessment of the estimated effect of a risk factor; this confusion is referred to as “confounding” (59). The variables that might distort the understanding of the relationship between the diet acid load and osteoporosis included: age, gender, family history of osteoporosis, physical activity, calcium intake, vitamin D status, body mass index and a change of body mass index, hormone status among women, kidney disease, smoking, and the use of thiazide diuretics and bisphosphonates.

2.1 Risk factors of Osteoporosis

2.1.1 Age

Age is a major risk factor for osteoporosis (3, 60, 61). The risk of osteoporosis fractures increases with age, due to losses of BMD over time and to a higher risk of fracture for a given BMD with age (62-64) and an increased propensity to fall with age (65).

2.1.2 Gender

Gender is an important risk factor for osteoporosis (66), since women have lower peak bone mass relative to men in early adulthood and then have a period of greatly accelerated bone loss for 10 to 15 years around and after menopause. After the menopause transition, women resume a slower rate of loss, however 25% of the skeletal mass can be lost soon after menopause (67). For men, bone loss is usually a more constant process and usually of a lower magnitude relative to women.

2.1.3 Family history of Osteoporosis

A family history of osteoporosis among first degree relatives (parents, siblings and/or children) may indicate a genetic predisposition for lower BMD (68) and for fractures (69). The mechanism for osteoporosis occurring in families could be due to inherited traits and may also be due to family lifestyle habits. Osteoporosis is under diagnosed (70) so people are not always aware of the presence of this disease in themselves or a family member, and therefore this variable, regarding a family history of osteoporosis, may not be accurately reported.

2.1.4 Physical activity

Physical activity is an important modifiable prevention factor for osteoporosis, that likely works through two mechanisms: preventing or decreasing age-related bone mineral loss, and decreasing the risk of falling. A meta-analysis of randomized controlled trials indicated that exercise programs can prevent bone loss in adults (71). Recent work has attempted to determine which types of physical activities are the most effective at maintaining bone (72, 73). For adults, physical activities that involve resistance and/or impact prevent bone mineral loss more effectively than stretching or non-impact exercises (74-76). An additional benefit of being involved in physical activity for older adults is improved dynamic balance (77) which may explain a reduced risk of falling associated with exercise programs (78, 79).

2.1.5 Hormonal status among the women

Exogenous estrogen taken by women after menopause is effective at preventing the usual postmenopausal bone mineral loss (80, 81) that is characteristic of this phase of life (82).

2.1.6 Calcium intake and vitamin D status

Expert Committees that make recommendations regarding the prevention of osteoporosis, the Scientific Advisory Council of the Osteoporosis Society of Canada (83), the American Association of Clinical Endocrinologists (84), the Institute of Medicine (85) and the National Institutes of Health Consensus Development Panel on Osteoporosis (86), all recommended that adequate intakes of calcium and vitamin D be consumed to promote the attainment of a dense peak bone mass and to prevent the subsequent loss of bone, due to calcium or vitamin D deficiency, in later life. The intakes of calcium that are recommended for adults by these Committees are listed in Table 1.

RECOMMENDED CALCIUM INTAKES (MG/DAY)	ADULTS $\leq$ 50	OVER 50 YEARS
Institute of Medicine, 1997	1000	1200
National Institutes of Health Consensus Development Panel on Osteoporosis prevention, diagnosis and therapy, 2001	1000 to 1500	1000 to 1500
Scientific Advisory Council of the Osteoporosis Society of Canada, 2002	1000	1500
American Association of Clinical Endocrinologists, 2003	500 to 1000	500 to 1000

Table 1- Recommended Calcium intakes

RECOMMENDED VITAMIN D INTAKES (INTERNATIONAL UNITS/DAY)	ADULTS ≤ 50	OVER 50 YEARS
Institute of Medicine, 1997	200	400 to 600
National Institutes of Health Consensus Development Panel on Osteoporosis prevention, diagnosis and therapy, 2001	400 to 1000	400 to 1000
Scientific Advisory Council of the Osteoporosis Society of Canada, 2002	400	800
American Association of Clinical Endocrinologists, 2003	400	800

Table 2 - Recommended Vitamin D intakes

Vitamin D's role in the prevention or treatment of osteoporosis is not fully understood but work in this area has expanded recently. The current recommended intakes of this vitamin are listed in Table 2, however, recent studies have brought more interest to the question of the vitamin D requirements (87-91). A recent Cochrane review concluded that vitamin D along with calcium marginally reduced hip fractures, but not vertebral fractures in older people, while alone without supplemental calcium, vitamin D was not effective in reducing any fractures (92). These results are in contrast with another meta-analysis that concluded that vitamin D supplementation was associated with a decrease in vertebral fractures (93). This latter meta-analysis found that the fracture studies of vitamin D mostly used the hydroxylated version of vitamin D; therefore their conclusions refer to this less common formulation of the vitamin. A more recent meta-analysis determined that vitamin D reduces the incidence of hip fractures, but only when taken with calcium supplementation (94). As well as difference in calcium intake between the studies, it has been proposed that the difficulties experienced determining the importance of vitamin D in the prevention of

osteoporotic fractures has been due to the different doses, forms (vitamin D_2 versus D_3), short follow-up times, and other differences between studies (95).

The recommendations for vitamin D intakes are currently under question. It has been proposed that the biomarker of vitamin D sufficiency, 25-hydroxy-vitamin D, should be set at around 75 (96) to 80 (97) nmol/l rather than the 30 nmol/l that was used to derive the current recommended intakes for this vitamin (85). Heaney at al recommend that 80 nmol/l for 25-hydroxy-vitamin D be used based on the level associated with the lowest level of parathyroid hormone (98). Bischoff-Ferrari, Willett, Dawson-Hughes and others reviewed the evidence regarding vitamin D status and bone health (parathyroid suppression, fracture prevention, lower extremity function and falls) as well as colorectal cancer and dental health and concluded that the optimal serum 25-hydroxy-vitamin D levels are likely in the range of 75 to 100 nmol/l (99).

2.1.7 Body Mass Index and change of Body Mass Index

Low body weight or low BMI (BMI) (weight (kilogram(kg))/height squared (meters^2)) (kg/m2) and losses in weight or BMI over time have been associated with higher osteoporosis risk (100, 101), particularly among women (102). The factor in BMI that is important as a risk factor in osteoporosis is the fat mass and change in fat mass (103, 104).

2.1.8 Kidney Disease

Decreased kidney function is a risk factor for osteoporosis since it has been associated with hip fracture in women (105), and in men with a faster loss of BMD at the hip (106). As well, decreased kidney function has been associated with lower serum pH and bicarbonate concentrations, among adults aged 17 to 74 years (60). The authors assumed that these associations were due to age-related decline in renal function and acid excretion (60).

2.1.9 Parathyroid hormone

Parathyroid hormone acts to elevate a low serum calcium via bone resorption, enhanced renal calcium reabsorption and increased gastrointestinal absorption of calcium, the latter effect is through the conversion of vitamin D (25-OH vitamin D) to the active form (1,25-OH vitamin D) (107). Elevated parathyroid hormone is a risk factor for osteoporosis due to its bone reabsorbing action. It is possible that parathyroid hormone may be an intermediate in the mechanism of the acid-ash hypothesis, or it may be a confounder of the association. The data on this hormone in acid-ash clinical trials is mixed (108-111) so that it is not clear what role it may play and whether this hormone is important or not. If parathyroid hormone is an intermediary for the mechanism of the hypothesis, then it should not be included into the models as a potential confounder.

2.1.10 Smoking

In a recent meta-analysis, smoking was found to be detrimental to BMD among postmenopausal women but not among those who are still menstruating (112). There was evidence among men of a detrimental effect of smoking on BMD; however the available data did not permit examination for an age effect. hip fracture risk was increased in both sexes due to smoking (113).

2.1.11 Medications

There is some evidence that as well as decreasing urine calcium, thiazide diuretics may improve BMD over time (114), however, not all studies have documented this effect (115). Prospective observational studies have recognized improved fracture rates among subjects taking thiazide diuretics (116, 117), however, this effect may not be lasting once the medication has been discontinued (117).

Bisphosphonates are considered “first-line therapies for the treatment and prevention” of osteoporosis since they are effective at both inhibiting bone resorption and reducing fractures (118). Based on the results of a rat study, there

may be an effect of some bisphosphonates on blood acid-base equilibrium when renal function is impaired (119).

2.2 Issues of acid measurement: in food and in urine

2.2.1 Urine measures of diet acid load may be superior to food measures

Over the past century, attempts have been made to predict the dietary acid load of foods from foods (40, 48) and from urine (35, 51, 57, 120). Urine acid analysis may be a superior method to food measures for the measurement of the dietary acid load for three reasons. These reasons include: food composition tables only approximate the composition of any sample of food, food intake measurement tools are prone to random and systematic errors, and urine measures reflect the actual amount of minerals that are absorbed rather than the mineral intake.

2.2.2 Measurement of food intake

The measurement of food intake is a challenging science. First, the measurement of nutrient intake from foods in most studies is imprecise due to the reliance on food composition tables. Most studies that measure food intake calculate nutrient intakes based on the data from food composition tables. The foods consumed are assumed to have the composition of the published values. Some studies have analyzed a few selected nutrients in identical collections of the foods consumed by the study subjects (Table 6). Only selected nutrients were usually measured since these measurements are time consuming and expensive. Food varies in composition due to growing conditions, genetic variation, food preparation, and storage (121, 122). Food composition tables reflect the food samples that were measured during development of these tables, which may or may not reflect the composition of the foods consumed in a study.

Second, when food frequency questionnaires are used to estimate food intake of research subjects, errors are incorporated into the estimates due to random and systematic errors (123). Food frequency questionnaires ask the

subjects to specify their food intake by type and quantity over a time period, usually the past year. Random errors result from recall bias and the challenges subjects incur in estimating which foods were eaten and the amounts, on average, over a time period. As well, people tend to report their diets favourably (124) and tend to underreport their total intake (125), which leads to systematic errors or bias of food intake. The accuracy to which people report their food intake is known to be related to covariates such as gender, age, body weight status, and the type of food (e.g. desserts tend to be underreported more than vegetables) (126). Consequently, the measurement of food intake is not precise due to both random and systematic errors in recall and reporting.

Some nutrients are measured with more accuracy than others. Potassium, a nutrient of primary interest in studies of dietary acid (127), is not accurately measured in food frequency questionnaires (128, 129), due to random and/or systematic errors. For example, the precision of food frequency questionnaire measurement of potassium intake, a crucial nutrient in the calculation of diet base load, is low (128, 130).

Two studies examined the correlation between potassium intake as measured using a food frequency questionnaire and 24-hour urinary excretion levels (128, 131). These studies determined that the correlation between intake estimated from the food frequency questionnaire and from the urinary excretion ranged from 0.18 to .048, and concluded that the estimates from the food questionnaire were weak or poor (128, 132). A third study that examined the correlation between a 159-item food frequency questionnaire administered among eight year old children with a single overnight timed urine found the correlation for potassium was 0.14 (133). The poor precision for the measurement of potassium by food frequency questionnaires would lead to misclassification of subjects as to whether their potassium intakes were high or low, and lead to an attenuation of any estimated effect of potassium effect on bone.

Third, the absorption of nutrients by the gastrointestinal tract varies between foods, depending on the other foods consumed at the same time, the chemical form of the nutrient in the food, and the nutritional status and physiological state of the individual (134). In studies of acid-base balance, usually the variable of interest is the amount of absorbed nutrient rather than the intake. Fourth, dietary ions can vary in terms of the degree of protonation (for example phosphate as K_2HPO_4 versus H_3PO_4 (135) and possibly potassium from fruit versus from grain or dairy products (136)). The hydrogen ion content of these molecules may vary and their potential contribution to acidity also varies. The measurement of food and nutrient intake is imprecise for studying the diet acid load due to the need to rely on food composition tables, random and systematic errors for food intake measurement, variable absorption of nutrients in different foods by the gastrointestinal tract, and variation in the degree of acidity/alkalinity of the ions.

2.2.3 Urine measures of diet acid load

Due to these errors inherent in estimating nutrient intake from self reported food intake, urine measures of acid load, including urine pH and urine mineral or ion content, may provide better estimates of diet acid load than measures of food intake. Urine measures of nutrient intake do not suffer from these sources of error and provide an estimate of the quantity of nutrients absorbed, provided that the subjects are in approximate nutrient balance.

Urine measures of acid load are objective measures that reflect the absorbed nutrients rather than the amount consumed. Although Remer and Manz have proposed an algorithm to take the imperfect absorption of ions from food into account, absorption is variable from food to food, and compound to compound (134) . The measurement of acid excretion based on food intake has been criticized because food is a complex mixture of compounds, and nutrient absorption depends on the availability of nutrient and also on interactions with other foods eaten at the same time (137).

The body maintains tight control over systemic pH through excretion of hydrogen and/or bicarbonate ions via the lungs ($H+ + HCO_3- \rightarrow CO_2 + H_20$) and the kidneys ($H_2PO_4$, NH_4+, and HCO_3-) (43, 138). The kidney is an important route of acid excretion, and urine pH reflects the excretion of excess hydrogen ions (43). Generally, when the body excretes hydrogen ions the urine pH is less than 7.4. Therefore, hydrogen ion excretion, as measured as urine pH, may be a useful way to estimate the diet acid load.

In terms of minerals, generally healthy adults are in a nutrient balance (30), that is, the amount of a nutrient they absorb from the diet is usually very close to the amount they excrete in the urine. Studies of sodium and potassium excretion have documented that healthy adults excrete most of the sodium and potassium they consume in the urine (approximately 80 to 90% of intake or 90% of once absorbed ions (139, 140). The correlation between sulfate and protein intake has been documented to range from 0.77 (141) to 0.98 (142). Therefore it is possible that the urine measures of acid load excretion, that is, urine pH and the absorbed and excreted ions (PO_4^{---}, SO_4^{--}, Cl^-, Na^+, K^+, Ca^{++}, Mg^{++}) may be more directly related to the actual absorbed diet acid load than are the estimates calculated from food consumption records.

In conclusion, due to the numerous difficulties quantifying absorbed nutrients, the estimates of acid load obtained from urine may be superior to the estimates obtained from measures of food.

2.2.4 How the acidity of urine is measured

In the medical literature, the quantification of diet acid load has been described in three ways: from the estimation of the acid load of the food consumed (35, 143-145), through the measurement of urine acid content, or the urine pH (35, 51, 144). The urine acid load excretion has been measured using two approaches. a) Early studies estimated net acid excretion from the sum of urinary titratable acidity and ammonium minus bicarbonate (35, 143), and urine pH. These former components (titratable acidity and ammonium minus

bicarbonate) are considered to be inaccurate measures of acid load for two reasons. First, ammonium and bicarbonate are not stable in urine since they are volatile and altered by bacterial growth (138, 146, 147). Consequently they are difficult to quantify accurately, particularly in stored urine. Second, the measurement of titratable acidity is not a precise method because some neutral substances that are not part of the renal acid excretion mechanism in urine, such as creatinine, titrate as well as the compounds of interest (137, 148).

b) Net acid load excretion has been measured indirectly through the measurement of urine pH (35, 51, 57, 144, 149, 150). In these studies, changes in urine pH was used as an indicator of a change in the diet acid load and associated with a larger quantity of calcium excreted in the urine.

2.2.5 Effect of storage of urine samples on pH

Urine pH may change slightly with storage as carbon dioxide (CO_2) and ammonia (NH_3) evaporate, and the changes are in opposite directions. The CO_2 that is present in the urine as it is produced can evaporate without any change in pH, however, additional CO_2 can be formed from hydrogen ions combining with bicarbonate ions, causing an increase in pH through the loss of the hydrogen ions (H^+ and HCO_3^- <--> $H_2O + CO_2$) (146). pH is a measure of the hydrogen ion concentration (H+) concentration, thus the incorporation of hydrogen ion into H_2O will decrease the hydrogen ion concentration, which increases the measured pH (pH is the negative antilog of hydrogen ion concentration). This reaction will take place in proportion to the HCO_3^- concentration, therefore it would be greatest among those with more HCO_3^-, which are those with more alkaline urines (138). For those with acidic urine and little or no bicarbonate present, this reaction will occur only to a very small degree or not at all. Therefore this reaction will cause the alkaline urine to become somewhat more alkaline.

Ammonia (NH_3) in urine can evaporate, from the equilibration state with ammonium, and in doing so, hydrogen ions are liberated from ammonium (NH_4^+ <--> $H^+ + NH_3$) and therefore the measured hydrogen ion will increase and thus

the pH will decrease. This loss of ammonia would be greatest among those with more NH_4^+, which are those with the more acidic urine. Therefore, the net effect of the loss of CO_2 and NH_3 will increase the spread in the pH range, since alkaline urines will increase in pH and acidic urines will decrease in pH as these compounds are lost to the air. Therefore, these effects are not likely to contribute to errors in the classification of urine pH as acidic urine samples will become more acidic and alkaline urine samples will become more alkaline.

2.2.6 Fasting morning urine samples

Several cross-over trials of acid loading demonstrated that urine reflects the acid load of the diet in terms of net acid excretion (35, 57, 143, 145, 151, 152) and urine pH (35, 51, 57, 144, 149, 150). When the dietary acid load is changed, there is a short delay before the urine pH reflects the change in acid load (57, 152, 153). Although urine measures of acid load are quite sensitive to the composition of the diet, this delay suggests that the urine excretion of the acid load is not immediately sensitive to changes in the diet. The reason for this delay is not clear; however it may be due to the time required for absorption, metabolism and renal clearance of acid and alkaline compounds. Because of this delay, urine measures of acid excretion appear to reflect the food intakes over more than one day and urine measures of acid excretion may be a valuable measure of overall diet acid load.

Most analyses of urine acid load excretion have been based on 24-hour urine collections however, fasting morning urine could be as sensitive to the diet acid load as 24-hour collections. Three cross-over studies examined acid load excretion in both 24-hour and fasting urine samples and found that the collections had similar differences of magnitude in the comparisons of urine pH, net acid excretion and calcium excretion despite the differences of duration in collection times (51, 55, 152)(Thierry Buclin, personal communication, January 10, 2005). In a study of potassium deprivation of adults consuming formula diets, Lemann et al saw most of the same directional changes in urine composition whether fasting or post-breakfast sample versus 24-hour collections were used

(152). As well, a cross-sectional study that examined urine minerals in a time overnight urine sample with respect to eight year old children's BMD found a correlation between urine potassium and BMD at all bone sites (femoral neck, lumbar spine, and total body) (133). Further, in a cross-sectional study to examine whether estimates of acid excretion predicts urine pH in healthy adults, Michaud et al found the correlations between urine pH and acid load estimates from diet were similar whether the urine collections were full or partial (144). In summary, the literature provides enough evidence that less than 24-hour urine collections should provide similar results for the measurement of urine acid load excretion.

This study will use stored samples of fasting morning urine to estimate the diet acid load. The pH was used as a proxy for dietary acid load and related to changes in BMD over 5-years. These urine samples were collected from the second void of the day, while a fast was maintained, and have been stored in a –70 degree Celsius freezer from within hours of collection in 1996-7 to 2006. The samples have been stored for a decade. The storage of urine samples will result in some changes, which are not expected to lead to misclassification errors.

2.2.7 To quantify dietary stability, as measured in urine, over time

The interpretation of relationships between a predictor variable and an outcome variable "requires some knowledge of the error structure and its likely impact" on the association of interest (154). In other words, unstable predictor variables (i.e., those with large measurement errors over time) tend to decrease their apparent associations with the outcome of interest (155). Thus, it is important to quantify the level of dietary stability over time and the measurement error to determine whether a single measure of urine acid load can be used as an index of long-term diet acid load excretion. Predictor variables can be assessed for their stability by collecting two measures at different time points on a sample of individuals, and then quantifying the level of agreement for individuals and measurement error between these two measures. High levels of agreement and low levels of variability indicate stability over time (156).

Chapter Three: A meta-analysis of the acid-ash hypothesis of osteoporosis

The purpose of this section of the literature review is to conduct a meta-analysis of the acid-ash hypothesis to examine the evidence about the role of the modern diet in the development of osteoporosis in apparently healthy people. Specifically this section aims to conduct a meta-analysis to:

- Assess whether there is a mathematical association between net acid excretion and calcium excretion

- Estimate the quantity of calcium excretion in the urine associated with the modern diet

- Assess whether the quantitative difference in calcium observed between acidic and alkaline urine might be due to the lower solubility of calcium in alkaline urine (157, 158).

3.1 Methods for the meta-analysis

3.1.1 Literature search for the meta-analysis

Literature relating to the acid-ash hypothesis was identified through computerized searches using, but not limited to, keywords/textwords: acid-base equilibrium, bone or bones, and bone density as well as the text words: calciuria, calcium, excretion, net acid excretion, acid excretion, biopsy, fracture(s), and bone mineral density. The databases searched included PubMed back to 1966, Cochrane Database of Systematic Reviews, CINAHL back to 1982, EMBASE back to 1980, and the Cochrane Controlled Trials Register, up to July 2007. Reference lists were reviewed for additional relevant studies.

3.1.2 Selection criteria for the meta-analysis

Studies which examined the acid-ash hypothesis were included if they manipulated subjects' acid-base intake through foods or supplemental salts such as potassium bicarbonate and reported the change of net acid excretion and the outcomes of either calcium excretion or bone health changes (changes in bone mineral density (BMD), fractures or bone biopsy) in healthy adult subjects. The included studies were limited to those with a manipulation of the subject's acid-base intake, and therefore the design was limited to clinical trials or cross-over studies. Since the aim of this review was to study the potential for the acid-ash hypothesis to have a role in the development of osteoporosis in apparently healthy people, studies were not included if the subjects had chronic conditions such as renal diseases, diabetic keto-acidosis, spinal cord injury or acute effects of drug abuse or poisoning. Studies of infants and children were not included, nor were those in which the subjects were in conditions such as fasting or weight loss. Studies of people predisposed to renal stone formation were only included if there was a group without renal stones that could be included in the meta-analysis. To accurately estimate the quantity of calciuria only studies that collected urine over a 24 hour time period were used. Since the Hill criteria for Temporality cannot be met in cross-sectional studies, these studies were not included. The meta-analysis was not limited to English language articles.

3.1.3 Description of studies for the meta-analysis

The literature search identified 94 studies of which 26 met all the inclusion criteria (Table 3) (35, 39, 41, 54, 55, 57, 108-111, 149, 150, 159-172). All of the studies reported net acid excretion as the exposure, as determined in the selection criteria, and all except one measured calcium excretion as the outcome. This latter study measured a change of BMD after 12 months of potassium-citrate or potassium-chloride in an randomized controlled trial (172). This study is discussed below under Outcomes. Of the remaining 25 studies, two were randomized controlled trials (110, 167), one was a non-randomized clinical trial (111), 21 studies had a cross-over design, ten of which randomized the order

of treatments (39, 54, 108, 109, 149, 166, 168-171) and 11 that did not randomize the order (35, 41, 55, 57, 150, 159-165). Acid-ash interventions included alteration of food or nutrient intakes and/or administration of acidic or alkaline salts, such as potassium bicarbonate or ammonium chloride. The manipulations to alter diet acid load included: changes in food intake (35, 39, 108-110, 149, 150, 160-166, 168-171), sulfur-containing amino acids (35, 162), supplements of potassium bicarbonate (41, 54), ammonium chloride (55, 159), or potassium citrate (111, 167), substitution of sodium/potassium-chloride with the bicarbonate salts (57, 150), or a combination of food and salts. The majority of investigators (20/25 studies) controlled calcium intakes of their subjects, in an attempt to prevent differences in calcium excretion due to changes in calcium intake (Table 3). None of the foreign language papers met the criteria for acceptance.

Many studies, including some which have been well quoted, were not included in the meta-analysis for the following reasons: No numerical results (143, 152, 173, 174); no quantification of net acid excretion (51, 175-178); or more than one intervention was performed at the same time (145). Numerous studies were observational and were not included since there was no manipulation of acid intakes (36, 37, 46, 52, 133, 136, 144, 179-188). Some studies did not qualify for the meta-analysis since the urine collection was for time periods that were shorter than 24 hours (120, 172, 189-191).

	Study	year	Intervention	N	Design	Random	Concealed	Blind	Accounted for losses	Ca days	Ca intake (mg/d)
1	Weber (159)	1976	NH_4Cl	6	CO	no	no	no	no	0	1000
2	Schuette(160)	1980	Amount of protein	11	CO	no	no	no	no	0	800
3	Hegsted (161)	1981	Amount of protein	6	CO	no	no	no	no	0	500
4	Lutz (150)	1981	Amount of protein	8	CO	no	no	no	no	0	700
5	Schuette (162)	1981	Amount of protein	8	CO	no	no	no	no	0	500
6	Lutz (163)	1984	$NaHCO_3$	6	CO	no	no	no	no	8	500
7	Lemann (55)	1986	NH_4Cl	5	CO	no	no	no	no	0	1300
8	Breslau (108)	1988	Type of protein	15/10	RCO	yes	no	no	no	9	400
9	Lewis (164)	1989	Ca sources	8	LSD	no	no	no	no	0	1600
10	Trilok (165)	1989	Amount of protein	8	CO	no	no	no	no	1	800
11	Remer (35)	1994	Amount of protein	6	CO	no	no	no	no	3	?
12	Sebastian (41)	1994	$KHCO_3$	18	CO	no	no	no	no	12	650
13	Dahl (166)	1995	Lentils	10	RCO	yes	no	no	no	14+	usual
14	Frassetto (54)	2000	KHCO3	19	CO	no	no	no	no	-	usual
15	Sellmeyer (167)	2002	Kcitrate	60	RCT	yes	no	yes	no	18	500
16	Maurer (57)	2003	HCO3-	9	CO	0	no	no	no	5	1000
17	Roughead (168)	2003	Amount of protein	15	RCO	0	no	no	yes	20	600
18	Ince (149)	2004	Amount of protein	42	RCO	?	no	no	yes	5	same

19	Marangella (111)	2004	Potassium citrate	52	Trial	no	no	no	no	?	?
20	Gettman (169)	2005	Cranberry juice	12	RCO	yes	no	no	no	5	400
21	Kerstetter (39)	2005	Amount of protein	13	RCO	yes	no	no	no	10	800
22	Roughead (170)	2005	Meat/soy	13	RCO	yes	no	no	yes	21	700
23	Spence (171)	2005	Soy vs. milk protein	15	RCO	yes	no	yes	no	14	1100
24	Jajoo (110)	2006	Grains/Fruit&veg	20	RCT	yes	no	no	no	13	600added
25	Kerstetter (109)	2006	Amount of protein	20	RCO	yes	no	no	no	14	800
26	Jehle (172)	2006	Kcitrate vs KCl	161	RCT	yes	no	yes	yes	-	usual

CO = cross over study, RCO = randomized cross over study, LSD = Latin square design, RCT = randomized controlled trial, Trial = non-randomized trial, Concealed refers to concealment of allocation to groups, Ca days = number of days subjects received calcium intake prior to outcome measurement, Ca intake (mg/d) = calcium intake in milligrams per day

Table 3 - Studies included in the meta-analysis regarding change of net acid excretion and changes of calcium excretion or BMD

3.1.4 The methodological quality of the studies of the acid-ash hypothesis studies

The studies were assessed for the following eight indicators of methodological quality: randomization to groups or order of treatments, concealment of randomization, blinding of intervention, complete follow-up, blinding of outcome measurement, intent-to-treat analysis, calcium intakes, and duration of control of the subject's calcium intakes prior to urine measurements. Efforts were made to contact investigators for additional information and/or clarification when necessary. Subjects were allocated to treatment groups or to the order of treatment by randomization in 13 of the 26 studies included in this review. None of the studies described any concealment of allocation to groups. Only three studies mentioned any attempt to mask or blind subjects to their group allocation (167, 171, 172). None of the studies reported using an intention-to-treat analysis. Additionally, only nine (109, 111, 149, 166-168, 170-172) of the studies reported whether or not all of the subjects completed the interventions. The methods used to control calcium intakes are described below. In summary, the methodological quality of the studies was limited and therefore, it is possible that the findings from these studies may provide biased estimates (192) of the effect of the acid load on calcium excretion.

3.1.5 Methods of the meta-analysis

The net acid excretion of the modern diet was estimated from the weighted average of the control arm of the studies. The intervention studies that measured the change of urine calcium and change of net acid excretion were entered into a database (Table 4) and regression analysis (weighted by study sample size) was used to assess whether there was evidence of a relationship across the studies and to estimate the change of urine calcium for every unit of net acid excretion (193). For those studies that measured the outcomes from each intervention at multiple times, the outcomes were averaged together to provide one set of acid excretion and calcium values (168, 170). Some studies reported more than one intervention, and each comparison to the control was

included in the meta-analysis; in all 34 comparisons and 509 observations were included (Table 4).

Two variables were considered possible confounding variables, calcium intakes and urine acidity. Higher calcium intakes lead to increased urinary calcium excretion (85). Calcium forms insoluble salts in alkaline urine that are not measured when urine calcium is measured (157, 158) and it is possible that there could be a measurement error for the association between the diet acid load and calcium excretion in the studies that did not acidify alkaline urine prior to calcium measurement. Therefore the potential confounding variables considered were whether the urine was acidic and the subjects' calcium intakes.

Studies were categorized as acidic if a) the urine was treated with acid prior to analysis or, b) if the mean urine pH was < 6.5 (157) in both treatment arms. Researchers were contacted to clarify whether the urine samples were treated with acid prior to analysis if this detail was not clear in the report (35, 149, 150).

To assess whether the three outlying values were influential, the regression analysis was repeated after removal of those three studies.

Study	Intervention	n	Control Ca	Change NAE mEq/d	Change uCa mmol/d	Acid treat	Max upH	Acidic urine
Weber (159)	NH_4Cl	6	yes	216	9.1	no	5.97	yes
Schuette (160)	Amount Protein	11	yes	37	2.15	yes	-	yes
Hegsted (161)	Amount Protein	6	yes	38.1	2.48	yes	-	yes
Lutz (150)	Amount Protein	8	yes	56.0	2.05	yes	-	yes
Schuette (162)	Amount Protein	8	yes	32	1.20	yes	-	yes
Schuette (162)	Amount Protein	8	yes	46.5	3.56	yes	-	yes
Lutz (163)	$NaHCO_3$	6	yes	-60	-1.5	yes	6.9	yes
Lutz (163)	Amount Protein	6	yes	39	2.25	yes	6.1	yes
Lemann (55)	NH_4Cl	5	yes	209	7.3	no	6.7	no
Breslau (108)	Vegetarian	10	yes	-27.1	-1.1	no	6.55	no
Breslau (108)	Ovo-vegetarian	15	yes	-13	-0.7	no	6.32	yes
Lewis (164)	$CaCO_3$ vs. Milk	8	yes	21.3	-0.6	yes	6.67	yes
Lewis (164)	$CaCO_3$ vs. CaCl2	8	yes	28.0	0.6	yes	6.67	yes
Trilok (165)	Amount Protein	8	yes	16.46	1.39	no	6.67	no
Remer (35)	Methionine	6	no	42.9	0.9	no	6.7	no
Remer (35)	Medium Protein	6	no	45.6	2.0	no	6.7	no
Remer (35)	High Protein	6	no	111.4	2.4	no	6.7	no
Sebastian (41)	$KHCO_3$	18	yes	-58.1	-1.6	yes	-	yes
Dahl (166)	Lentils	10	yes	3.1	-0.9	yes	-	yes
Frassetto (54)	$KHCO_3$	19	no	-38	-0.7	no	-	no
Sellmeyer (167)	Potassium citrate	60	yes	-53	-1.25	no	-	no
Maurer (57)	HCO_3^- vs Cl-	9	yes	-71	-0.6	no	7.07	no

Study	Intervention	n	Control Ca	Change NAE mEq/d	Change uCa mmol/d	Acid treat	Max upH	Acidic urine
Roughead (168)	Amount Protein	15	yes	23.1	-0.08	no	6.02	yes
Ince (149)	Amount Protein	42	yes	-21.5	-1.1	yes	-	yes
Marangella (111)	Kcitrate	52	no	-21	0.275	yes	6.33	yes
Gettman (169)	cranberry	12	yes	8.6	0.4	yes	5.97	yes
Kerstetter (39)	Amount Protein	13	yes	68.9	1.66	no	-	no
Roughead (170)	Meat/soy	13	yes	-11	0.05	no	6.33	yes
Spence (171)	Soy/milk protein	15	yes	1.6	1.03	yes	-	yes
Jajoo (110)	Grains	20	-	17	0.09	no	-	no
Jajoo (110)	Fruit/veg	20	-	7.8	0.49	no	-	no
Kerstetter (109)	Meat/soy	20	yes	-24	-0.07	no	-	no
Kerstetter (109)	Amount soy	20	yes	28.6	0.83	no	-	no
Kerstetter (109)	Amount meat	20	yes	18.4	1.52	no	6.41	yes

Control Ca refers to control of calcium intake, NAE = net acid excretion, Acid Treat refers to whether the urine was acid treated, Acidic urine refers to whether the urine was acidic due to either treatment with acid or naturally acidic in both arms of the study, uCa = urinary calcium upH = urinary pH. mEq = milliequivalents, mmol/day = millimoles per day.

Table 4 - Interventions of altered acid/base intake and change of net acid excretion and the outcome of calcium excretion

3.2 The results of the meta-analyses

Although five of the 25 studies did not demonstrate greater calcium excretion with higher net acid excretion (111, 164, 166, 168, 170), there was a significant relationship between net acid excretion and calcium excretion for both acidic and alkaline urine for all of the studies once combined together in the meta-analysis. One of the studies demonstrated an opposite direction for the changes of urinary calcium relative to the acid-ash hypothesis since the substitution of soy protein with lentils led to a non-significant increase in net acid excretion and a significant decrease in urinary calcium ($p < 0.01$) (166).

The approximate quantity of net acid excretion from the modern diet was 45 milliequivalents (mEq)/day, based on the weighted average of 481 twenty-four-hour urine measures of the control arm in the studies (35, 41, 54, 108, 111, 149, 159, 168-172, 194) that provided an estimate. In the regression analysis, whether or not the urine was acidic (acidic or acid-treated) significantly modified the relationship between net acid excretion and calcium excretion. There was a significant difference in the rates of increase in urinary calcium with a change in renal net acid excretion dependent on whether the urine was acidic ($p < 0.001$, Figure 1).

For the more alkaline urine, the change in urine calcium for the 45 milliequivalent change in net acid excretion was 1.4 mmol (57 mg) compared to 2.0 mmol (81 mg) for those with acidic urine (acidic or acid-treated) (p-values both < 0.001). Change in urine calcium (mmol/day) in acidic urine = 0.323 + net acid excretion x .0380 ($R^2 = 0.8208$). Change in urine calcium (mmol/day) in alkaline urine = 0.218 + net acid excretion x .0266 ($R^2 = 0.9056$).

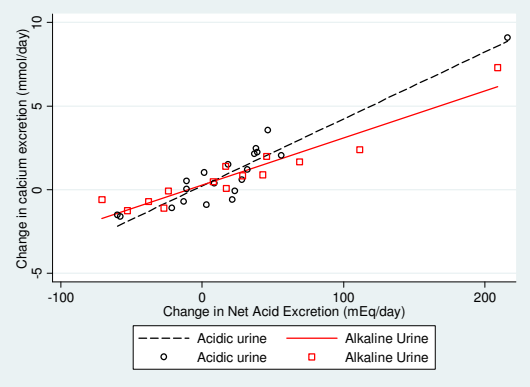


Figure 1 - The effect of diet acid load on calcium excretion, stratified by whether the urine was treated with acid.

After removal of the three outlying study results, the significant changes in urine calcium in response to changes in net acid excretion, which depended on the acid treatment of the urine, were still apparent (Figure 2).

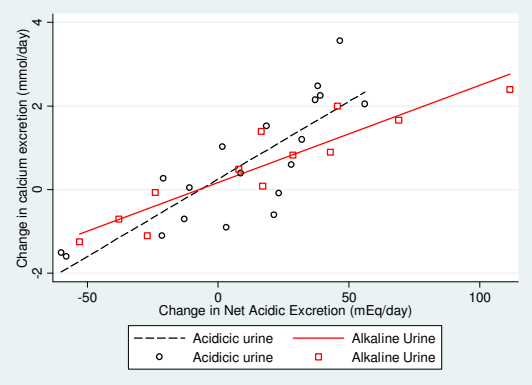


Figure 2 - The relationships remain after omission of two outlying results.

The results of this meta-analysis can be used to assess the third objective, to assess whether the quantitative difference seen between acidic and alkaline urine calcium might be due to the lower solubility of calcium in alkaline urine. These findings of a relationship between net acid excretion and calcium excretion in both acidic and alkaline urine demonstrate that calcium insolubility does not explain all of the higher concentration of calcium in acidic urine. The significant difference seen between the acid-treated and non-acid treated urine

demonstrates that there is a difference in the quantity of calcium measured in the urine depending upon whether the urine is acid treated.

Chapter Four: Does the modern diet promote bone weakness? Hill's criteria of causation as a framework for analysis of the acid-ash hypothesis

The purpose of this section of the literature review is to evaluate whether the modern diet, under the acid-ash hypothesis, as a cause of osteoporosis using Hill's criteria. Hill's criteria of causation assist the assessment of whether of causal relationships exist between an exposure and a disease (195-197). These criteria consider the Strength of the evidence, the Consistency of findings between the various studies of various designs with subjects from different populations, whether there is evidence that the exposure precedes the disease in time (Temporality), whether the concept is Biologically Plausible, whether there is evidence of a dose-response or Biological Gradient for the relationship, and whether Experiments have been done to determine whether altering the exposure results in changes in disease frequency. These criteria can be used to assess the strength of the evidence from a variety of sources and are useful to assess the relationship between acid excretion and osteoporosis.

4.1 Methods

Literature selected for the meta-analysis (Chapter 3) was used and additional literature searches were conducted to find literature regarding prospective cohort studies of the intakes of fruit and vegetables and changes in BMD and intervention studies regarding the effect of changes in phosphate intake on changes in BMD or calcium excretion. These studies were identified through computerized searches using but not limited to keywords/textwords: fruit, vegetables, phosphorus, phosphate, bone or bones, and bone density as well as the text words: calciuria, calcium, excretion, balance, biopsy, fracture(s), and bone mineral density. Only studies in which the intervention or exposure

occurred prior to the measurement of the outcome were included in the review to ensure that the criterion of Temporality was met. The databases searched included PubMed back to 1966, Cochrane Database of Systematic Reviews, CINAHL back to 1982, EMBASE back to 1980, and the Cochrane Controlled Trials Register, up to July 2007. Reference lists were reviewed for additional relevant studies.

4.2 Biological gradient (dose-response relationship)

Hill's criterion of causation regarding a Biological Gradient requires that when the dose of an exposure is increased, the risk of the outcome should also increase. The studies of the acid-ash hypothesis exposed subjects to a wide range of acid or base treatments, specifically from a change in net acid excretion from a decrease of seventy-one (57) to an increase of over 200 (55, 159) mEq/day. The finding of a significant linear relationship between net acid excretion and calcium excretion for both acidic and alkaline urine demonstrates that there is a biological gradient for this relationship.

4.3 Strength

The Strength criterion requires that the putative cause of an illness be of sufficient strength of association to cause the disease. Is the magnitude of excess calciuria induced by the modern diet sufficient that it could lead to the development of osteoporosis? The studies of the acid-ash hypothesis suggest that the daily excess calciuria from excess diet acid load could be 80 mg/day (2.0 mmol/day) (See the Meta-analysis section 3.2). A continuous loss of 80 mg/day would lead to 29 grams/year or 580 grams over 20 years. Adult humans have about 1150 grams of calcium in their skeletons (198). A loss of 580 grams is equivalent to about half of the skeleton calcium which would be a substantial loss of bone mineral and would be considered rapidly progressing osteoporosis. Therefore, the calciuria associated with the modern diet is sufficient in quantity that it could explain the progression of osteoporosis. In other words, the acid-ash

hypothesis has been shown to have sufficient strength in terms of calciuria to be a cause of osteoporosis, if the excess calcium is derived from bone.

4.4 Plausibility

Hill's criterion regarding plausibility requires that a theory fit with current biological knowledge. Although Hill included this criterion in his list, he also stated that "this is a feature I am convinced we cannot demand" since at any point current understanding of the biological world is limited. Under the plausibility criterion, a theory needs to be examined for any difficulties or lack of fit with current existing biological knowledge (197).

4.4.1 The plausibility for the mechanism of bone involvement in the acid-ash hypothesis

The acid-ash hypothesis purports that acid from the modern diet promotes urinary calcium loss and bone mineral resorption. However, the mechanism for the mineral resorption at the bone is not well described. Some researchers assert that bone is dissolved, releasing skeletal calcium and bicarbonate to neutralize the systemic acidosis (41, 54, 110, 111, 145, 167). Others hypothesize that at the kidney, calcium is lost in the urine when urinary bicarbonate is reabsorbed from the distal nephron to compensate for the excretion of acid (111, 160). In vitro studies of animal bones by Arnett et al. and others have demonstrated higher rates of bone demineralization when exposed to pH below the physiological range ($\leq$ 7.3) (199-202). However, in vivo systemic pH changes only minimally (between 0.0014 and 0.02 pH units (41, 51, 57)) in response to the experimental changes to diet or acid-base supplementation. Whether these minute changes in systemic pH can influence the activity of osteoclasts in vivo has not been established.

4.4.2 The plausibility regarding the internal consistency of the acid-ash hypothesis model

The components of the model used to calculate the acid load of foods (phosphate (PO_4^{---}) + sulfate (SO_4^{--})+ chloride (Cl^-) + organic acids (OA)) minus

(sodium (Na^+) + potassium (K^+) + calcium (Ca^{++})+ magnesium (Mg^{++}) do not necessarily act in the way the model assumes. The acid-ash hypothesis model states that phosphate, sulfate, chloride and organic acids reflect acid intake and increase calciuria and are detrimental to bone health (35). In contrast, sodium, potassium, calcium and magnesium are considered to reflect base intake, to decrease calciuria, and exert a positive effect on bone (180). However, as will be discussed below, the evidence regarding the effect of the individual cations and anions on urinary calcium and bone outcomes does not follow this simple concept.

4.4.2.1 Phosphate:

Under the acid-ash hypothesis, phosphate is one of the anions considered to contribute to acid excretion and hence increase urinary calcium losses (35, 45). However, contrary to expectations under the hypothesis, additional phosphate actually caused no significant change (135, 189) or a decrease in urinary calcium excretion (162, 203-211) (Table 5).

Thirteen studies of supplemental phosphate were found (135, 162, 189, 204-212). All of the studies assessed the effect of supplemental phosphate salts on urine calcium; 11 administered these salts orally, two intravenously. Five of the eight studies employed randomized study designs (randomized cross-over and randomized block designs) while the remainder used non-randomized cross-over (n = 7 studies) and one non-randomized trial designs.

The phosphate ion exists in four degrees of protonation: H_3PO_4, H_2PO_4, HPO_4 or PO4. The eight studies that provided subjects with “near neutral” phosphate (valence at systemic pH of 7.4 is -1.8; at pH = 7.4 80% is in the divalent form HPO_4^{-2} and 20% is in the monovalent form: $H_2PO_4^-$ (43)) demonstrated a significant decrease (162, 204, 207-210, 213), or a non-significant decrease (189) in calcium excretion in response to the phosphate supplement.

Study	Year	Design	Phosphate source	n	Outcomes		
					Change in Net acid excretion	Urinary pH	Change in Calcium excretion
a) Oral phosphate							
Spencer (173)	1965	CO	Glycerophosphate	5	-	-	decrease
Goldsmith (174)	1976	CO	K_2HPO_4 KH_2PO_4 ratio 1.5:1	7	-	-	decrease
Spencer (175)	1978	CO	$NaGlycPO_4$	29	-	-	decrease
Schuette (132)	1981	CO	KH_2PO_4 (monobasic)	16	increase	-	decrease
Spencer (176)	1986	CO	$NaGlycPO_4$	10	-	-	decrease
Silverberg (177)	1986	Trial	Neutral PO_4	13	-	-	decrease
Heaney (178)	1987	CO	Neutral PO_4 vs. $NaHCO_3$	8	-	-	decrease
Whiting (159)	1997	RBD	NaH_2PO_4	10	NS increase	-	NS decrease
Whybro (179)	1998	RCO	Near neutral PO_4: NaH_2PO_4 + Na & K HCO_3	22	-	-	decrease
Heaney (105)	2001	RBD	$H3PO_4$ vs H_2O	30	NS increase	no change	no change
Kemi (180)	2006	RCO	Na_3PO_4 + Na_2HPO_4	14	-	-	decrease
b) Intravenous phosphate							
Krapf (181)	1995	CO	Neutral sodium phosphate vs NaCl	6	increase	increase	decrease
Berkelhammer (182)	1998	RCO	TPN: PO_4^- vs Cl^- as K^+ salts	7	-	-	decrease

CO = cross over study, Trial = non-randomized trial, RBD = random block design, RCO = randomized cross over study, NS = non statistically significant

Table 5 - Phosphate studies

No significant change in either urine calcium or net acid excretion were observed by Heaney et al. in subjects provided with the most protonated version of phosphate (2.5 mmol/day of H_3PO_4) in 567 ml of caffeine-free cola for five hours post ingestion compared to water (135). Spencer et al. provided the most alkaline form of phosphate (sodium glycerophosphate) and noted a decreased calcium excretion (206) among the higher phosphate phase. All of these studies except the one that compared cola to water (135) controlled the calcium intakes of the subjects (162, 189, 204-211, 213).

Regarding acid excretion, four of the phosphate studies measured net acid excretion. The effect of additional phosphate on net acid excretion did not follow the expected pattern under the acid-ash hypothesis. The net acid excretion increased statistically significantly in two studies (162, 213) and non-significantly in two others (135, 189) in response to the phosphate supplementation. The two studies that demonstrated the statistically significant increase had provided their subjects with near neutral sources of phosphate (162, 213). The non-significant increases in net acid excretion had provided the most protonated from of phosphate in the cola (135) or a near neutral form (135). Therefore the effect of additional phosphate on net acid excretion did not follow the predicted pattern of an increased acid excretion with the addition of the acidic form of phosphate and no change in response to the neutral form.

The two phosphate supplementation trials measured urine pH also did not see the expected effects. Heaney and Rafferty observed no statistically significant change in urine pH in response to the cola, in comparison with water (135). Krapf et al observed an increase in urine pH in response to neutral phosphate in comparison to the chloride ion (213). The acid-ash hypothesis would have predicted that the cola would induce a lower pH urine while the neutral phosphate should have had no effect.

The intravenous studies had the advantage of avoiding the gastrointestinal tract and therefore the complexing of calcium with orally administered phosphate.

Karpf et al administed either neutral sodium phosphate or sodium cholide to orally feeding subjects, while Berkelhammer varied the potassium anion in parenteral nutrition between the phosphate and chloride ions to subjects nourished intravenously. Both of these studies observed a decrease of calcium excretion in response to the phosphate salt, which suggests that the mechanism for decreased calcium excretion is not due to complexes of calcium with phosphate forming in the gut, and subsequent reduced absorption.

Therefore, in spite of the prediction under the acid-ash hypothesis that phosphate should increase urinary acid and calcium excretions, the evidence revealed that in spite of an increase of acid excretion, supplemental phosphate caused urinary calcium to decrease. This hypocalciuric effect of phosphate may have a confounding effect in whole food studies of protein and calcium excretion since phosphate is highly correlated to protein intakes (214).

4.4.2.2 Organic acids:

Organic acids (including acetic, acetoacetic, citric, formic, hippuric, hydroybutyric, lactic, and uric acids) exist in urine as protonated and anion forms. Some fruits such as cranberries and plums contribute hippuric acid which is subsequently excreted in the urine. The acid-ash hypothesis dictates that a factor for organic acid based on body surface area be added in when estimating the diet acid load (35, 215). The consumption of one litre of juice compared to deionized water was associated with a significant decrease in urine pH and a non-significant ($p = 0.06$, $n= 12$) increase in urinary calcium (169).

However, there are no studies that assess whether bone mineral is lost when cranberries are consumed. Studies of plums, which also contain hippuric acid, found a positive association between the consumption of plums and measures of bone health. A randomized trial of 100 grams per day of dried plums versus dried apples added to the diet of post menopausal women, for three months, found improved markers of bone mineralization in the women consuming the plums (216). Improved bone density and microstructure of the

bones of rats fed dried plums as part of their food has also been reported (217). Therefore, the prediction of the acid-ash hypothesis that organic acid excretion is associated with loss of bone mineral is not completely understood. At this point there is very little information to guide clinicians regarding which individual foods may be supportive of bone health or what components may confer the protective effect.

4.4.2.3 Sodium:

In the acid-ash hypothesis model, sodium is one of the cations that has been associated with base excretion and would theoretically protect against urinary calcium losses. In the model, sodium is considered to be equivalent to potassium, calcium, and the other cations, however, evidence indicates that sodium and potassium salts do not equally affect urinary calcium (145, 189). Some experts consider that high sodium intakes are a risk factor for BMD loss (218). Perhaps the differences in relationship between these cations and calcium excretion relates to their differing roles in renal hydrogen ion excretion: Sodium is involved in neutral $Na^+/HCO3^-$ exchange and potassium is involved in the H^+, K^+ ATPase pump (45). It may be too simplistic under the acid-ash hypothesis model to categorize sodium, potassium and the other cations as equivalent ions.

4.4.2.4 Protein:

The studies regarding calcium excretion with varying protein diets have not produced consistent findings, in terms of the source of the calcium in the urine. According to the acid-ash hypothesis, as protein intake increases, the acid load increases and, bone mineral is dissolved to neutralize acids and avoid systemic acidosis. Increased calcium in the urine has been considered confirmation of this theoretical effect (35, 108, 149, 160, 180). Whether this calcium comes from the bones has been brought into question by a recent study that used superior methods to measure calcium flux in the body, specifically calcium isotopes, administered two ways simultaneously, intravenously and orally (equilibrated with the calcium in the milk) (39). Kerstetter et al, determined

that the source of the calcium was improved absorption induced from a higher protein intake (39). Contrary to expectations under the acid-ash hypothesis, the amount of calcium excreted from bone origin, using a multicompartmental model of calcium kinetics between an oral and an intravenously administered calcium tracer, was lower when the subjects were consuming the higher protein intakes. The other protein studies of acid excretion had mixed results in terms of calcium absorption with the increase of protein intake. In response to the increased protein intake, two of the studies documented that the majority of the subjects experienced increased absorption (150, 160), while two other studies saw no change in calcium absorption (161, 163). It is possible that the differences in findings regarding protein are due to the methods of the balance studies. Further discussion regarding calcium absorption can be found below under Consistency. It can be acknowledged that discrepancies exist in the results between the protein diet studies regarding the source of the excreted calcium.

Therefore, based on biological knowledge of the day, the biological plausibility of the acid-ash hypothesis has weaknesses in terms of the biological mechanism, and how the phosphate, organic acids, sodium, and protein components of the model act in the human body.

4.5 Consistency

Hill's criterion regarding consistency requires that there be evidence from numerous studies that differ in study design elements that support the acid-ash hypothesis. The acid-ash hypothesis has been studied among humans in a variety of different ways to achieve altered acid-base intakes. Study designs implemented include cross-overs, randomized cross-overs, randomized controlled trials, non-randomized trials and one with a Latin squares design. The interventions to alter the diet acid load included changing food intake (35, 51, 108-110, 120, 149, 150, 160, 162-165, 168-171, 189, 219), by providing supplements of potassium bicarbonate (54, 189, 190, 220), ammonium chloride

(55, 159, 191, 221), or potassium citrate (111, 167); and substituting sodium/potassium-chloride with the bicarbonate (57) or the citrate salts (172).

4.5.1 Calcium excretion

Of the 24 studies of calcium excretion in relation to significant changes in net acid excretion, four studies did not reveal increased calciuria in response to significantly higher dietary acid loads (111, 164, 168, 170). The interventions in these studies included changes of food intake in well-controlled metabolic studies (168, 170), calcium carbonate versus milk (164), and a potassium citrate supplement (111).

4.5.2 Changes in Bone mineral density

Some studies have used changes in BMD as the outcome in studies of the acid-ash hypothesis. So far only one randomized control trial used change of BMD as an outcome. This trial revealed a positive effect of potassium citrate supplements on BMD at the femoral neck and lumbar spine among postmenopausal women (172). Prospective observational studies have also considered changes in BMD as the outcome while examining associations with either fruit and vegetable intakes or nutrients that come from these foods, such as vitamin C and/or potassium intakes (52, 181, 182, 222). The findings from these studies are mixed. Within the studies, for each group in which a positive finding was identified there were other groups or bone sites for which the finding did not apply.

Specifically, the Framingham Osteoporosis study found that potassium intake was associated for men with the loss of BMD at the femoral neck and trochanter but not at the radius and there were no associations found for women for potassium (52). Fruit and vegetables were not significantly associated with the changes of BMD at any site for men or women in the Framingham study (52).

Vitamin C was found to be associated with less loss of BMD at the total hip among elderly women but not elderly men in the EPIC-Norfolk study, and

only after the vitamin C intakes were divided into tertiles (222). The Aberdeen Prospective Osteoporosis Screening study demonstrated that intakes of calcium and phosphorus (positively) and fat (negatively) were associated more strongly with the loss of BMD over time than was potassium (181).

The Saskatchewan Bone Mineral Accrual Study found that each serving of fruit and vegetables to the change of BMD over 7 years in older children (8 to 20 years) contributed the same degree toward the growth of bone mineral content as each milligram of calcium for boys but there was no association noted for fruit and vegetables or calcium for girls (182).

Considering that the acid-ash hypothesis is assumed to be the likely cause of osteoporosis (32, 223), there was little consistency among the findings among the prospective observational studies of changes of BMD over time. Indeed, there were more associations that were found not to be of importance between food measures of the acid-ash hypothesis and changes in BMD than those that were found to be important in the Framingham and EPIC-Norfolk studies (52, 222). Even if the acid-ash hypothesis was found to be important in the prospective observational studies, this type of study cannot provide proof of the hypothesis since this type of study can be confounded by other variables. Therefore the studies that use changes of BMD as the outcome do not provide consistent or conclusive proof of the acid-ash hypothesis.

4.5.3 Food estimates of acid load

Some researchers have attempted to define food sources of acid and base. Early work was done prior to 1915 (47, 48, 175) and Sherman published tables listing the acid and base contributions of 64 foods based on the food's mineral content (48) in 1912. in 1995, Remer and Manz published tables of foods (40) in which they used correction factors for each mineral to take imperfect absorption into account (35). Consistent between these 1912 and the 1995 food lists is the premise that sodium, potassium, calcium and magnesium reflect base while phosphate, sulfate, and chloride reflect acid (40, 47, 48). As discussed

above, neutral or acidic phosphate supplements cause either no change (135) or an increase (213) of urine pH, and urine calcium to decrease (135, 162, 206-209, 213). This finding of opposite to the expected effects by phosphate makes the estimated food acid-load tables of Remer and Manz invalid, especially for grains and protein foods, since these foods are both good sources of phosphate. For example, Remer and Manz estimated that milk has a small but acidic load (40). In contrast, studies found that milk contributes a small alkaline load (48, 135, 224). Grains have not been evaluated for their hypothesized acidogenic and calciuric responses (110). To measure the effect of one food, and not the omission of another, the comparison diet needs to use fats and/ or sugars (which have no appreciable mineral content and therefore no acid or base load) to replace omitted foods while keeping energy intakes constant. Others have recognized that there are difficulties estimating the diet acid load from food intake due to the problems inherent with measuring food intake (50) and with estimating absorption of the nutrients which depends on the food composition and foods eaten concurrently (137).

4.5.4 Urine measurements

The measurement of the acid load in urine is imprecise. Studies have both measured urine pH and quantified the titratable acidity, ammonia and bicarbonate as a measure of the diet acid load (35, 51, 150). The measurement of the diet acid load based on these latter three components is not a precise measurement (137). Ammonium (as ammonia) and bicarbonate (as CO_2) molecules may be lost due to volatility prior to their measurement. The measurement of titratable acidity will include additional molecules that can donate a proton during the titration which can cause an overestimation of titratable acidity (137, 148). Therefore the measurement of urinary acid excretion may not accurately reflect the exposure to dietary acidity.

In summary regarding consistency, the estimations of the high diet acid loads have not consistently shown an association with calcium excretion. As well, the link between food intake and loss of BMD has also not been established.

The reasons for these lacks of consistency could be due to the differences among the studies in methodological quality, control for calcium intake and the various ways the acid load is measured in both food and urine.

4.6 Experiment

Hill's criterion regarding experiment suggests that, when possible, experiments be conducted to determine whether the frequency of a disease is altered by an exposure (196). When experimentation induces or prevents the disease based on a hypothesis, support for causation is demonstrated. In terms of the acid-ash hypothesis, although a majority of studies found that calcuria is elevated when the diet acid load is increased, there is no evidence that the acid load of the diet induces bone demineralization. The only clinically practical measures of bone fragility in osteoporosis are the typical low trauma fractures or biomechanical testing of bone biopsy material (225, 226). No prospective studies to date have examined osteoporosis fracture risk or biomechanical testing of bone related to measures of the whole picture of the acid load of the diet. The outcomes that have been used in tests of the acid-ash hypothesis are changes in calcium excretion (35, 39, 41, 54, 55, 57, 108-111, 149, 150, 159-162, 165-171), bone markers of formation and resorption (41, 55, 57, 109-111, 149, 162, 167, 168, 170-172) and BMD (172), all of which are surrogate measures or correlates of osteoporosis fracture risk.

4.6.1 Outcomes - Changes in bone mineral density (BMD)

Changes in BMD are a better measure of osteoporosis than is calciuria, since BMD changes reflect the actual gain or loss of bone mineral; urine calcium reflects only the amount excreted and not the amount absorbed and retained. BMD changes do not, however, measure the quality of the bone, the degree of bone cross-links or bone strength, and therefore BMD is a surrogate measure of osteoporosis. To date, only one trial of an intervention to alter the acid load has used a change in BMD as their outcome variable (172).

This first intervention trial of base administration that has used a change of BMD as the outcome was a 12-month comparison of potassium citrate (considered a source of alkaline) versus potassium chloride (each 30 mEq/day) in postmenopausal women with osteopenia or osteoporosis (172). This trial used a double blind randomized controlled study, a rigorous study design. The authors did not report their method of randomization and whether they had used allocation concealment. After 12 months, the women in the potassium citrate group had significantly higher BMD of 1.4 to 2 percent at the femoral neck and the lumbar spine compared to the group receiving potassium chloride (172). Two variables changed between the groups, the omission of citrate and the inclusion of chloride. Although encouraging, further work will be needed to confirm this finding, identify the important factor, determine the extent of bone benefits that may be achieved from potassium citrate supplements, and whether these benefits could be derived from other treatments that fit the acid-ash hypothesis such as changes in food intake.

As discussed above in the section on Consistency (section 4.5.2), some prospective observational studies have examined changes in BMD with specific aspects of the diet, either with fruit and vegetable intakes or nutrients that come from these foods such as vitamin C and potassium and/or magnesium intakes (52, 181, 182, 222). None of these studies measured net acid excretion in urine. Although the findings were not consistent between the sub-groups or bone sites examined in the studies, it is possible that higher fruit and vegetable consumption was associated with better BMD as it may reflect other behaviours that support bone health, such as physical activity. Or, perhaps there are active compounds in some fruit and vegetables that promote bone health through a mechanism separate to the acid-ash hypothesis (217, 227, 228).

4.6.2 Outcomes – Calcium excretion

The outcomes that have been most consistently associated with a change in diet acid load are changes in the excretion of calcium in the urine and bone resorption markers. These measures are surrogate measures of bone

mineralization. Calcium excretion is not a direct measure of osteoporosis, because it is possible that there are differences in absorption that offset any change in excretion (39, 136). Although superior to calcium excretion, calcium balance measurement is also not a measure of osteoporosis since changes in calcium balance do not measure changes at the bone. A further limitation to balance studies is that they are typically short terms studies and therefore they may not measure adaption to a dietary regimen.

4.6.3 Calcium absorption

The question of whether diets or supplements that alter calcium urinary excretion also change intestinal calcium absorption is critical factor in ascertaining whether the change in urinary calcium simply reflects a change in calcium absorption. If calcium absorption is increased with an increase in the acid intake, then this influx of calcium from the intestine could explain the source of the subsequent observed increase in urinary calcium excretion. Conversely, if there is no change of calcium absorption with changes in acid load, then the change in urine calcium excretion likely reflects a loss of bone calcium. Twelve studies of acid excretion assessed calcium absorption and twelve assessed calcium balance (Table 6). One study assessed calcium balance (using calcium isotopes) and not absorption (170), while another assessed absorption but not balance (108). Of the studies that assessed absorption, six found no change in calcium absorption, two studies demonstrated improved absorption for milk calcium compared to calcium carbonate (229) and lentils versus soy isolate protein (166), and three observed mixed results or a partial compensation of calcium absorption (150, 159, 163).

Study information		Design		Calcium Absorption	Calcium Balance	Balance study methodology (see text)				
Study	Intervention	n	Design	Significant change	Significant change	Days on Ca intake	Days on metabolic diet	Food weighted	Food lab analysis	Usual Ca intake
Weber (48)	NH4Cl	6	CO	yes	yes	0	0	no	no	no
Schuette(49)	Amt protein	11	CO	no	yes	4	4	no	no	no
Lutz (51)	Amt protein	8	CO	no	no	0	0	yes	N, Ca, Mg, P, Na	no
Hegsted (50)	Amt protein	6	CO	no	yes	3	3	yes	Ca, Mg, P	no
Lutz (53)	Amt protein	6	CO	no	no	7+	8	yes	N, Ca, Mg, P	no
Breslau (55)	Veg/veg-ovo	15	RCO	no	-	7+	8	no	Ca, Mg, P, Na, K	no
Breslau (55)	Animal/veg	10		no	-					
Lewis (56)	Ca sources	8	CO	no	yes	5	5	yes	Ca, Mg, P	no
Sebastian (40)	Kbicarb	18	RCO	no	yes	4	6	no	no	yes
Dahl (59)	Lentils	10	RCO	no	no	7+	14	yes	Ca, Na, K	yes
Roughead (63)	Amt protein	15	RCO	no	no	7+	14	yes	Ca	no
Spence (68)	Milk/soy pro	15	RCO	no	yes	7+	7	yes	Ca	no
Roughead (67)	Meat/soy pro	13	RCO	-	no	7+	21	yes	Ca	no
Kerstetter (38)	Amt protein	13	RCO	yes	no	7+	7	yes	no	no

Table 6 - Absorption and balance details of the acid-ash studies

Experts in calcium metabolism recommend rigorous methods be used in balance studies to obtain valid measures of calcium retention (85). The suggested methods include: initiate absorption studies at least seven days after starting the diet in order for subjects to achieve a steady state; provide all food to the subjects; accurately measure the amount consumed; determine nutrient composition by lab analysis; and when possible; have study subjects consume their usual calcium intakes (85).

Only one of the studies of calcium balance used ideal methods in terms of the recommended procedures (85), the study by Dahl et al which evaluated the effect of replacing soy protein with lentils (166). (Table 6) Only seven of the studies randomized the order of the interventions (39, 41, 108, 168, 170, 171). Seven studies had the subjects on the study diet for at least seven days (39, 108, 163, 166, 168, 170, 171), and only two kept their subjects on their usual calcium intake (41, 166). All of the studies provided all of the food and any supplements to the subjects (referred to as a metabolic diet), but only nine measured exact quantities of food consumed and analyzed the food to provide an estimate nutrient intake. One balance study, which used appropriate methods, reported very low calcium absorption (means of 6 to 7%), which raises the question of the availability of their calcium carbonate supplement (41). The number of subjects in each study was small, ranging from six to 18 subjects.

Research groups differed in approaches used to compensate for the differences in energy intakes between study arms. When the intervention included higher protein intakes in one study arm, the other study arm needed some other food added to keep the energy intake constant since weight loss is associated with losses of BMD (230). For example, Kerstetter noted that additional fats and simple sugars were used to keep energy intakes constant

between the study arms, however they also report that fibre (28%) intakes were higher and phosphorus intakes were lower (8.4%) in the low meat diet (39). Both fibre (231) and phosphorus (161) influence calcium absorption. Therefore the compensation for changes in energy intake between the study arms may have confounded the association between change in protein intake and calcium outcomes if these compensatory foods altered the calcium absorption.

In summary, due to the limitations in the balance study methodologies employed and the differences in study design, at this point in time it is not clear whether changes in diet acid load alter intestinal calcium absorption to account for the calcium in the urine.

4.6.4 Outcomes – Bone resorption and formation markers

Three studies reported a significant increase in bone resorption markers in response to increases in protein intake (149), changes in food intake (51), or bicarbonate replacement of chloride (57) each of which raised acid excretion and increased calcium excretion. The markers they reported changes in were N-telopeptides (149), C-telopeptides (51), and N-telopeptide, pyridinoline, and deoxypyridinoline (57). However, two studies that administered potassium citrate and saw a reduction in urine calcium (167) or an improved BMD over 12 months (172) reported no change in the bone resorption markers: N-telopeptides (167) and C-telopeptides (172), respectively.

Alterations in the surrogate measures of calcium in the urine and/or changes in bone resorption markers are not proof that alterations in the diet acid load cause bone demineralization. For proof of the acid-ash hypothesis, adequately powered studies are needed with direct measures that reflect the development of osteoporosis as the outcome measures of bone fragility, such as

biomechanical testing of bone or the incidence of fragility fractures. The scarcity of prospective studies using direct bone outcomes (fractures, biopsy, BMD) evaluated against measures of the complete and complex picture of the acid load of the diet remains a major weakness in the acid-ash hypothesis.

Chapter Five: A prospective population-based cohort study of the association of urine measures of diet-derived acid excretion with bone loss and fractures

Given the importance of bone health to overall health, the need to find ways to delay bone loss to prevent osteoporotic fractures, questions about the importance of the acid-ash hypothesis for bone health, and the need to make dietary recommendations based on the best quality evidence, further research regarding this hypothesis is needed. The quantity of excess calcium in the urine associated with the modern diet is sufficient in quantity that the acid-ash hypothesis could explain the bone loss that results in osteoporosis (See section 4.3). This thesis will examine the importance of urine measures of the dietary acid load in terms of bone mineral loss and fractures among adults.

5.1 Objectives

1) To determine whether low urine pH predicts five-year loss of BMD in adults.

2) To determine whether measures of excess urine acid load (as reflected by urine ions: potassium, sodium, calcium, magnesium, sulfate, phosphate, chloride, and organic acids) predict five-year loss of BMD in adults.

3) To determine whether low urine pH and associated hypercalciuria predict fragility fractures in adults over seven years.

4) To determine whether within-subject diet is stable by testing the levels of urine pH and acid load (the ions: phosphate, sulfate, chloride, potassium, calcium, magnesium, sodium) in fasting morning urine specimens collected at baseline and at five-years.

5.2 Methodology

5.2.1 CaMOS, an opportunity for osteoporosis research in Canada

CaMOS is a longitudinal cohort study designed to study skeletal health and risk factors among a random sample of Canadian adults 25 years of age and above (232). Trained interviewers collected comprehensive data from the subjects at baseline (1996-8) through an extensive in-person interview, measurement of BMD, and for some subjects in Quebec City, collection of blood and urine samples. At the five year time point; the subjects were interviewed again with a slightly modified version of the baseline questionnaire and their BMD was assessed a second time on the same machine. Fracture data was collected annually from all of the participants for seven years.

The Quebec Nutrition Survey documented a wide range of intakes of protein, fruit and vegetables among the population in Quebec (233, 234). Specifically, protein intakes in Montreal, Quebec among adults of French Canadian heritage averaged 90 grams with a standard deviation of 36 for men and averaged 75 grams for women with a standard deviation of 32 for women (235). Fruit and vegetable intakes among adults in the province of Quebec ranged from 0 to 25 servings a day among the 2000 participants of the Quebec

Nutrition Survey (236). Potassium intakes were wide ranging among the Montreal adults, with mean intakes of approximately 2000 mg a day and standard deviations of over 1000 mg (237). Given the wide range of intakes of the foods and nutrients of interest by the population under study, this population was an appropriate choice for this study.

5.2.2 Study design:

Canadian Multicentre Osteoporosis study (CaMOS) is a prospective cohort study in which subjects were entered into the study and then followed for their loss of BMD over five years and the occurrence of fractures over seven years (238).

A cohort study design has numerous advantages, which include large numbers of subjects, followed for long time periods, and minimal selection bias (239). These advantages of cohort studies are related to the prospective design of these studies. Exposures are measured before the occurrence of the disease, and therefore the study has temporality, and the order of events is apparent. Since subjects are not selected on the basis of their exposure or the disease, cohort studies are not usually initially at risk of selection bias. However, if subjects self selected into this study by contributing urine or are lost to follow-up in a way that is related to the exposure and/or the disease, there is a potential for selection bias to invalidate the conclusions of this study (240).

In this study not all of the subjects contributed a urine sample. If the subject's contribution or lack of contribution of a urine sample was related to the urine composition and the progression of osteoporosis, then the lack of contribution could cause a bias in the estimates of association. The

characteristics of the subjects who did and did not contribute a urine sample were evaluated for statistical and clinically important differences.

5.2.3 Target population

The target population of CaMOS was non-institutionalized Canadian adults, 25 years and older.

5.2.4 Study population

CaMOS participants recruited from Quebec City were included in this analysis. To increase compliance with follow-up, the source population of CaMOS was identified as non-institutionalized Canadian adults, 25 years and older who resided within 50 kilometres (km) of the 10 study centres (241). These geographical areas were estimated to include 37% of the Canadian population. InfoDirect provided random lists of telephone subscribers from lists of postal codes within the geographical areas from which subjects were selected (232). CaMOS inclusion criteria restricted entry into the study to those aged 25 years or older and the study was designed to include adequate representation among age and gender categories. This study excluded Canadians who did not live close to the study centres and those who did not have phone service.

5.2.5 Subjects:

Of the initial contacts made in national CaMOS (22,173) with individuals who were eligible to participate in the nine study centres, of which 42.5% agreed to participate in the study (Suzette Poliquin, personal communication, November 19, 2006). When those that were deemed eligible for the study did not choose to participate, these “non-responders” were asked some questions to enable description of this group. Those who chose to participate were very similar to the non-responders and their reasons for not participating were mostly unrelated to

osteoporosis risk, therefore, Kmetic et al felt that there was little response bias in CaMOS (242). Of all of the study centres, Quebec City centre had the highest participation; 1822 contacts were deemed eligible, and 62.2% (1133 subjects) agreed to participate in the study.

5.2.6 CaMOS database

CaMOS cohort data is stored on a mainframe computer at McGill University, where it is backed up daily on alternating tape drives. All of the variables of interest were reviewed for possible coding errors in graphical and tabular displays.

5.3 Exposure variables:

5.3.1 Urine samples

The fasting urine samples were collected in the morning after an initial void and a wait of two hours, while subjects maintained a fast from the evening before. Within two hours of collection, the urine samples were aliquoted, frozen and stored at -70 ^{o}C in 2 millilitre plastic vials.

5.3.1.1 Urine pH

Urine pH was measured by the PhD student using a Radiometer PHM82 Standard pH Meter (Copenhagen, Denmark) in the urine samples immediately after thawing. The machine was calibrated at the beginning of each session to pH 4.0 and 7.0 and recalibrated to pH 7.0 after every 10 samples, as recommended by the manufacturer.

5.3.1.2 Urine ions

Urine sodium, potassium, calcium, magnesium, phosphate, chloride, sulfate, and organic acids were used to define the estimate of acid load excretion (40, 144, 180, 243). The components of excess urine acid load were measured in three laboratories. The majority of the calcium (77.2%), phosphate (76.9%) and creatinine (89.6%) measurements were done in Quebec City using a Vitros 950 (Vitros Chemistry product, Ortho-Clinical Diagnostics, Johnson and Johnson Company, Rochester, NY, USA), which used a dry chemistry enzyme immunoassay system. The remainder of the calcium, phosphorus and creatinine were measured by Calgary Laboratory Services using Cobas Integra 700 analyzer (Roche Diagnostics, F.Hoffmann-La Roche Ltd, Basel, Switzerland). The calcium analysis was a colorimetric assay using o-cresolphthalein complexone, the creatinine analysis was an enzymatic colorimetric assay using creatininase, and the phosphate analysis was an ammonium-phosphomolybdate reaction (Danielle Panchuk, Personal communication, November 30, 2007). Sodium, potassium, chloride, and magnesium, were measured by Calgary Laboratory Services using the same machine. Sodium, potassium, and chloride were measured using ion-selective electrodes while magnesium was tested using a colorimetric method with chlorophosphaonazo III. According to the manufacturer, the coefficient of variation of these tests for Na, K, Cl and Mg range from 1.2 to 3.0 percent. The initial reports from Calgary Laboratory Services reported 121 potassium results as >80 millimole per litre (mmol/litre). Upon inquiry, it was revealed that Calgary Laboratory Services reports potassium to this limit; however more detailed results were obtained from the analysis history files for all except 15 of the samples. Eleven of these 15 samples were reanalyzed, and so only 4 of the samples remained capped at 80 mmol/l.

BDS Laboratory in Qu'Appelle, SK measured the urine sulfate content using an automated methylthymol blue method, which has a coefficient of variation of 2.4% in water. For the sulfate testing, the samples were diluted through a series of dilutions beginning with 1:5, 1:10 and up to 1:100 as necessary. The sensitivity range for the sulfate analysis was 0.10 to 3 mmol/l (John Blachford, personal communication, November 22, 2004).

Each component of urine acid load (sulfate, phosphate, chloride, estimated organic acids, sodium, potassium, calcium and magnesium) was added individually into the regression models rather than as one summary measure as is usually used to quantify the diet acid load (35). Adding each component in separately allows a comprehensive evaluation of the impact of each component on the change in BMD.

5.3.1.3 Organic acids

Organic acids, and/or the conjugate bases of organic acids, were estimated using the method proposed by Remer (35, 215). The calculation of urinary organic acids has recently been defined by Berkemeyer and Remer as "[(body surface area x 41)/1.73] where 41 is the median daily organic acid anion excretion (mEq/d) at an average body surface area of 1.73 m^2 for healthy subjects" (body surface area was used by these authors as [0.007184x *height* $(cm)^{0.725}$ x *weight* $(kg)^{0.425}$] (215). Although referred to as organic acids, these compounds could be in the form of their conjugate base, carboxylates, in the urine depending on the pH.

5.4 Outcome variables:

The outcomes of interest in this study were change of BMD over five years (at the femoral neck (FN), the lumbar spine (LS), and the total hip (hip)) and fractures (confirmed fragility fractures and total fractures) over seven years of follow-up. Fractures were confirmed with radiology reports.

5.4.1 Changes in bone mineral density (BMD)

Changes in BMD were calculated as the difference between the two Dual Energy X-ray Absorptiometry (DEXA) readings of BMD on each subject that were taken at baseline and five years later on the same DEXA machine (Hologic 2000). The BMD measurements were performed on the same DEXA machine to decrease measurement error. The baseline reading was subtracted from the five-year reading, to provide a positive value for a gain of BMD and a negative value for a loss of BMD. The BMD assessment sites used were the lumbar spine (L1-4), femoral neck, trochanter, and total hip.

5.4.2 Fractures

Fractures were initially self-reported annually in the CaMOS Follow-up Questionnaire; however, radiology reports were obtained to confirm the fractures. A fracture was only recorded as a fragility fracture if it occurred spontaneously, if it was a no fall injury, or if it was preceded by a fall from a standing height or less. Data on fractures have been collected and confirmed for seven years of follow-up in the CaMOS study.

Both confirmed fragility fractures and total fractures (confirmed and unconfirmed) were modeled as outcome measures in the fracture study.

5.5 Potential confounding variables

The following variables were considered to be potentially confounding of the association between diet and the loss of BMD or fractures based on the literature. Variables collected primarily by interviewer administered questionnaire included: age, gender, reported family history of osteoporosis among first degree relatives (parents, siblings and/or children), physical activity, smoking, calcium intake, hormonal status among the women, medication (thiazide diuretics, bisphosphonates, and estrogen) use. Copies of the Baseline and 5-year questionnaires are included in Appendix 1. Blood collected at baseline was used to measure vitamin D status, serum creatinine (which was used to assess renal function), and parathyroid hormone (PTH). Body mass index and change in body mass index were based on measurements and recall.

Baseline measures were used to define the potentially confounding variables with the exception of the change in BMI (between baseline and 5 years). Change of weight status over the study period is a potential confounder (244-246), which required use of the measurement of weight at baseline and at 5-years.

5.5.1.1 Age and Gender

The age and gender were asked of the subjects and recorded at the baseline interview of the subjects.

5.5.1.2 Hormonal status among the women

Menopause status was determined at baseline from the question “Have your menstrual periods stopped for more than one year? (No period one year or

more after last menstruation)". Similar questions asked at three and five years were used to examine the chance of misclassification.

None of the women who were coded as menopausal by the question about their last period had been pregnant or lactating within the past year. Women coded as menopausal included those who had both ovaries removed. Seventeen women were coded as pre-menopausal at baseline, but possibly menopausal at baseline by the 3 or 5 year questions. One of these women responded at 3-years that it had been 10 or more years since her last period, but at 5-years she was coded as "near the end of the process". The other 16 women who at 5-years were coded that they had "completed the process" were coded at 3-years as having a period within the previous 2 years. All 16 women were kept coded as premenopausal since two of their three answers suggested that they were pre-menopausal.

Women were coded at baseline as estrogen deficient or estrogen replete as follows: those who were premenopausal were considered estrogen replete, those who were on supplemental estrogen (n= 159) and postmenopausal or premenopausal were considered estrogen replete; those who were postmenopausal and not on exogenous estrogen were considered estrogen deficient. Men were coded as not estrogen deficient since this gender does not suffer from estrogen deficiency.

5.5.1.3 Physical activity

Physical activity was quantified in the baseline CaMOS questionnaire with questions about strenuous (e.g., jogging, tennis) vigorous (e.g. manual labour, shovelling) and moderate (e.g. housework, gardening, brisk walking) activities in terms of hours per week over the last year. Activities performed while sitting

(eating, playing cards, sitting in a car or bus or watching TV) and the usual duration of sleeping were quantified as the average number of hours per day over the past year. The questions regarding physical activity were adapted from a questionnaire from the Cancer Research Center in Hawaii (247). Validation data for these questions has not been published.

Physical activity was quantified in terms of the number of Metabolic Equivalents (METs) per day, based on the Compendium of Physical Activities (248). A MET is defined as the amount of energy consumed, expressed in terms of basal metabolic rate multiples, in kilocalories per day. The basal metabolic rate is estimated to be approximately 1 kilocalorie per kilogram per hour (248). Physical activity was quantified in two ways: as the total daily METs from physical activity only (exercise physical activity), and as total daily energy expenditure (METs of all activities including sitting and sleeping).

To obtain the measures of physical activity for each subject, first, an average MET was calculated for each category of activity by calculating an average estimated energy cost (248, 249) of the example activities provided to the subjects in the questionnaire for each category. The average energy costs used for the categories were 7.1 MET for strenuous, 6.5 MET for vigorous, and 4.1 MET for moderate activities, 1.0 to 1.5 MET for sitting (1.5 MET for sitting at work, at meals or while sewing or playing cards, and 1.0 MET for sitting in a car or bus or watching TV) and 0.9 MET for sleeping (248, 249). Second, the average MET for each category was multiplied by the subject's estimated time spent in each category. Third, the activities quantified in hours per week were converted to hours per day. Fourth, the 'exercise physical activity' variable was obtained by summing the duration of strenuous, vigorous, and moderate activities and the result was multiplied by the subject's baseline body weight.

Fifth, all of the METs for strenuous, vigorous, moderate, and sitting activities, as well as sleeping were summed for the 'total physical activity' variable. The result was multiplied by the subject's baseline body weight to obtain the estimated energy expenditure in kilocalories, expended in physical activity in 24 hours for each subject.

The rationale for examining two measures of physical activity was based on the following: 1) the MET method was developed to estimate total energy expenditure, whereas 2) strenuous, vigorous, and moderate activities are likely associated with the maintenance of BMD over time, 3) the quantity of energy expended for sleeping and sitting is not likely associated with maintenance of BMD, 4) therefore the energy used in physical activity is more likely associated with the maintenance of BMD than is total energy expenditure.

The two versions, total physical activity and exercise physical activity, of the physical activity variable were tested as potential confounders.

Some of the subjects reported very low activity ratings, so that their total energy expenditure was less than expected (250). To determine whether this could have been due to reporting their activities for fewer than 24 hours, the number of hours reported by each subject was examined.

The data was examined for occurrences of subjects estimating greater or less than 24 hours of activity as an assessment of data quality. To allow for rounding errors, only those with total hours greater than 25 were examined. Five subjects' hours summed greater than 25 hours in a day and two subject's hours summed greater than 27 hours. These highest two with estimates over 27 hours also had recorded the highest number of hours of sitting in a day (both with 18.5 hours). Since sitting hours make little difference in the energy use estimates, it

was assumed that overestimation of hours of activity was not a problem in this dataset.

5.5.1.4 Calcium

The calcium intakes from food and supplements by the Quebec City sample at baseline were examined. The variable of interest as a potential confounder was the total calcium intake from all sources. CaMOS included the following variables: calcium from milk, calcium from dairy products not including milk, calcium from all dairy products, non-dairy calcium, supplemental calcium, and total calcium from food and supplements. The calcium variable that was used in the models was the total calcium from food and supplements.

5.5.1.5 Body Mass Index and change in Body Mass Index (BMI)

The body mass index (BMI) was defined as (weight (kg)/height squared (m2)). BMI at baseline, BMI at 5-years and the change of BMI over the 5-years was calculated for this study using baseline height along with the weights reported for baseline and 5-years. The reason baseline height was used for both measures was that the quality of the height data was questionable, since 3 subjects “grew” (5 year height was greater than the baseline height) more than 2.5 cm (average = 3.8 cm, median = 4.0, maximum = 4.4 cm). All of the subjects were 25 years of age or older at baseline. According to the CaMOS interview guides, height and weight were to be measured at baseline and at 5 years whenever BMD was not obtained. The interview guide does not state whether height and weight should have been measured when the BMD was measured. It is possible that at least some of the height data was self reported and therefore may be biased. Since the important aspect of the change of BMI as a risk factor in osteoporosis is the fat mass and change in fat mass (251, 252), only baseline

height was used to calculate BMI at both baseline and at 5-year. The range of the change of BMI over 5-years varied from a loss of 9.5 to a gain of 11.9 BMI units.

5.5.1.6 Vitamin D

The blood samples were analyzed for vitamin D (25-hydroxy vitamin D) using the Octeia kit which uses an enzyme immunoassay followed by colorimetric detection. The coefficient of variation for the vitamin D assay ranges from 4.1 to 8.4%. The blood samples were collected in each season.

5.5.1.7 Kidney disease

The Kidney Disease Outcomes Quality Initiative (K/DOQI) (253) developed a method for calculating the GFR from serum creatinine. Chronic kidney disease (CKD) has been defined as a "GFR less than 60 mL/min/1.73 m^2 for greater than three months, with or without kidney damage" (254). The K/DOQI calculation of GFR controls for age, gender and the black race since these factors effect the association between serum creatinine and actual GFR.

5.5.1.8 Smoking

Subjects were defined as a current smoker or a non-smoker at baseline based on reported current smoking status.

5.5.1.9 Medications

The CaMOS participants were asked to show their medication bottles at baseline to document their use of prescription medications. As well as estrogen, discussed above, thiazide diuretics, and bisphosphonates were considered

potential confounders due to the association between these medications and bone health. An experienced Pharmacist reviewed the medications list to identify which pharmaceutical products contained these medications. Only eight subjects in this study were on bisphosphonates, which was too low a number to be able to estimate the influence this medication may have on the relationship between measures of acid load and the outcomes, therefore bisphosphonates were not included as a potential confounder.

5.6 Statistical analysis

5.6.1 Statistical modeling

Multiple linear regression analysis was used for the BMD study and logistic regression analysis was used for the fractures study. The purpose of the regression analyses was to develop the most parsimonious model (balanced between simplicity and fit (255)) regarding the association of the measures of exposure to diet acid load with change of BMD or the occurrence of fractures over five years.

Variables were considered potential confounders based on associations with a change of BMD or fractures in the literature. The modeling process involved assessment for confounding (256, 257), using backwards elimination. Age and gender were retained in the models, except when estrogen deficiency had a larger effect on the coefficient than did gender, in this case estrogen deficiency was retained instead of gender. Baseline BMD was included in the models as a covariate to control for the baseline BMD (258). As well, for the fracture study, urine calcium was also assessed as a potential confounder.

Variables were kept in the model as confounding variables when the coefficient for the exposure variable(s) showed a clinically important change on its elimination. Assessment for possible multicollinearity was made in the modeling process for those variables with high correlations ($\geq$ 0.6).

Effect modification was not assessed since the goal of this study was to acquire estimates of the effects of the urine measures of diet acid load on bone outcomes. According to Kleinbaum and Klein, the assessment of effect modification is not appropriate in a study with this goal (257).

There were frequent missing values for the questions regarding current smoking (44.9%) and osteoporosis family history (26.8%) and only half of the subjects had their serum 25(OH) vitamin D measured. To retain the sample size, two indicator variables were used for each of these variables with frequent missing values. For the vitamin D indicator variable, vitamin D status needed to be used in a binary version. Vitamin D status was assigned as adequate if the serum 25-hydroxy vitamin D was 80 nmol/l or greater and inadequate if it were less than 80 nmol/l (259). The first indicator variable for each set was assigned the value of one for the positive response (smoker, family history, and adequate vitamin D status) or a zero for all other subjects (non-smoker, no family history, low status of vitamin D or missing values). The second indicator for each set was assigned the value of one for the negative response (non-smoker, no family history of osteoporosis, or low status of vitamin D) or a zero for either a positive or a missing value. Therefore the missing values were the reference category, since both indicator variables were coded as a zero for missing.

Each component of urine acid load (potassium, sodium, calcium, magnesium, sulfate, phosphate, chloride, and estimated organic acids) was

included in regression models versus the change of BMD to allow an evaluation of the impact of each component on the changes in BMD. For this analysis, potassium was modeled as the priority among these potential exposures once early analyses indicated its importance and this ion is emphasized in the literature (30, 41, 182, 260). The other urine ions were removed from the analyses since they were not statistically significant nor did they show evidence of confounding the association between the change of spine BMD and urine potassium.

Additionally, the calculated Acid Excretion (phosphate + sulfate + chloride + organic acids) minus (sodium + potassium + calcium + magnesium in mmol/l) was modeled as an exposure.

The use of the logistic regression was reasonable for the estimation of the relative risks of fractures under the rare disease assumption (261) since the frequency was 5.8% for fragility fractures and 10.9% for all fractures. Recently it has been proposed that since the propensity to fracture is increased in osteoporosis, any fracture incurred by an adult may be indicative of osteoporosis (Personal Communication, Jacques Brown, Rheumatologist, Quebec City, April 28, 2007), so total fractures over 7-years of follow-up were modeled as well as fragility fractures.

5.6.1.1 Control for baseline BMD

Baseline BMD is a potential confounder of the relationships under question since change scores are likely associated with the baseline differences. For this reason, baseline BMD was included in the models with change of BMD as the outcome to control for potential confounding (258).

5.6.1.2 Potential multicollinearity

When a confounder has strong collinearity or a close relationship with the exposure, it is not possible to estimate the effect of one of these while including the other in the model (255). Strong collinearity produces wide confidence intervals for the estimates (255). The exposure and potential confounder variables were assessed for signs of non-linearity and for possible multicollinearity using scatterplots and correlation matrices with the guideline for potential multicollinearity when the correlations were greater than 0.6.

When high correlations occurred, the variables identified were removed from the models, to see if their exclusion altered other variable's coefficients or standard errors. Redundant variables were dropped to achieve the most parsimonious model.

5.6.1.3 Regression criticism

The assumptions of regression analysis (Linear: linearity, homoscedasticity, and normality, and no influential outliers) were assessed by examining the residuals for relationships not explained by the models. The area under the receiver operator curve for any significant finding was examined after logistic regression.

5.6.2 Stability of within-patient urine pH and acid load in fasting morning urine specimens collected at baseline and at five-years

For the stability study, 200 subjects were randomly selected to have their urine ions measured at 5-years. This random selection was performed using computer generated random numbers. Urine calcium and phosphorus were measured at both baseline and at 5-years by CaMOS Quebec City.

Two hundred measurements of the 5-year urine were made since this number was estimated to be a sufficient sample size to demonstrate fair to good stability at a two-sided 5% significance level (262, 263).

To characterize the level of agreement between the baseline and five year urine samples as an indirect measure of diet stability. The intraclass correlation coefficient (ICC) was calculated using the following formula ICC = $\frac{S_B^{\ 2}}{S_B^{\ 2} + S_W^{\ 2}}$, based on the between ($S_b^2$) and within-subject ($S_w^2$) variances. The ICC compares the between-subject variance with the total variance, which is a sum of between and within-subject variances. When two measurements from a subject are very similar relative to the variance of the between-subject variance, then the calculated ICC would be close to unity. In contrast, when the within-subject measures are as variable as the between-subject measures, the ICC would be close to 0.5. When the ICC is less than 0.5, then the within-subject variability is greater than the between subject variability, and there is low stability of the measures over time.

The following criteria were used to classify the ICC: According to criteria by Landis and Koch, if the ICC was less than 0.2 then the stability was Slight, if it exceeded 0.2, then the stability was Fair, if the ICC exceeded 0.4, then was Moderate, if the ICC exceeded 0.6, stability was concluded to be Substantial, and if it was greater than 0.8 it was concluded to be Almost Perfect (264).

The measurement error was quantified by the common within subject standard deviation (265). Bland-Altman plots (of the difference between the two measures (5-year – baseline) versus the average of these two measures(266))

were constructed to examine how the differences between the baseline and 5 year measurements were related to the magnitude of the baseline values.

5.7 Results

5.8 The study cohort

Eleven hundred and thirty-three subjects completed the baseline questionnaire and became the Quebec City sub-cohort (Figure 3). Seven hundred and ninety-five (71.0 %) of these subjects contributed urine samples and formed the fracture study. Five years later 651 (81.9%) of these subjects had their 5-year BMD measurements measured for a second time so they formed the BMD study. At year seven, 776 (68.5%) subjects remained in the study and have complete fracture data recorded for the 6804 person years of data collection. At the time of the seven year data collection, 102 subjects had died (9.0%) and 255 (22.5%) had withdrawn from the study. The withdrawals were less than three percent each year (years 1 through 7), except for year 6 which had a loss of 13.1% (n = 148) in the one year.

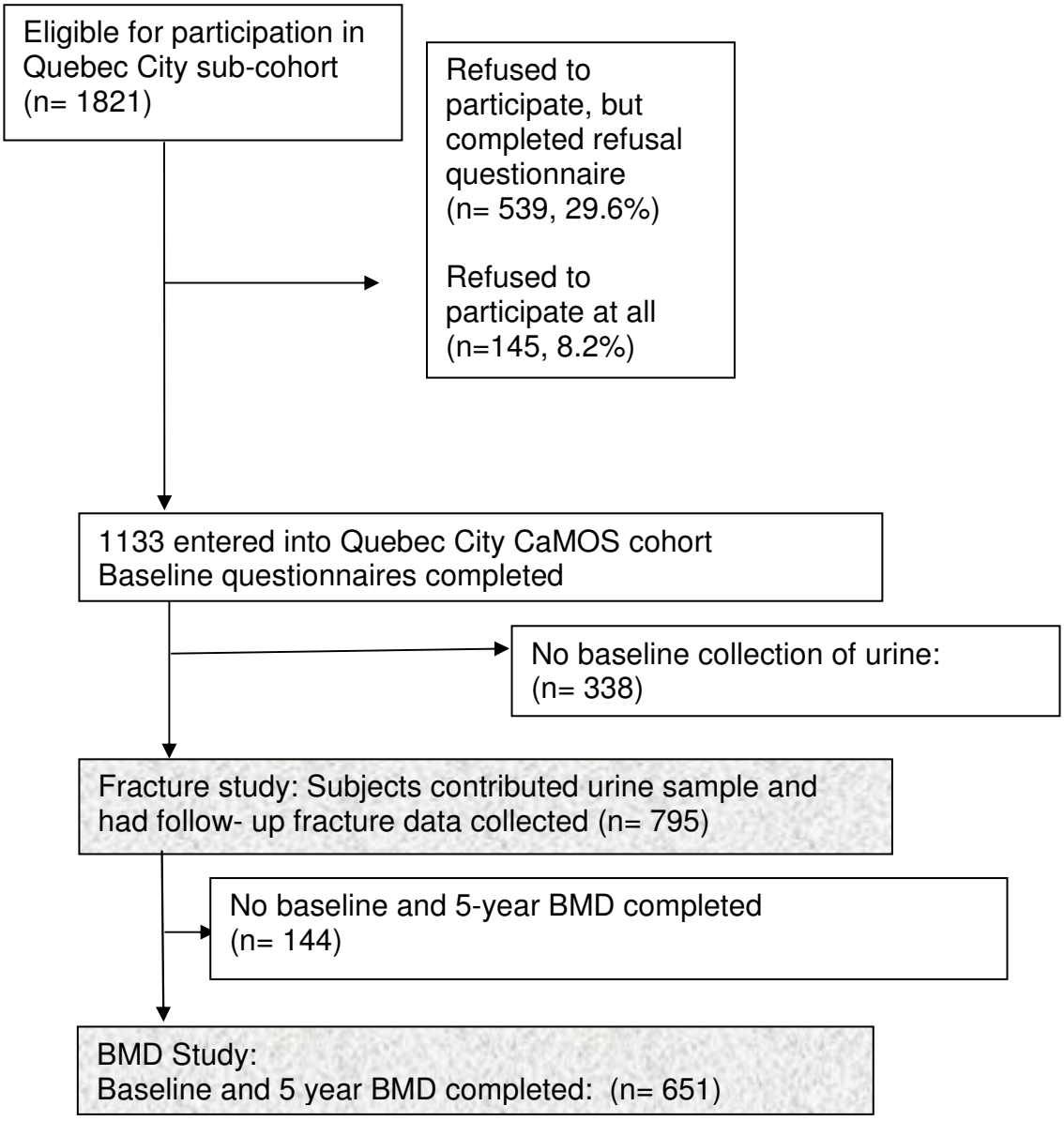

Eligible for participation in Quebec City sub-cohort (n= 1821)
Refused to participate, but completed refusal questionnaire (n= 539, 29.6%)
Refused to participate at all (n=145, 8.2%)
1133 entered into Quebec City CaMOS cohort Baseline questionnaires completed
No baseline collection of urine: (n= 338)
Fracture study: Subjects contributed urine sample and had follow- up fracture data collected (n= 795)
No baseline and 5-year BMD completed (n= 144)
BMD Study: Baseline and 5 year BMD completed: (n= 651)

Figure 3 - Flow diagram of the entry of subjects into the fracture and BMD studies

5.9 Characteristics of the subjects

The baseline characteristics of the subjects in both the fracture and BMD studies are shown in Table 7. The characteristics of the subjects in the two studies were similar.

DEMOGRAPHIC AND CLINICAL CHARACTERISTICS	FRACTURE STUDY	BMD STUDY
n	795	651
Age	59.8 +/- 12.8	58.6 +/- 12.3
Female gender, (%)	553/795 (69.6%)	455/651 (69.9%)
Women estrogen sufficient, (%)	261/553 (47.2%)	228/455 (50.1%)
Family history of osteoporosis, (%)	71/511 (13.9%)	60/415 (14.5%)
Kidney Disease, (%)	118/733 (16.1%)	119/632 (18.8%)
Smoking, (%)	154/438 (35.2%)	113/352 (32.1%)
Thiazide medications, (%)	66/795 (8.3%)	52/651 (8.0%)
Bisphosphonates, (%)	8/795 (1.0%)	6/651 (0.9%)
Physical activity, kcal/day Total physical activities Exercise physical activity Hours reported	 1548 +/- 589 498 +/- 467 16.0 +/- 3.0	 1576 +/- 806 521 +/- 484 16.1 +/- 3.0
Calcium intake, mg/day	889 +/- 550	885 +/- 550
Body Mass Index, kg/m2	26.1 +/- 4.7	26.0 +/- 4.7
Change of BMI, kg/m2	0.56 +/- 1.71	0.59 +/- 1.69
Vitamin D status, nmol/l	75.1 +/- 28.4	74.9 +/- 28.4

mean +/- sd nmol/l = nanomole per litre

Table 7 - Subject characteristics in the fracture and BMD studies

By design the majority of the study subjects were older women due to the purposeful over-sampling of this group. The age distributions of the men and women demonstrated slight negative skews toward the younger ages among both genders (Figure 4).

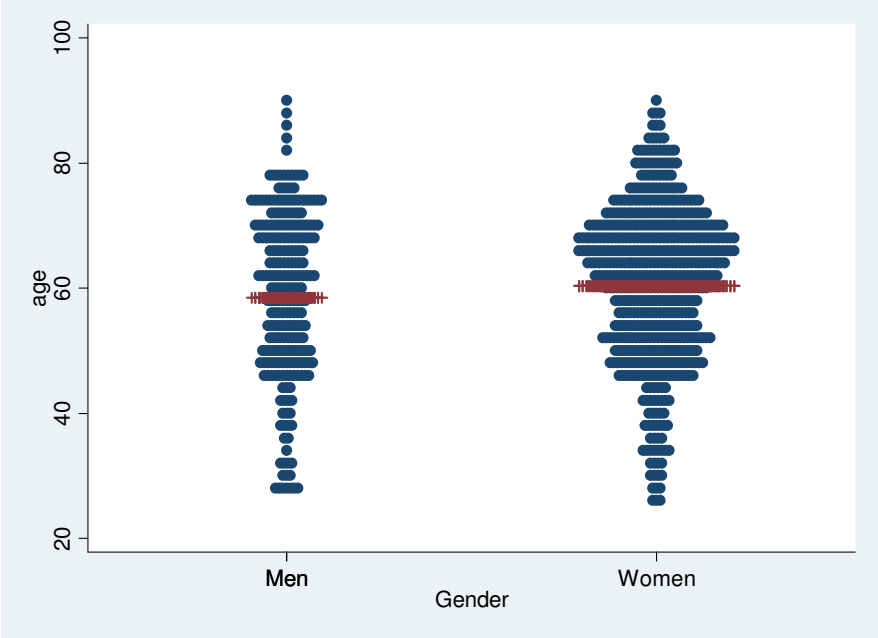


Figure 4 - Distribution of age by gender

Approximately half of the women were estrogen sufficient due to being on estrogen replacement or premenopausal. Missing values in the database were common for variables such as family history of osteoporosis (26.9%), smoking (46.0%), vitamin D status (49.7%), serum creatinine (19.9%), or parathyroid hormone (24.0%) while others were complete (e.g. age and gender).

Urine pH, sodium and chloride had distributions that were slightly skewed to the right with no outlying values (Table 8, Figure 5 & 6). All of the other urine constituents (potassium, calcium, phosphorus, sulfate, magnesium, creatinine and the calculated organic acids) had slight positive skews with some high outliers (Table 8, Figures 6 and 7).

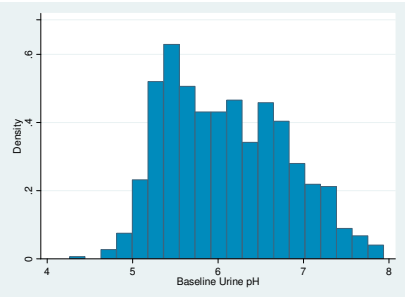


Figure 5 - Distribution of Urine pH

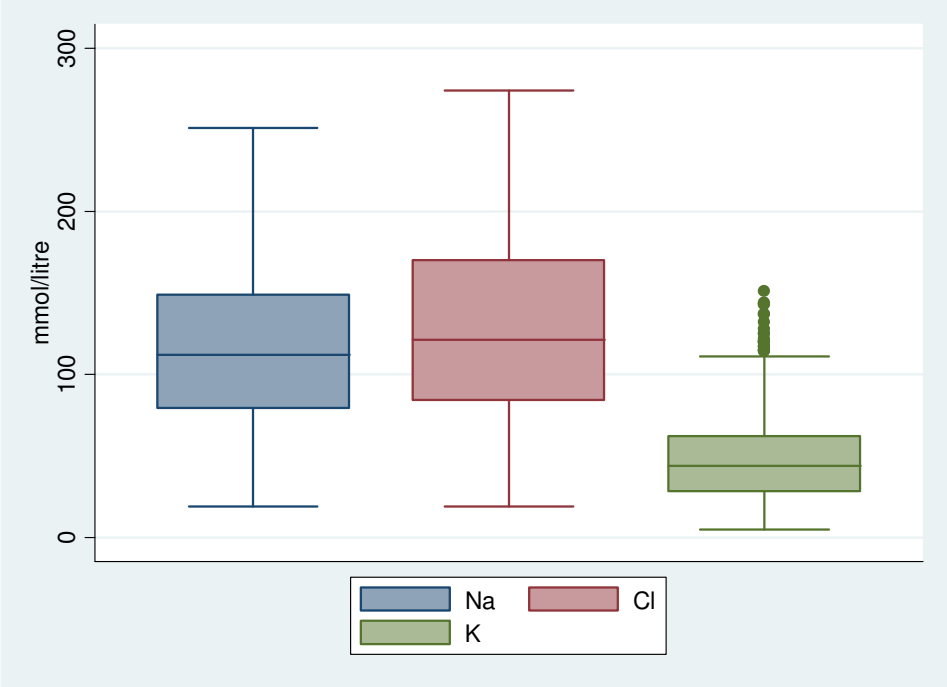


Figure 6 - Distributions of Urine sodium, potassium and chloride

LABORATORY VALUES	FRACTURE STUDY	BMD STUDY
Urine pH	6.1 +/- 0.70	6.1 +/- 0.71
Potassium, mmol/l	47.8 +/- 26.3	48.2 +/- 26.2
Sodium, mmol/l	115 +/- 50.0	116 +/- 49.6
Calcium, mmol/l	2.90 +/- 2.22	2.96 +/- 2.07
Magnesium, mmol/l	3.37 +/- 1.98	3.41 +/- 2.00
Phosphate, mmol/l	20.3 +/-11.8	20.9 +/- 11.9
Chloride, mmol/l	127 +/- 57.4	128 +/- 57.8
Sulfate, mmol/l	17.2 +/- 10.8	17.4 +/- 10.6
Organic acids, mmol/l	40.8 +/- 4.6	40.9 +/- 4.6
Urine creatinine, mmol/l	8.75 +/- 5.23	8.94 +/- 5.30

mean +/- sd mmol/l = millimole per litre

Table 8 - Laboratory results of the subjects in the fracture and BMD studies

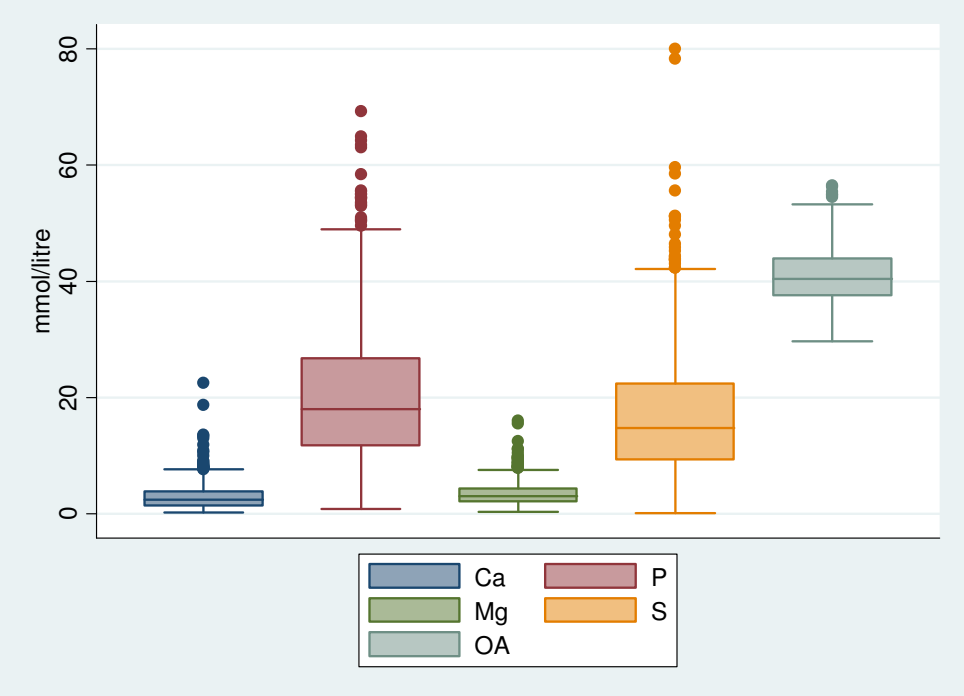


Figure 7 - Distributions of Urine calcium, phosphorus, magnesium, sulfate and the calculated organic acids

5.9.1 Bone Mineral Density (BMD)

The mean femoral neck BMD of the whole sample decreased significantly by 0.0052 g/cm^2 ($p < 0.001$) over 5 years (-0.71%) of follow-up, but the total hip BMD loss of 0.0006 g/cm^2 did not decrease significantly ($p = 0.66$) (-0.07%) (Table 9, Figure 8). In contrast, the lumbar spine increased significantly by 0.0180 g/cm^2 during follow-up ($p < 0.001$) (1.92%).

CHARACTERISTIC	FRACTURE STUDY	BMD STUDY
Baseline FN BMD, g/cm^2	0.727 +/- 0.128	0.731 +/- 0.126
Baseline LS BMD, g/cm^2	0.936 +/- 0.166	0.938 +/- 0.160
Baseline HIP BMD, g/cm^2	0.880 +/- 0.151	0.885 +/- 0.146
Change FN BMD, g/cm^2	-	-0.005 +/- 0.035
Change LS BMD, g/cm^2	-	0.018 +/- 0.054
Change HIP BMD, g/cm^2	-	-0.001 +/- 0.036
Change FN BMD, % of baseline (change over 5-years)	-	-0.57% +/- 5.0
Change LS BMD, % of baseline (change over 5-years)	-	2.1% +/- 6.0
Change HIP BMD, % of baseline (change over 5-years)	-	-1.1% +/- 4.2
All fractures	87 (10.9%)	n/a
Confirmed fragility fractures	46 (5.8%)	n/a

mean +/- sd g/cm^2 = grams per squared centimetres

Table 9 - Bone outcomes of the subjects in the fracture and BMD (g/cm^2) studies

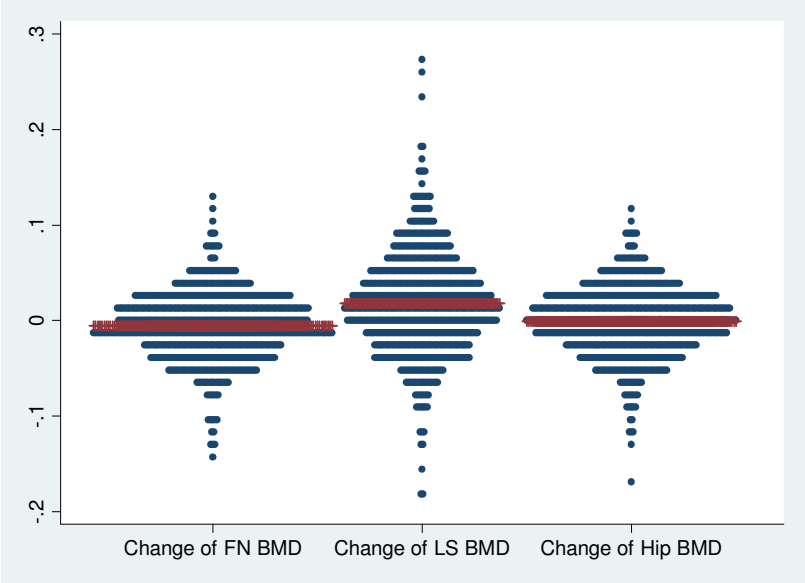


Figure 8 - Dotplot distribution of the change of BMD over 5-years at the three sites

When the subjects were examined by gender, a similar pattern of a small increase in spine BMD and slight decreases in femoral neck and total hip BMD existed among both genders. There were no significant differences between the genders for the changes in BMD at the three sites (p = 0.20, 0.41, 0.62, respectively for the FN, LS, and HIP sites).

Estrogen deficiency was associated with changes in BMD at the hip and spine. At the total hip, women who had estrogen deficiency at baseline lost BMD on average (-0.0049 g/cm2, sd = 0.039) while those estrogen replete on average gained some bone (0.0017 g/cm2, sd = 0.034) (p = 0.004) over the 5-years. The opposite effect of estrogen was seen at the lumbar spine; those with estrogen deficiency had a greater increase in BMD (0.0264, sd = 0.598) than those that were deficient (0.0134, sd = 0.494) (p = 0.004).

5.9.2 Fractures

There were a total of 159 fractures among the Quebec City cohort during the seven years of follow-up, and 112 occurred among those who contributed urine and became the fracture study. Of those 112 fractures, 25 occurred among those who already had a fracture, so there were 87 subjects who sustained at least one fracture (10.9%). Forty-six (5.8%) of those fractures were confirmed fragility fractures.

5.10 Potential confounding variables

There were no apparent curvilinear relationships between any of the exposure and covariate variables. There were seven potential concerns regarding multicollinearity between the exposure variables: sodium with chloride ($r = .880$), sulfate with potassium (0.727), potassium with chloride (0.632), and magnesium with three other ions (0.664 with sulfate, 0.617 with calcium, and 0.617 with phosphate) and between estrogen deficiency and age (0.663). As well, there was a strong correlation (0.773) between Body Mass Index (a covariate) and the calculated organic acids (an exposure). During the regression analysis, multicollinearity was observed between sodium and chloride, as well as BMI and organic acids, which led to the removal of one of each of the pairs. When chloride was removed from the model, the coefficient for sodium decreased to 20% and the standard error decreased to 43% of their former values. A similar effect occurred to BMI when the organic acid variable was omitted. Removal was decided based on the other variable in the pair's coefficients and/or standard errors changing by a factor of two or more.

5.10.1 Physical activity

The measures of physical activity, total physical activity and exercise physical activity were highly skewed to the right since few people reported high levels of activity. The median MET for physical activity was equal to 5.0 while the average was 7.3. A MET of 5 is equivalent to 1.2 hours of brisk housework, gardening, or brisk walking each day.

Seventy-five percent of the exercise physical activity came from moderate activities, 19% from vigorous activities and 6% from strenuous activities. Subjects' involvement in activities varied by the strenuousness of the activity. Eighteen percent of the subjects (143/795) reportedly engaged in strenuous activities, while 32% (255/795) engaged in some vigorous activities, while 98% (782/795) engaged in some moderate activities. Men reported higher physical activity levels, under both definitions, than women ($p < 0.001$ for both).

The total number of hours accounted for by the subjects averaged 16.0 hours (Figure 9). The subjects reported sleeping an average of 7.3 hours per night, exercising 1.8 hours (95 percentile range was 0.35 to 4.6 hours) and sitting an average of 6.8 hours per day.

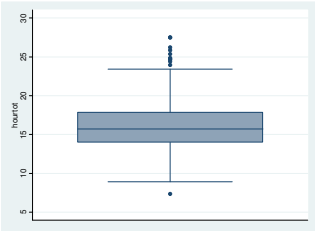


Figure 9 - Boxplot of total hours reported for physical activities

5.10.2 Vitamin D status

Fifty percent of the subjects in the fracture study had their serum vitamin D measured. When categorized into two groups, sixty-three percent (252/400) of the cohort were rated as having adequate vitamin D status (80+ nmol/l)(267).

5.11 Regression analysis

5.11.1 Urine pH and loss of BMD over 5-years

There was no relationship between urine pH and five-year loss of BMD for any of the three sites assessed, femoral neck, lumbar spine, or total hip (Table 10). Individuals' BMD equally increased and decreased for those with high and low urine pH. The findings were unchanged when all the potential confounders were controlled. Age and gender were retained in the models, except for the total hip where estrogen deficiency had a larger effect on the coefficient than did gender, and the latter was retained. The coefficients for urine pH were never statistically significant and the greatest amount of explained variance (adjusted R^2) for the best fit model for each bone site ranged from only four to ten percent.

Bone Mineral Density	pH			
Bone Site:	Coefficient	95% confidence interval	p-value	aR^2
Femoral Neck	0.0021	-0.0019 to 0.0060	0.31	0.036
Lumbar Spine	0.0015	-0.0045 to 0.0074	0.63	0.036
Total hip	0.0229	-0.0017 to 0.0062	0.26	0.100

*Baseline BMD, age and gender or estrogen deficiency were included in the models

Table 10 - Multiple linear regression analysis results: Change of BMD on urine pH*

The crude associations between the changes in BMD and urine pH, with no adjustment for potential confounders, showed no relationships (Figures 10 & 11). The associations were not confounded between urine pH and the change of femoral neck or lumbar spine BMD, however, the association between the total hip BMD and urine pH was confounded by the change of BMI, and therefore BMI was kept in the model.

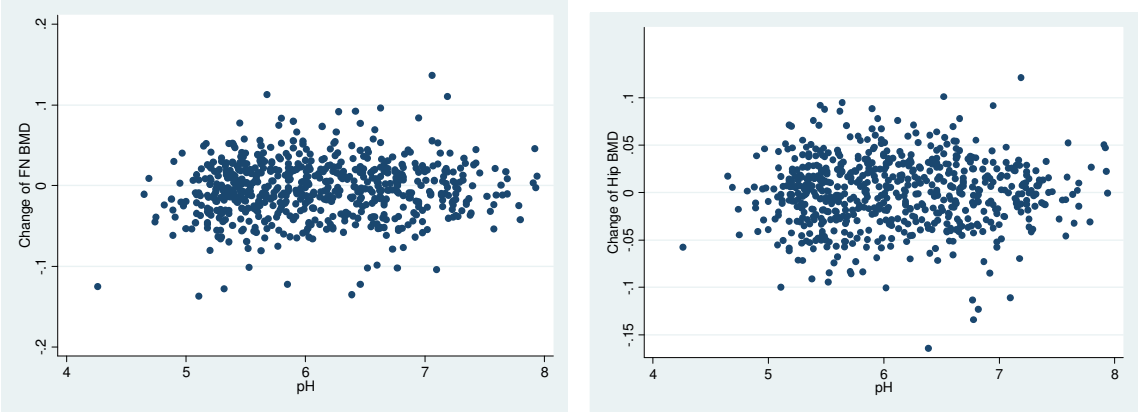


Figure 10 - Scatterplots for urine pH versus change in BMD: femoral neck and lumbar spine, with no adjustment for potential confounders

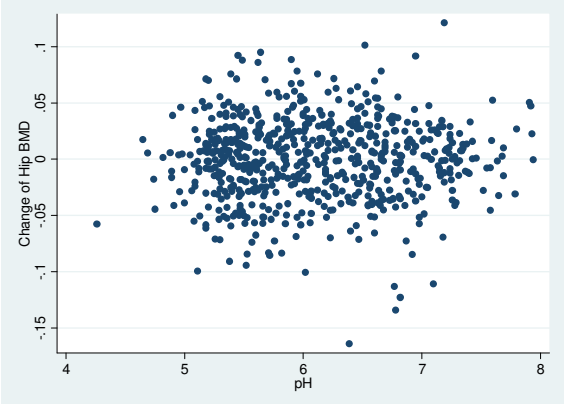


Figure 11 - Scatterplots for urine pH versus change in BMD: total hip, with no adjustment for potential confounders

Post estimation residual analysis did not identify any concerns with the assumptions of linear regression, including linearity, normality of residuals, homoscedasticity, and influential outliers (Figures 12 through 15). After defining the best models for each of the bone sites, the residuals were evenly distributed, with a close to normal distribution and had a reasonable quantity of outliers (<=5%) which were not likely to be influential given their placement and even distribution.

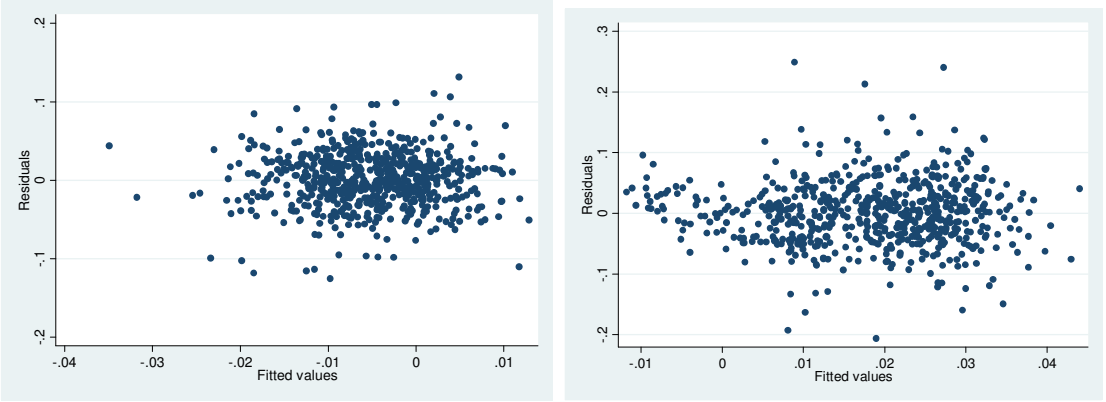


Figure 12 - Residuals versus fitted values for urine pH versus change in BMD for the femoral neck and lumbar spine

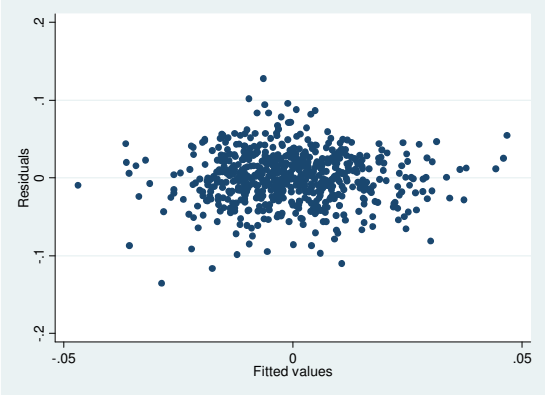


Figure 13 - Residuals versus fitted values for urine pH versus change in BMD for the total hip

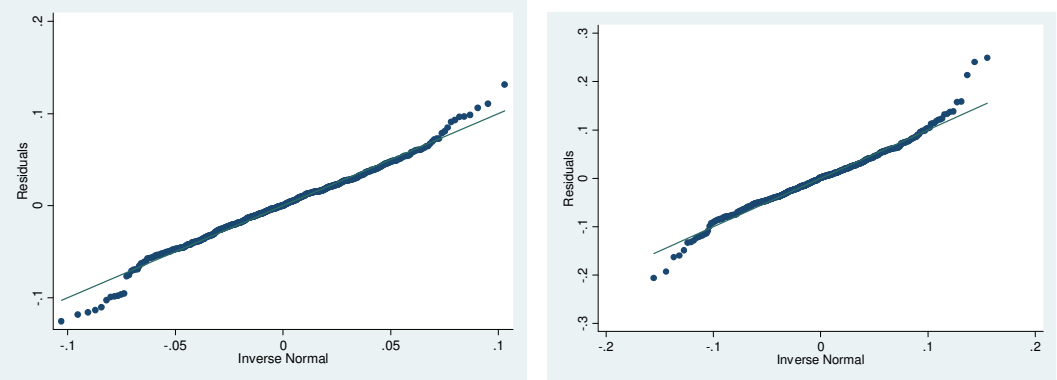


Figure 14 - Qnormal plots of the residuals for urine pH versus change in BMD for the femoral neck and lumbar spine

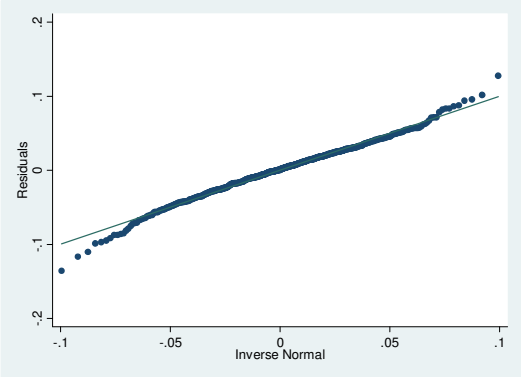


Figure 15 - Qnormal plots of the residuals for urine pH versus change in BMD for the total hip

5.11.2 Urine measures of excess urine acid load (as reflected by urine potassium, sulfate, phosphate chloride, sodium, calcium and magnesium and estimates of organic acids) with loss of BMD over 5-years

For the lumbar spine, urine potassium was significantly and negatively associated with an increase of BMD at the lumbar spine over 5-years (Table 11). The estimated rate of change of lumbar spine BMD was 0.0026 g/cm^2 for every ten mmol/l of potassium decrease in fasting morning urine (p = 0.011, aR2 = 4.2%, 95% confidence interval = -0.0046 to -0.0006). Post estimation residual analysis for the associations between urine minerals and changes of BMD, after

defining the best models for each of the three bone sites, did not identify any concerns with the assumptions of linear regression (Figures 16 through 19).

Bone Mineral Density	Potassium			
Bone Site:	Coefficient	95% confidence interval	p-value	aR^2
Femoral Neck	-0.0001	-0.0002 to 0.0001	0.31	0.034
Lumbar Spine	-0.0003	-0.0005 to -0.0001	0.011	0.042
Total hip	-0.0001	-0.0002 to 0.0001	0.23	0.015

*Baseline BMD, age and gender or estrogen deficiency were included in the models

Table 11 - Multiple linear regression analysis results: Change of BMD on urine potassium*

There was no relationship between urine ion concentrations and five-year loss of BMD for the other two three sites assessed, femoral neck and total hip (Table 11). Individuals' BMD for the femoral neck and total hip equally increased and decreased for those with high and low urine ions. The coefficients for the ions were never statistically significant, and the greatest amount of explained variance (adjusted R^2) for the best fit model for the femoral neck and total hip were three and fifteen percent, respectively. The findings were unchanged when all the potential confounders were controlled. Age and gender were retained in the models, except for the total hip where estrogen deficiency had a larger effect on the coefficient than did gender, and the latter was retained. Urine creatinine confounded the associations for the femoral neck and the lumbar spine. However, the inclusion or exclusion of urine creatinine did not alter the significance of the models.

The crude associations between the changes in BMD and urine potassium, with no adjustment for potential confounders, showed no relationship

(Figure 20 and 21). The associations between urine potassium and the change of BMD at the femoral neck and the lumbar spine were confounded by urine creatinine.

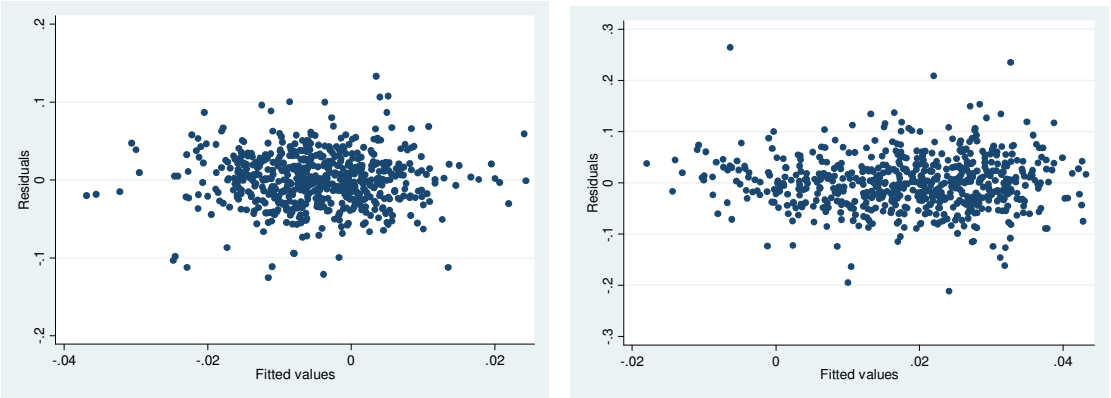


Figure 16 - Residuals versus fitted values for urine pH versus change in BMD for the femoral neck and lumbar spine

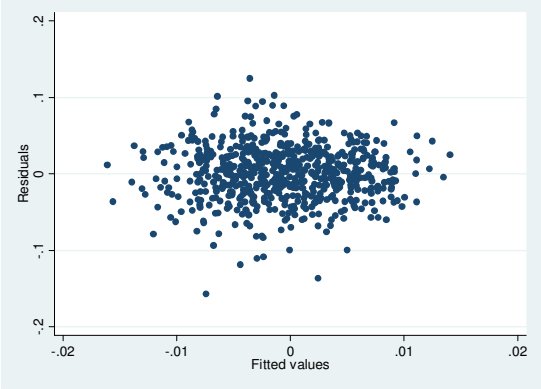


Figure 17 - Residuals versus fitted values for urine pH versus change in BMD for the femoral total hip

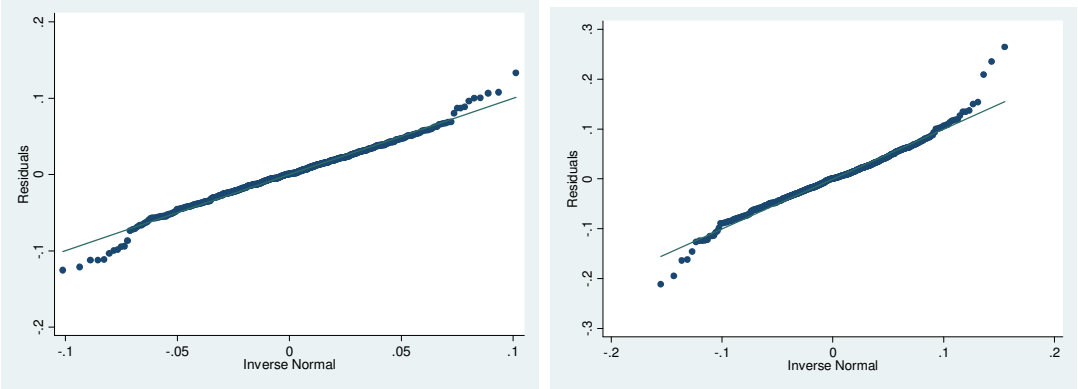


Figure 18 - Qnormal plots of the residuals for urine pH versus change in BMD for the femoral neck, total hip, and lumbar spine

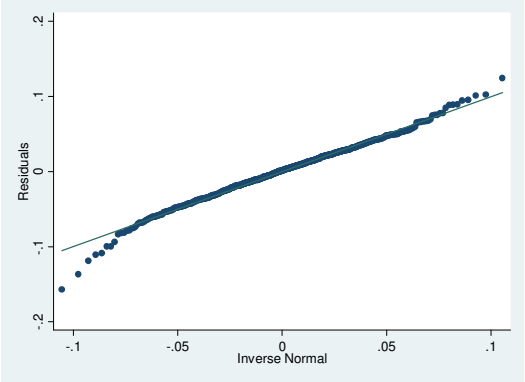


Figure 19 - Qnormal plots of the residuals for urine pH versus change in BMD for the femoral neck, total hip, and lumbar spine

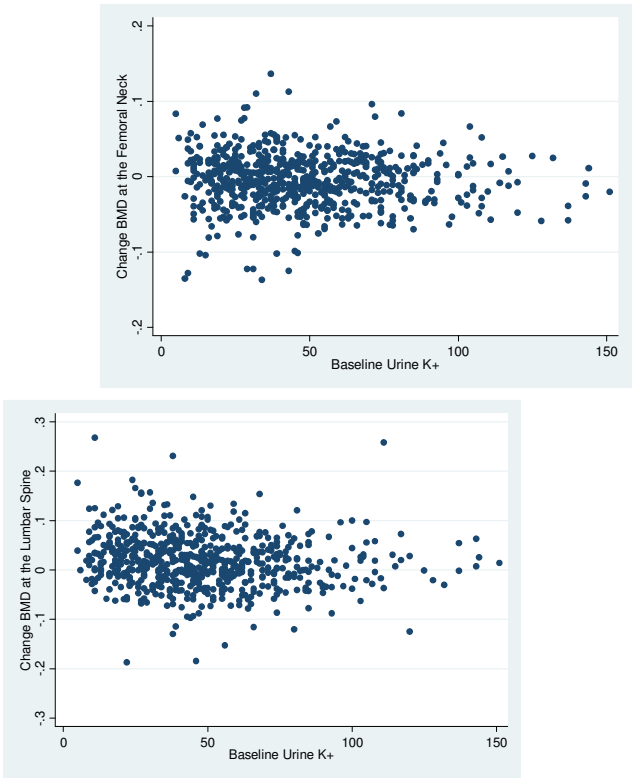


Figure 20 - Scatterplots for urine potassium versus change in BMD: femoral neck and lumbar spine, with no adjustment for potential confounders

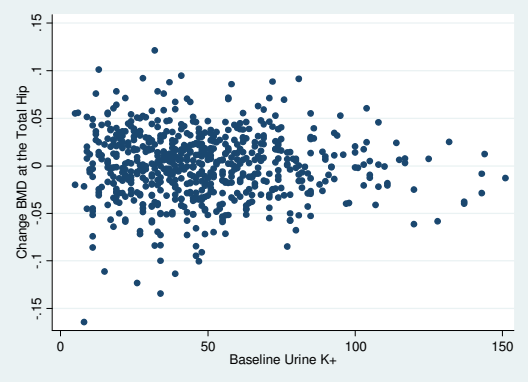


Figure 21 - Scatterplots for urine potassium versus change in BMD: total hip, with no adjustment for potential confounders

5.11.3 Urine Acid Excretion and loss of BMD over 5-years

There was no relationship between Acid Excretion and five-year loss of BMD for any of the three sites assessed, femoral neck, lumbar spine, or total hip (Table 12). Individuals' BMD equally increased and decreased for those with high and low Acid Excretion. The coefficients for Acid Excretion were never statistically significant and the greatest amount of explained variance (adjusted R^2) for the best fit model for each bone site ranged from only 0.3 to ten percent. Age and gender were retained in the models, except for the femoral neck and the lumbar spine where estrogen deficiency had a larger effect on the coefficient than did gender, and the latter was retained.

Bone Mineral Density	Acid Excretion			
Bone Site:	Coefficient	95% confidence interval	p-value	aR^2
Femoral Neck	0.0885	-0.0363 to 0.2132	0.16	0.094
Lumbar Spine	0.0001	-0.0002 to 0.0002	0.69	0.034
Total hip	0.0001	-0.0001 to 0.0002	0.20	0.005

*Baseline BMD, age and gender or estrogen deficiency were included in the models

Table 12 - Multiple linear regression analysis results: Change of BMD on Acid excretion*

The crude associations between the changes in BMD and Acid Excretion, with no adjustment for potential confounders, showed no relationships (Figures 22 & 23) for any bone site. The associations between acid excretion and the change of BMD for the femoral neck and the lumbar spine were confounded by the change of BMI, therefore BMI was kept in the model.

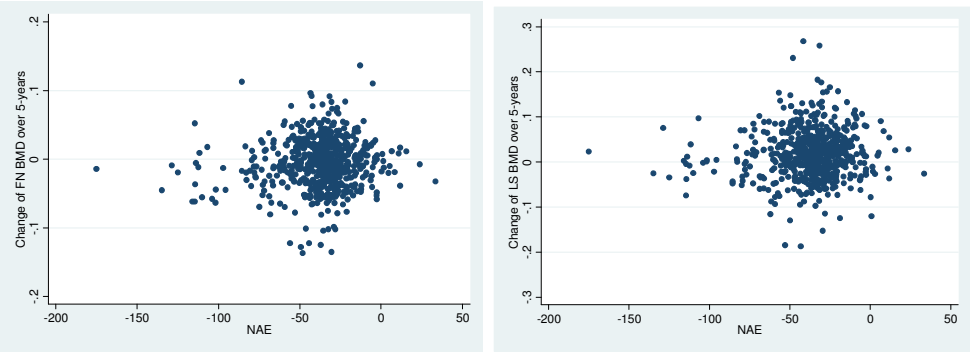


Figure 22 - Scatterplots for Acid Excretion versus change in BMD: femoral neck, lumbar spine, and total hip with no adjustment for potential confounders

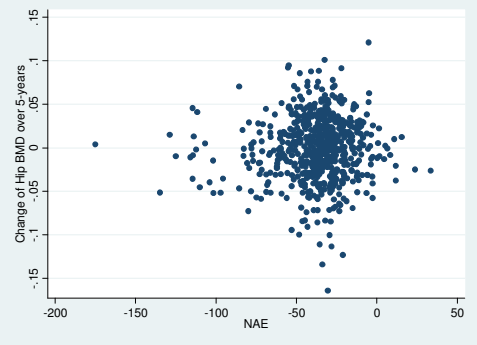
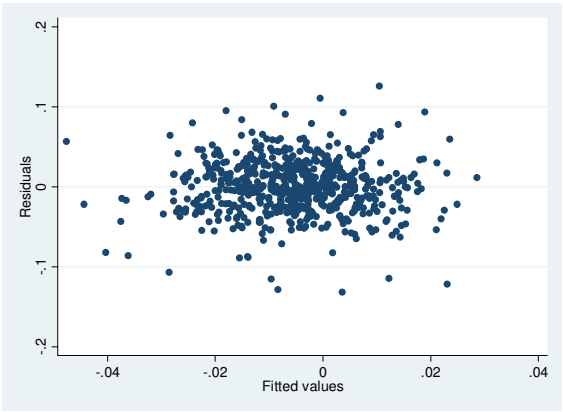


Figure 23 - Scatterplots for Acid Excretion versus change in BMD: femoral neck, lumbar spine, and total hip with no adjustment for potential confounders

After defining the best regression model for each of the bone sites, post estimation residual analysis did not identify any concerns with the assumptions of linear regression (Figures 24 through 27).



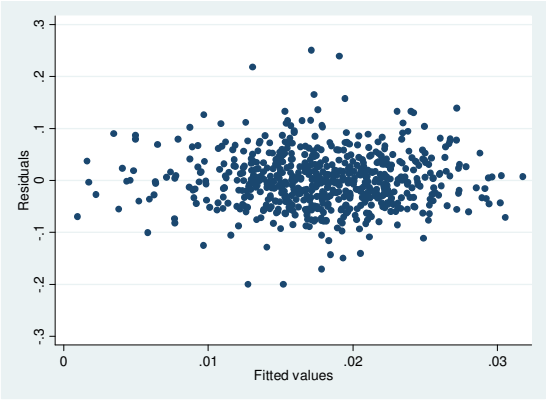


Figure 24 - Residuals versus fitted values for Acid Excretion versus change in BMD for the femoral neck and lumbar spine

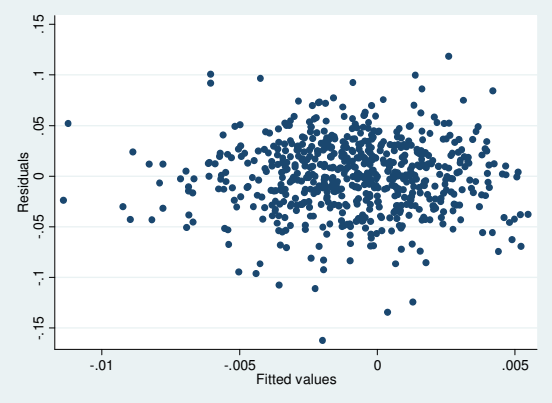


Figure 25 - Residuals versus fitted values for Acid Excretion versus change in BMD for the total hip

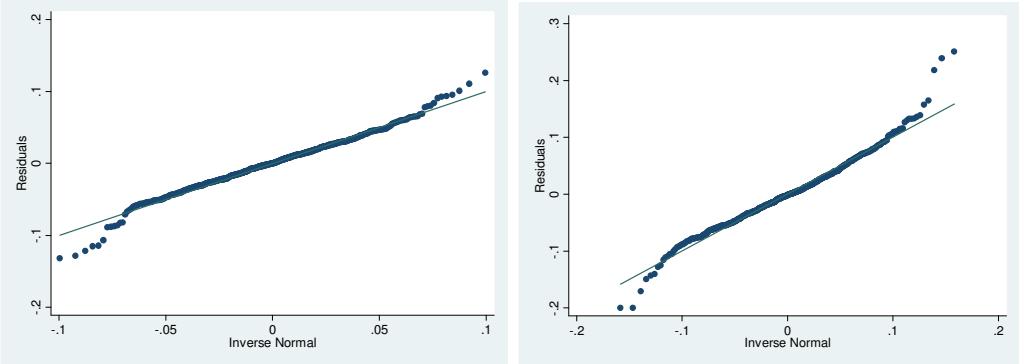


Figure 26 - Qnormal plots of the residuals for Acid Excretion versus change in BMD for the femoral neck and lumbar spine

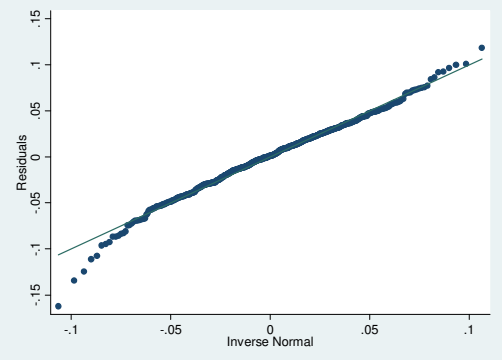


Figure 27 - Qnormal plots of the residuals for Acid Excretion versus change in BMD for the total hip

5.11.4 Power for the change of BMD over 5-years

The rate of BMD loss was less than expected based on cross sectional data of BMD at the different ages among the full CaMOS baseline cohort (3). Assuming a change of BMD of 0.01 g/cm^2 (1.4% for the femoral neck, 1.1% for the spine and total hip) over 5-years is clinically important, and using the standard deviations for changes of BMD from this study (Table 9), the resulting power of the analyses regarding the change of BMD in this study was estimated to be 94% for the femoral neck and total hip and 66% for the lumbar spine.

5.11.5 Urine pH and fragility fractures in adults over seven years

There was no association between urine pH and the occurrence of fragility fractures (relative risk (RR) = 0.88, 95% confidence interval = 0.57 to 1.36, p = 0.57) or total fractures (RR = 0.85, 95% confidence interval = 0.61 to 1.19, p = 0.35) during 7-years of follow-up (Table 13). Subjects had similar risks of fracture for those with low and high urine pH, whether or not potential confounders, including baseline urine calcium, age and gender, were controlled. Age and gender were retained in the models. The explained variances (Pseudo R^2) for the final models were five and one percent, respectively, and the relative risks for urine pH were never statistically significant. The association between urine pH fragility fractures was not confounded, while the association with total fractures was confounded by urine creatinine.

Fractures	pH			
	Relative Risk	95% confidence interval	p-value	Pseudo R2
Fragility fractures	0.88	0.57 to 1.36	0.57	0.047
Total fractures	0.85	0.61 to 1.19	0.35	0.014

*Baseline BMD, age and gender or estrogen deficiency were included in the models

Table 13 - Multiple logistic regression analysis results: Fractures on urine pH

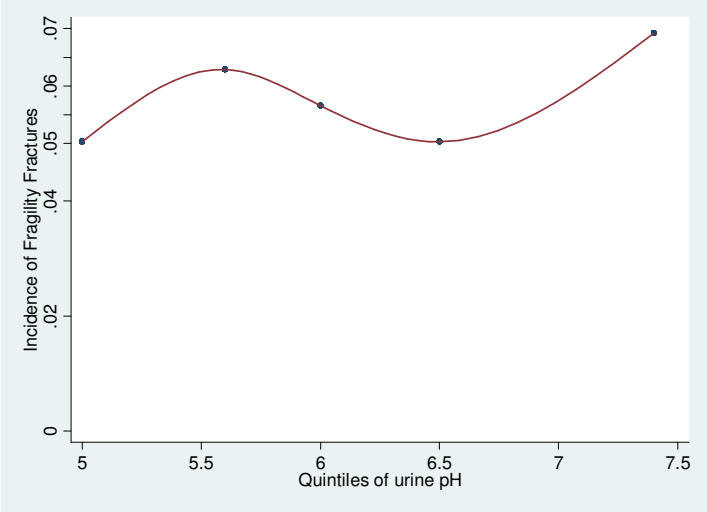


Figure 28 - Incidence of fragility fractures with respect to urine pH (median spline plot), with no adjustment for potential confounders

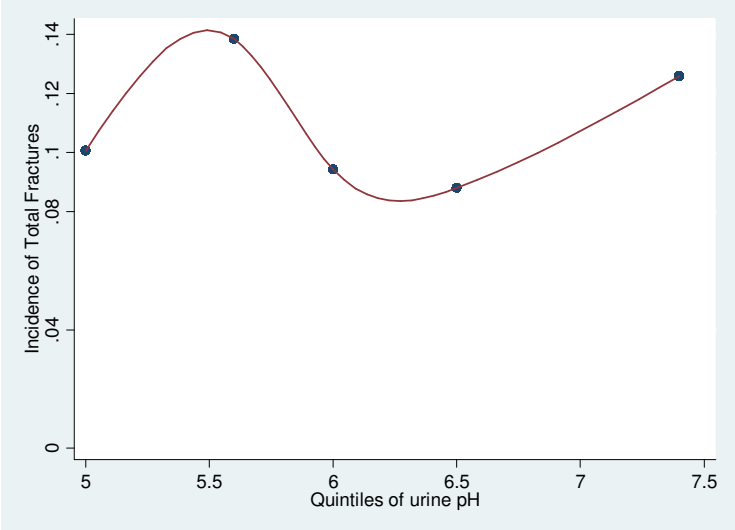


Figure 29 - Incidence of total fractures with respect to urine pH (median spline plot), with no adjustment for potential confounders

The crude incidence of fragility (Figure 21) or total (Figure 22) fractures with respect to urine pH, with no adjustment for potential confounders, showed no increase or decrease as urine pH increased.

Assuming that the clinically important difference in fracture reduction would be a decrease in the rate of fractures by one half, the estimated power of the analyses regarding fragility fractures was 43% and total fractures was 76%.

5.11.6 Stability of within-patient urine pH and acid load in fasting-morning-urine specimens collected at baseline and at five-years

The ICCs, which are the ratio of the between-subject variance divided by the total variance reflects the degree of stability of the within-subject urine measures over 5-years (Table 14) (Section 5.6.2). The stability of the urine ions over 5-years was highest for sulfate, which demonstrated good stability, however

the confidence interval was wide and compatible with poor to perfect stability. Potassium and phosphate demonstrated the next highest stability, which ranked as Fair stability. The remaining urine measures (pH, sodium, chloride, calcium and magnesium) had Slight stability (< 0.2) for the urine measures between the two measures taken 5-years apart.

	S_W	ICC	ICC 95% CONFIDENCE INTERVAL
Potassium	12.3	0.30	0.12 to 0.49
Sodium	47.0	0.15	0.00 to 0.38
Calcium	1.45	0.19	0.05 to 0.32
Magnesium	1.63	0.12	0.00 to 0.19
Phosphate	4.38	0.21	0.00 to 0.50
Chloride	54.7	0.15	0.00 to 0.38
Sulfate	5.61	0.64	0.00 to 1.00
pH	0.82	0.08	0.00 to 0.36

mmol/l = millimole per litre, Sw = SD within subjects, ICC = Intraclass correlation coefficient

Table 14 - Calculations of diet stability as measured in urine: pH and urine ions

The measurement error for ion excretion and urine pH was calculated as the within-subject standard deviation (Sw) to quantify the common measurement error of the variation within the subjects (Table 14) (265).

The Bland-Altman plots regarding the differences between the baseline and 5-year measurements versus the average of baseline and 5-year measurements all demonstrated that approximately 95% (94.8% for calcium to 96.9% for pH) of the differences fell within the 95% confidence limits of the plots (figures 30, 31, and 32). The plots all had some heteroscedasticity, since the variances were low for all of the plots near the y-axis where the average levels of the urine variables were low. At the upper end of the average levels, the plots

appear to follow two patterns. The plots for sodium, chloride and pH had a diamond shape, as the variance decreased at the higher average levels. Calcium, phosphorus, and magnesium appeared to have a fan shape and did not show the tendency for the variance to decrease at the higher levels. Potassium and sulfate seemed to have a mixture of these two shapes.

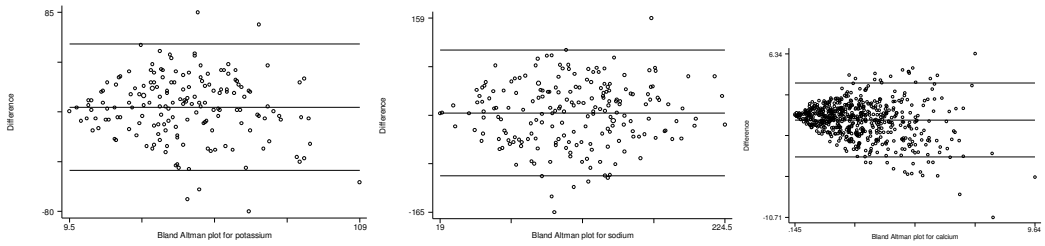


Figure 30 - Bland-Altman plots of differences between baseline and 5 year urine ions versus average of baseline and 5 year urine ions: Potassium (K+), Sodium (Na+), Calcium (Ca++

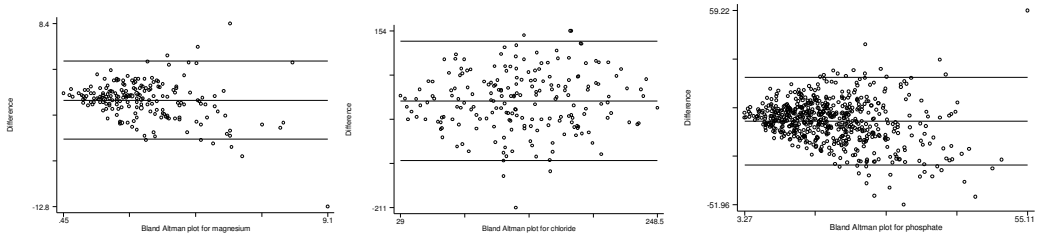


Figure 31 - Bland-Altman plots of differences between baseline and 5 year urine ions versus average of baseline and 5 year urine ions: Magnesium (Mg++), Phosphate (PO4--), Chloride (Cl-)

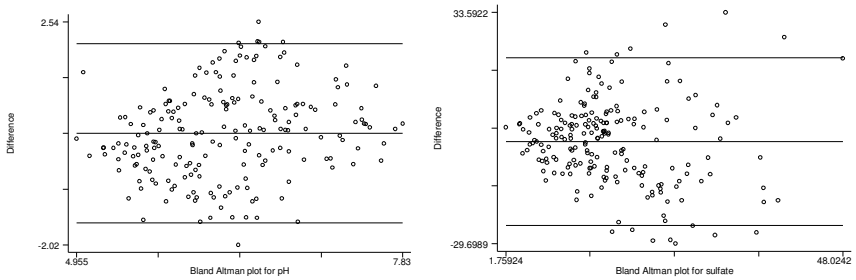


Figure 32 - Bland-Altman plot of differences between baseline and 5 year versus average of baseline and 5 year for urine pH and urine sulfate

This heteroscedasticity was illustrated with the Bland-Altman plots since the range of y-axis differences (between the baseline and 5-year values) varies across the range of the x-axis averages (of the baseline and 5-year values). For example, for average urine sodium of 50 mmol/l average of the baseline and 5-year values, the difference between the 5-year and baseline urine sodium ranged from 40 to +40 mmol/l, while those with average urine sodium of 100 mmol/l, the difference between the 5-year and baseline intakes ranged from -50 to +50 mmol/l.

For calcium, as represented in the Bland-Altman plots, the differences between the baseline and 5-year measurements ranged from approximately -2 to +2 mmol/l for the average of the two equal to 2, and ranged from about -7 to +4 at the average level of 5 mmol/l (Figure 23).

5.11.7 Evaluation of selection bias due to no urine sample provided

The subjects who contributed a urine sample at baseline were compared to those who had not contributed (Tables 15 and 16). Those who did not contribute were statistically significantly older, had lower vitamin D status, reported less family history of osteoporosis, lower levels of physical activity and calcium intakes, but had similar a gender distribution.

DEMOGRAPHIC AND CLINICAL CHARACTERISTICS	FRACTURE STUDY	NO URINE AVAILABLE	P-VALUE
n	795	338	
Age	59.8 (12.8)	65.9 +/- 15	<0.001
Female gender, (%)	553/795 (69.6%)	246/338 (72.8%)	0.28
Family history of osteoporosis, (%)	71/511 (13.9%)	12/242 (5.0%)	<0.001
Kidney Disease, (%)	118/733 (16.1%)	2/34 (5.9%)	0.11
Smoking, (%)	154/438 (35.2%)	70/177 (39.6%)	0.31
Thiazide medications, (%)	66/795 (8.3%)	37/338 (11.0%)	0.16
Bisphosphonates, (%)	8/795 (1.0%)	2/338 (0.6%)	0.49
Physical activity, kcal/day Total physical activities Exercise physical activity Hours reported	 1548 +/- 589 498 +/- 467 16.0 +/- 3.0	 +/- 555 369 +/- 393 15.9 +/- 3.0	 0.001 <0.001 0.61
Calcium intake, mg/day	889 +/- 550	810 +/- 535	0.026
Body Mass Index, kg/m2	26.1 +/- 4.7	26.2 +/- 5.0	0.75
Change of BMI, kg/m2	0.56 +/- 1.71	0.22 +/- 2.6	0.010
Vitamin D status, nmol/l	75.1 +/- 28.4	62.2 +/- 31 (n=16)	<0.001

mean +/- sd nmol/l = nanamole per litre

Table 15 - Subject characteristics of those who contributed a urine sample with those who did not contribute

Those who did not contribute urine samples had statistically significantly higher BMD at the femoral neck at baseline, and less change of BMD over 5-years at the lumbar spine, compared to those who contributed urine samples. There were no statistically significant differences between the groups in terms of their baseline lumbar spine and hip BMD and changes to their hip BMD over time (Table 16).

CHARACTERISTIC	BMD STUDY	NO URINE AVAILABLE	P-VALUE
Baseline FN BMD, g/cm^2	0.731 +/- 0.126	0.756 +/- 0.141	0.005
Baseline LS BMD, g/cm^2	0.938 +/- 0.160	0.967 +/- 0.176	0.08
Baseline HIP BMD, g/cm^2	0.885 +/- 0.146	0.904 +/- 0.155	0.06
Change FN BMD, g/cm^2	-0.005 +/- 0.035	-0.007 +/- 0.036	0.40
Change LS BMD, g/cm^2	0.018 +/- 0.054	0.011 +/- 0.047	0.045
Change HIP BMD, g/cm^2	-0.001 +/- 0.036	-0.002 +/- 0.034	0.66
Change FN BMD, % of baseline (change over 5-years)	-0.57% +/- 5.0	-0.67 % +/- 4.9 (n= 90)	
Change LS BMD, % of baseline (change over 5-years)	2.1% +/- 6.0	1.4% +/- 5.0 (n= 90)	
Change HIP BMD, % of baseline (change over 5-years)	-0.04% +/- 4.2	-0.01% +/- 4.0 (n= 90)	

mean +/- sd g/cm^2 = grams per squared centimetres

Table 16 - Bone outcomes of those who contributed a urine sample with those who did not contribute

Chapter Six: Discussion

The purpose of our study was two-fold: to critically evaluate the evidence in the literature regarding the acid-ash hypothesis and to determine whether the

acid-ash hypothesis is an important clinical predictor of individuals at risk of increased rates of bone loss or osteoporosis-related fractures over time, using fasting morning urine samples.

6.1 Comments on the study results

6.1.1 Bone mineral density (BMD) and fracture outcomes

In the primary analysis, we found no association between urine pH and a change in BMD at any of three bone sites, femoral neck, lumbar spine or total hip. All analyses were controlled for age, gender, reported family history of osteoporosis, physical activity, smoking, calcium intake, vitamin D status, hormonal status among the women, medications (thiazide diuretics and estrogen), renal function, urine creatinine, change of body mass index. Additionally, there were no associations between acid excretion, as measured in fasting morning urine samples, and changes in BMD at the three bone sites. Nor were there any associations between urine pH and the occurrence of fragility or total fractures. Among 11 measured associations between urine measures of dietary acid load and bone outcome measures, only one association was statistically significant. Low urine potassium was associated with increased BMD in the lumbar spine over 5-years, however, urine potassium was not associated with changes of BMD at the femoral neck and total hip.

The association between urine potassium and the change of lumbar spine BMD was a negative relationship in which spine BMD changed in the opposite direction to the change of urine potassium. Specifically, for each ten millimole decrease of urine potassium the estimated change in the change of spine BMD was a 0.003 g/cm^2 increase ($p = 0.011$)(section 5.11.2). The average lumbar

spine BMD of this cohort increased slightly over the five years. Although usually BMD decreases in adults over time, an increase in BMD at the spine can be a consequence of degenerative changes (from osteoarthritic changes and vertebral fractures) (268, 269). Degenerative changes in the spine have been associated with osteoporosis of the femoral neck and total hip in published studies, and have been considered indicative of osteoporosis (270). Consequently the association of urine potassium was in the direction expected by the acid-ash hypothesis, given that an increase in spine BMD (which is associated with osteoporosis) was associated with decreased urine potassium. However, this finding should be considered hypothesis generating rather than confirmation of the acid-ash hypothesis since the relationship was weak (only 4% of the variance was explained by this relationship), and there was no association between urine potassium and changes of BMD at the femoral neck and total hip.

6.1.2 Previous cohort studies of the acid-ash hypothesis

Not one of the previous prospective cohort studies regarding the importance of fruit and vegetables for the maintenance of bone mineral density over time has demonstrated consistent results between the various bone sites and sub-groups tested. Some associations between fruit and vegetable intakes, or the nutrients found in them, with the maintenance of BMD over time have been noted to be of statistical significance: with fruit and vegetable (52, 182), potassium (52, 181), and vitamin C (181, 222) intakes, however the majority of the associations tested between these factors and bone loss were not found to be important (section 4.5.2).

Due to the inconsistencies in the results from both previous prospective studies and this one, the few findings of importance of potassium or fruit and

vegetables should be considered as hypothesis generating rather than confirmation of the acid-ash hypothesis. Even if the associations between fruit and vegetable intakes, or the nutrients found in them, with the maintenance of BMD over time were strong and consistent, alone these findings would not provide proof of the acid-ash hypothesis since it would not be possible to attribute one specific mechanism to association noted in observational studies. Findings that there is an association between fruit and veg, or nutrients found in these foods, to be associated with better outcomes in observational studies do not confirm a hypothesis that refers to a specific mechanism since this study design does not differentiate about the mechanism. It is possible that an extraneous factor that was related to the consumption of fruit and vegetables accounts for the protective effect on bone, and the positive finding is due to uncontrolled confounding. It is also possible that a bone protective substance in fruit and vegetables that operates on a mechanism separate to the acid-ash hypothesis explains the statistically significant findings. It is also possible that the positive findings are due to chance. Therefore, the positive results regarding the acid-ash hypothesis from observational studies remain hypothesis generating rather than confirmation of the hypothesis.

6.1.3 Stability of urine measures

Urine samples from baseline and 5-years from the same subjects were examined to determine whether the urine measures of dietary acid load (pH, potassium, sodium, calcium, phosphorus, magnesium, chloride, and sulfate) remained stable or changed for the individual subjects, presumably due to changes in diet, over that time. The data revealed that the stability of the urine measures of dietary acid load were slight to substantial, with only one ranked as

substantial, and none were ranked as almost perfect. On the whole, the urine measures of diet acid load were not stable over the 5-years.

This low stability of urine ions over the 5-years reflects the inability of a single measure of urine to approximate the subjects' actual long term urine composition. This lack of stability may have been of importance to this study. When a risk factor has low stability over time, the estimation of the effect of the risk factor would be attenuated and the estimate of risk would be underestimated (155, 271). It may be possible that there was an association between the acid-ash hypothesis and osteoporosis that was not apparent in this study due to the inability of fasting morning urine samples to reflect the long term diet acid load of the subjects.

The Bland-Altman plots allow a check of the assumptions of studies of agreement. The assumptions of studies of agreement include a constant mean and variance of the differences and that these differences are normally distributed (272). These plots of the urine pH and ions indicate concerns with the constant standard deviation assumption since all of them show heteroscedasticity; the differences between the baseline and 5-year measures depended on the average value of measurements. At low average urine ion or pH levels the differences between the measures were smaller than those with higher average levels. Sodium, chloride and pH also demonstrated smaller differences at high average levels, while calcium, phosphorus and magnesium did not. The uneven variance demonstrated that the urine pH and ion stability was not uniform, but was related to the average urine measure. Subjects with low average urine ions and pH had more stability than those with higher levels. Sodium, chloride and pH also demonstrated more stability for subjects with the highest urine levels. Therefore, the stability of urine measures was dependent on

the average urine levels for all of the ions and pH. Overall, urine pH and most of the urine ions had low stability over the 5-years which reflect a poor description of long term diet acid load by one measure of fasting morning urine.

6.1.4 Other stability studies regarding repeated measures of food intake over time

Of particular interest for nutritional epidemiology is the change of food intakes of the individuals over time. Work that was done to determine the reproducibility of food intake measurement between two time periods has been published (273, 274). This work can be compared with our stability measures of the urine measures of diet acid load. We found good stability for sulfate (0.64) , but fair to slight stability (< 0.4) in our urine measurement of the diet acid load in fasting morning urine samples (section 5.11.6). In comparison to our findings from fasting morning urine, food measurement estimates of reproducibility were superior. ranging from moderate to almost perfect for food records (275) and from fair to substantial for food frequency questionnaires (276, 277).

Our study of urine stability and the studies of food intake reproducibility differed in a number of aspects. In terms of time between measures, Goldbohm et all had an identical time frame to ours, 5-years (278). The time frames in the Rimm et al. study were much shorter for the two tools they assessed: six months for the food records and one year for the food frequency questionnaires (279). Another difference was the purpose of the studies. The food measurement studies assessed the food frequency questionnaire's ability to measure what they assumed to be the underlying long-term food intake, which was an assessment of the tool itself. Our study assessed the stability of the underlying long-term urine measures of diet acid load, assuming that the laboratory measurements

were precise. In spite of the differences between the food intake measurement studies and our assessment of the stability of urine measures of diet acid load, it was clear from our findings that there was poor stability of most of these urine measures (except sulfate) over time. The poor stability documented in this study suggests that fasting morning urine may be a poor representation of the long term diet acid load.

6.1.5 The meta-analysis to estimate the difference of calcium excretion in response to acid-ash interventions

There is evidence of a relationship between the change of calcium excretion and the change of net acid excretion, based on the averages from 25 published studies. This relationship existed despite five studies that did not demonstrate this relationship (section 3.2). The relationships between calcium excretion and net acid excretion remained significant after removal of the three outlying study results, which indicated that the linear association was not due solely to the outlying cases.

Some of the difference in calcium concentration between acidic and alkaline urine was due to the lower solubility of calcium in alkaline urine, however, this difference in solubility did not appear to explain all of the calciuria seen. It is possible that the addition of acid to the urine after collection in these studies was insufficient to make all of the calcium soluble and that some measurement error remained. The meta-analysis of published studies does not provide any evidence regarding the source of the urine calcium or the potential for this effect to be indicative of the progression of osteoporosis.

6.1.6 The systematic review of the literature regarding the acid-ash hypothesis using Hill's criteria

The literature review based on the meta-analysis and Hill's causation criteria (Temporality, Biological Gradient, Strength, Experiment, Consistency, and Biologically Plausibility) was undertaken to evaluate whether there is evidence from the literature that the modern diet, via the acid-ash hypothesis, causes osteoporosis.

The findings from the meta-analysis suggest that that there is evidence of a linear association in the literature between study average results for calcium excretion from the changes of net acid excretion, based on the averages from 25 studies, which supports the criterion for a Biological Gradient for the calciuria outcome (Chapter 3). Hill's criteria for Strength was met by the acid-ash hypothesis in terms of the quantity of calcium excretion documented in response to acid loads is sufficient that over a lifetime the loss of calcium could explain the development of osteoporosis.

However, here are several important limitations to the published evidence and hence for the acid-ash hypothesis. The primary limitation to the acid-ash hypothesis is the inability to demonstrate that the diet acid load contributes directly to osteoporosis since Experiments have failed to include the direct measures of osteoporosis (bone strength as measured by fragility fractures or bone biopsy). The only clinically practical measures of bone fragility in osteoporosis that are currently available are low trauma fractures and or biomechanical testing of bone biopsy material (280, 281). The evidence that fits the acid-ash hypothesis is almost solely limited to results from studies that measured surrogate measure of bone loss, calcium excretion, which is not a

direct measure of the progression of this disease. Further, results from the prospective observational studies of the association between fruit and vegetables or their nutrients with changes in BMD have been inconsistent.

Further in terms of Consistency (section 4.5), there is evidence to the contrary regarding the hypothesis's purported roles for phosphate (detrimental) and sodium (protective) in terms of calcium excretion. As well, there are inconsistencies in terms of the food components (e.g. milk and grains) that are included together to form the acid-ash hypothesis, and in terms of the measurement of acidity in urine through titration. As well, whether fruits that contain substantial amounts of organic acids or protein are detrimental or supportive of bone mineralization has not been established.

Additionally, Biological Plausibility of the acid-ash hypothesis is weak based on the lack of a clear mechanism for bone demineralization by the hypothesis that can occur at physiological pH. The in-vitro studies that indicated an increased bone demineralization occurs at low pHs have been conducted at pHs below the physiological range, therefore there is no proposed mechanism for the acid-ash hypothesis that could occur at physiological pH.

In conclusion, based on the review of the literature to date using Hill's criteria it is not possible to draw a causal conclusion between the acid-ash hypothesis and osteoporosis. The literature does not contain proof that the acid-ash hypothesis is a determinant of bone health.

6.2 This study results in context of the previous studies of the acid-ash hypothesis

This study contributes important information in terms of the acid-ash hypothesis, and a caution about imprudent acceptance of this hypothesis. In

general the findings from this cohort study were in concordance with those of the other cohort studies on this topic and with the current systematic review based on Hill's criteria. In contrast, our findings were predominately not in support of the acid-ash hypothesis since 10 of the 11 hypothesized associations were found not to be predictive of bone health. Therefore this study did not provide unqualified support for the acid-ash hypothesis. This lack of support for the hypothesis could have been due to the type of urine samples (fasting morning) used and their poor ability to describe the long term diet acid load, or due to the hypothesis not describing an important cause of osteoporosis.

The systematic review based on Hill's criteria (Chapter 4), documented important concerns with the acid-ash hypothesis. Although the estimates of calcium loss in the urine from the literature are sufficient to be able to explain the progression of bone loss that becomes osteoporosis (section 4.3), there are six important weaknesses in the evidence regarding the acid-ash hypothesis. The weaknesses include the lack of studies that use more direct measures of this disease (bone strength as measured by fragility fractures or bone biopsy), the lack of consistency of results among the longitudinal cohort studies, and the lack of a mechanism that could take place at physiological pH. As well there are internal consistency problems with the hypothesis due to the conflicting roles of phosphate, sodium, and milk, since the evidence about these components differs from the purported roles by the hypothesis with respect to osteoporosis. The published evidence regarding the acid-ash hypothesis is not conclusive at this point.

The majority of the published intervention studies regarding the acid-ash hypothesis utilized urinary calcium changes as confirmation of bone demineralization (35, 41, 54, 55, 57, 108-110, 149, 150, 159-163, 165, 167, 169, 171). Importantly, if calcium absorption changes in response to the interventions

imposed, there may not have been any changes in bone mineralization and these studies' conclusions may be in error. The studies that investigated calcium absorption to attempt to determine the source of the urine calcium did not employ the recommended techniques (85), so it is not possible to combine the results in a firm conclusion about the effect of the diet acid load on calcium intestinal absorption to determine whether increased absorption might account for the calcium in the urine.

The published studies (52, 172, 181, 182, 222) that focused on more direct measures of osteoporosis (changes in BMD and fractures) demonstrated inconsistent support for the hypothesis (section 4.5.2), as did this study. Each of these studies, as well as this one, found only a minority of the tested associations to be important.

Between the concerns identified through the use of Hill's criteria combined with the inconsistent results from cohort studies, at this point the acid-ash hypothesis should be considered exploratory and not confirmed. A definitive study is needed to clarify whether or not the acid-ash hypothesis is important for long term bone health. Further research regarding the acid-ash hypothesis is needed to identify the source of the excess urinary calcium noted in the short term trials, to determine whether this mineral is being mobilized from the bone at physiological pH, or whether the interventions alter gastrointestinal calcium absorption.

6.3 The need for a definitive study of the acid-ash hypothesis

This study contributes information about the utility of fasting morning urine as a measure of the diet acid load and its importance in terms of fractures and the loss of BMD. The predominance of negative findings (finding of no associations) could be due to the hypothesis being flawed or due to the

measures in fasting morning urine are not measures of the diet acid load. Definitive studies are needed at this point to determine whether more resources (research dollars) need to be devoted to the acid-ash hypothesis, or whether these ideas need to be laid to rest while efforts for additional nutrition-related therapies to preserve bone health are sought elsewhere.

The definitive study to determine whether or not the acid-ash hypothesis is important for bone health should be a well-controlled, adequately powered randomized controlled trial designed to prevent bias from influencing the results. This study would require three arms with subjects two of the arms ingesting the “modern” diet , i.e. similar to the North American average intakes of protein, grains and fruit and vegetables. This modern diet could be defined to be similar to the average findings from nutrition surveys completed within the recent 35 years (282, 283). This time frame for the reference “modern” diet is arbitrary, however the concept of the “modern” diet causing osteoporosis by the acid-ash hypothesis has been in the literature for the past 90 years (49).

The comparison diet arm of the study (second arm) should receive the estimated “alkaline” diet as defined by leading researchers in the field (40, 41, 51, 189). The third arm of the study would receive the “modern” diet plus 60 mmol/day of potassium salt (citrate or bicarbonate) (41, 145), which should neutralize the estimated 45 milliequivalents (mEq)/day of net acid excretion from the modern diet (section 3.2).

To ensure that deficiencies of calcium intakes and vitamin D status do not cause any effects on BMD that will be observed in the study, i.e. to prevent confounding by these variables, intakes of these nutrients must be controlled throughout the study. The Scientific Advisory Council of the Canadian

Osteoporosis Society' recommendations of 1000-1500 mg/day of calcium and 400-800 IU of vitamin D could be used (section 2.1.6).

Ideally the primary outcome of this definitive study should be a direct measure of osteoporosis and therefore a measure of bone strength. The only clinically practical measures of bone fragility are low trauma fractures or biomechanical testing of bone biopsy material (284, 285). Biomechanical testing of bone is a direct measure of bone strength that requires samples of bone be taken through biopsy. Biopsies are invasive (require penetration of the skin, underlying tissues including bone) so some study subjects may not cooperate with this procedure. For the study to use fragility fractures as a measure of bone strength, the subjects would be required to be at risk of fracture (i.e. with established osteoporosis) and large numbers of subjects would be needed to power the study. The study with fractures as an outcome would require older subjects at higher-risk who might find changing their diet more difficult and could have a higher loss to FU through illness or death. The definitive study of the acid-ash hypothesis could use changes in BMD as the outcome measure. Although changes in BMD are not ideal measures of osteoporosis, they are generally accepted surrogate measure of osteoporosis (286) that are readily available and non-invasive. Additionally, the acid-ash hypothesis purports that the diet causes a loss of BMD, so the use of changes in BMD as the outcome would be measuring the parameter in question, i.e. the loss of BMD) for this study.

No further studies of the acid-ash hypothesis should be conducted with the outcomes limited to urinary calcium and/or bone resorption and formation markers. Urine calcium is not a measure of the progression of osteoporosis since changes in calcium absorption could confound the results and the interpretation of the results may be erroneous. Bone resorption and formation markers are

surrogate measures of osteoporosis that are suggestive of the mechanism at the cellular level (287), and so provide some hypothesis generating and potentially useful information as a secondary measure of outcome. Without more direct measures of osteoporosis, urine calcium and bone resorption changes do not provide definitive information about the progression of this disease.

The study subjects should be from the population at risk of bone loss under the acid-ash hypothesis: adults of approximately 25 years of age or older (3). Although some studies have suggested that the effect of the acid-ash hypothesis is a factor that determines bone mineralization of growth children (46, 184), the difficulties inherent in the estimation of bone mineral accrual in childhood (288) would add complexities to the study, so children would wisely be excluded.

The definitive study must be defined and conducted with care to prevent biasing the estimate of effectiveness of the interventions (289). Therefore, the participants must be randomly allocated to the study arms in a concealed fashion to ensure that the known and unknown confounding variables are distributed evenly among the groups. Subjects would be stratified by age groups ($\leq$ 50 and > 50 years) and by gender to ensure that the study groups are balanced for age group and gender. Within these strata, subjects would be randomized into the three groups using a method to keep the allocation concealed until the intervention is assigned. Allocation could be concealed by having a clerk not involved with the subjects keep the allocation plan in a separate location, and the investigators could contact to find the group assignment by telephone contact. Permuted blocks of varying size could be used in the randomization to ensure to avoid imbalance of the groups.

Randomization should distribute the potential confounding variables (age, gender, hormonal status of the women, amount of physical activity, etc) evenly between the groups of the definitive study. Although the study would not be adequately powered to assess the efficacy of the two interventions among age and gender subgroups, the subgroups groups could be examined for differences non-statistically under secondary objectives.

The sample size required for the definitive study would need to be based on the subjects' estimated change of BMD, and the standard deviation, as well as the alpha (usually 0.05) and beta (often 0.20) (290). If a randomized cross-over study design were selected, the estimated sample size would be smaller if the estimated variability were lower (291). Cross-over trials, which have been frequently used to assess this hypothesis, have advantages of further decreasing variance and hence the required sample size. However, a cross-over study design would require a very long follow-up to follow the subjects for their change of BMD through the three arms, and retention of subjects in the study would likely become inadequate, making this study design infeasible. Interventions to alter bone strength require some time to cause measurable changes in bone. Even with a parallel study design with different subjects in the three arms, the definitive study would require a follow up of at least two years to be able to detect changes in BMD to ensure that any effects of the diet changes or salt supplement would be measurable in spite of the measurement error of the DEXA (292).

Blinding of the diet intervention arms would not be possible, however the salt supplements could be blinded by providing the groups not allocated to the potassium salt with a placebo, containing an inert substance such as a sugar. Both the potassium citrate and the placebo should be provided in capsules, and a bitter substance such as quassin may be added to discourage tasting of the

contents (293). Both the salt and the placebo should be contained in identical jars assigned a random number for each of the three study arms. Study investigators would also be blinded to the salt and placebo study arms to prevent accidental or unintentional sharing of group assignment information with the subjects. Study analysts would be blinded to the group assignment of all three groups to prevent any unintentional bias during analysis.

The analysis of this definitive study should be an intent-to-treat analysis since this method maintains the benefits of the randomization in terms of control of confounding variables. Efforts should be made to encourage the subjects to remain in the study since a loss of subjects during the study decreases the sample size and therefore the power of the study (290).

Efforts to keep participants engaged in the study and increase subject adherence to the study protocol would help to decrease losses to follow-up and a loss of power due to subject drop-out (294). Numerous approaches can be used to encourage subject adherence, and these approaches are related primarily to establishing relationship with the subjects and making adherence as pleasant and easy for the subjects as possible (294). Additionally, it is useful to report the subjects degree of adherence to the protocol (294) to improve the estimated effectiveness of the alkaline diet or the potassium salt supplement (238), so efforts should be made to document the subjects' actual food intake. A food frequency questionnaire such as the one developed for the Nurses' Health Study (295) could be used to document food intakes.

To implement the diet of the definitive study, ideally all food would be provided in a controlled fashion, such as in a metabolic ward, to ensure that the subjects comply with the study and consume the assigned food. However, this is

not possible for humans to ignore the socio-cultural aspects of food and eat in an experimental setting for long periods of time. Therefore, an alternative approach would be to could counsel the “alkaline” diet group to consume nine to ten servings of fruit and vegetables each day. Consumption of these larger intakes of fruit and vegetables would likely be more successful if these foods are provided to the subjects and their families free of charge. The free fruit and vegetables could be provided to the whole family of the subjects’ so the sharing of food would not cause a dilution of the intervention for the study subjects. To ensure generous protein and grain intakes of the “modern” diet groups, these subjects could be provided with free of charge protein and grain foods.

If the acid-ash hypothesis is determined to be important in terms of bone health in a definitive study, the next step would be to identify the critical aspects of the diet. Further studies would be needed to determine what specific aspects of the diet were protective or harmful for bone health. Further research questions to be explored include: What is the ideal intake of protein and phosphorus to support life-long bone health? Are grains protective or detrimental, and what level of intake and which types of fruit and vegetables are most protective of bone health? Additionally, if the hypothesis is found to be important for bone health, 24-hour urine samples could be studied in association with changes in BMD over time to determine if these urine samples might provide a measurement tool for the assessment of the diet acid load.

6.4 Could the acid-ash hypothesis be harmful?

The acid-ash hypothesis recommends that people consume generous quantities of fruit and vegetables (8 to 10 servings per day (182)) along with modest amounts of grain and protein foods (protein intakes equal to the

recommended intakes (149, 250) to maintain bone health (40, 296). Generous quantities of fruit and vegetables are not likely to be harmful and may have other health benefits (297). It is possible that fruit and vegetables are beneficial to bone health through mechanisms other than through a decrease in the diet acid load since there is some preliminary human and animal evidence that some fruits and some vegetables have supportive effects on bone (216, 228). One of these studies indicated that plums increased serum levels of insulin-like growth factor-I (216), a stimulus to bone formation, and a marker of bone formation (bone-specific alkaline phosphatase), yet plums provide an organic acid and increase urine acidity (175), which would suggest that they would be a detrimental food for bone health under the acid-ash hypothesis. At this point the importance of fruit and vegetables for bone health is not clear.

In terms of protein, recent research suggests that sufficient protein intake is important for the maintenance of bone integrity (39, 298), so advice to limit protein intake for bone health may be counterproductive. To date the advice to restrict grain foods under the hypothesis (40, 296) has not been evaluated (110). Along with generous servings of fruit and vegetables, restricting grain foods may increase food costs as grain foods tend to be less expensive than other foods such as fruit and vegetables.

The acid-ash hypothesis has been promoted to the public through the popular press in terms of the importance of maintaining an alkaline state as a requirement for both bone health and general good health (299-301). It is possible that the advice regarding the hypothesis may be detrimental to bone health since the optimum intakes of protein to support bone health have not been defined. Some sources of organic acid such as cranberries and plums, and so their consumption under the acid-ash hypothesis would be discouraged, could be

protective of bone health. The recommended decreased intakes of grain foods could raise the cost of eating, yet this advice is not based on any substantiated study. As well, due to the lack of identification of an association between urine measures of acidity and changes in BMD or fractures in this study, and the questions raised regarding the hypothesis, advice to the public in this regard is premature.

6.5 Selection bias due to whether or not subjects contributed urine

The cohort subjects who did not contribute a urine sample were statistically different in terms of some of the osteoporosis risk factors (older age, less reported family history of osteoporosis and physical activity, lower vitamin D status, higher femoral neck BMD). Those who did not contribute a urine sample had less change of lumbar spine BMD over the 5-years, but the changes in BMD at their femoral neck and hips were similar. Although some differences were statistically significant, the groups were similar in terms of clinical characteristics: Both groups were predominately women of mean age in their sixties, with low reported family history of osteoporosis, mean calcium intakes approximating the Adequate Intake (85), did not partake in much physical activity, had similar BMD measurements at baseline and changes of BMD. Although it is possible that the estimated effect of the diet acid load as measured in urine could have been different in those not included in the study due to their lack of contribution of a urine sample, the two groups were similar in terms of their clinical characteristics. Since their change of lumbar spine BMD was less than those who contributed urine samples, the inclusion of those who did not contribute urine may have decreased the one statistically significant estimated association between urine potassium on the change of BMD.

6.6 Study Strengths

This first cohort study to measure the diet acid load post-absorption and metabolism, i.e. in urine had six strengths. First, this population-based cohort study of the adult population that live in or near Quebec City was an appropriate choice for this study since wide ranges of intakes for protein, potassium, fruit and vegetables are consumed by this population (302, 303) (section 5.2.1). The wide range of intakes of the foods and nutrients of interest ensures that there was sufficient range of exposures to create the acid-ash differences, if the acid-ash hypothesis is effective.

Second, the use of urine as a vehicle to measure the diet acid load had advantages over the measurement of food measures of this exposure. The measurement of urine allowed us to avoid the random and systematic errors of inherent in food intake measurement. Some of the published intervention studies of food intake under the acid-ash hypothesis have estimated correction factors to account for imperfect absorption of nutrients from foods (35), however absorption of a given nutrient from foods varies from food to food, person to person and absorption is dependent on the combination of foods consumed together (Section 2.2.3). Therefore a single correction factor for each nutrient is not likely to be accurate. Urine may be a superior vehicle compared to food intake for measurement of absorbed nutrients since urine quantifies the nutrients after the absorption phase, assuming that the subjects were in approximate nutrient balance.

Third, the outcome measures used in this study, the change of BMD and the occurrence of fragility fractures are superior measures of osteoporosis relative to the measurement of urine calcium changes. Fragility fractures are

considered a direct measure of osteoporosis (304, 305). Changes of BMD are not a direct measure, but the change of BMD measures the aspect of bone that is hypothesized to be influenced by the diet, i.e. the minerals. Further, low BMD is a risk-factor for osteoporosis and a generally accepted surrogate measure of the progression of this disease (2).

Fourth, being a prospective study, this study had temporality, that is, the outcomes occurred after the collection of the urine samples. With temporality, there was no confusion regarding whether the composition of the urine, the exposure, was influenced by the changes in BMD or the fractures, the outcomes, that occurred over the latter 5-years.

Fifth, there was no suggestion of a strong bias due to the self-selection by contributing a urine sample (section 6.1.7).

Sixth, the measurements of BMD were relatively accurate. The calibration report on the DEXA machine in Quebec City found that adequate calibration was maintained over time (Appendix 2).

6.7 Study Limitations

This study had seven main limitations. The first limitation was related to the type of urine samples used, fasting-morning samples. We cannot generalize our findings to other measures of diet acid load, for example 24 hour urine samples. Fasting morning urine samples, as used in this study, may not quantify acid excretion and the long term diet acid load of the subjects. It is possible that a study of pH and urine ions in single or multiple 24-hour urine samples would find an association between urine measures of dietary acid load and measures of bone health. Although some previous studies suggested that fasting morning

urine samples provide useful measures of the diet acid load (51, 55, 133, 144, 152) a recent Canadian study noted only a weak association between the pH of the first-voided-morning urine (as opposed to our second-voided morning urine) with net acid excretion of the 24-hour collection (Pearson correlation = 0.342) (56). Although this current study adds knowledge regarding pH and ions measured in fasting-urine samples in association with changes of BMD and fractures, it cannot inform regarding associations between changes in BMD or fractures and 24-hour urine sample measures of diet acid load.

Second, the poor stability of the urine measures of the diet acid load means that it is possible that there was an association between the acid-ash hypothesis and osteoporosis that was not apparent in this study due to the inability of fasting morning urine samples to reflect the long term diet acid load of the subjects.

Third, cohort studies are prone to confounding and reporting bias. Confounding of the associations occur in cohort studies since the subjects select their diets and other health related behaviours and they are not exposed to one factor in isolation of others. Reporting bias occurs when the subjects misrepresent their health related behaviours, which leads to measurement errors. In this study the confounding variables were reported and therefore susceptible to bias. The measurement of physical activity is an example of a variable that suffered from obvious reporting bias. Although subjects were asked to report an estimate of their physical activity for 24 hour period, the average number of hours reported totalled 16.0. The error was not due to the omission of sleeping since the average time for sleeping was 7.3 hours per night. It was possible that the questions to measure physical activity were difficult to answer accurately, since the measurement of physical activity is difficult (306). Confounding and reporting

bias can both lead to confusion of results and perhaps biased estimates of effects. Fortunately, this study had objective measures of urine and bone outcomes, so the only variables influenced by reporting bias were the potentially confounding variables.

Fourth, due to multicollinearity between the variables, such as between two exposure variables or between an exposure variable and a potential confounder, it was not possible to assess the effect of all of the variables together in the model. The correlation between BMI and the calculated organic acids (Section 5.1) was likely due to the dependence of both of these variables on measures of height and weight. It was not possible to accurately estimate the effect of each of these variables due to their collinearities.

Fifth, this study had limited power for the analyses regarding two of the outcomes: the change of BMD at the lumbar spine and for the fragility fracture analyses. The consequences of low power are wide confidence intervals and lower chances of finding a statistical significant difference. In this case the estimated relative risk = 0.88 (95% confidence interval = 0.57 to 1.36, p = 0.57) for fragility fractures reflects a weak relationship and is not suggestive that a larger sample size would readily cause it to become statistically significant. Rather, estimates of weak relationships are usually due to uncontrolled biases and not due to a causal mechanism (197).

Sixth, the genetic component of the risk for osteoporosis may be a very important factor, but it was not adequately represented in this analysis. An attempt was made to include genetic factors, through the variable regarding family history of osteoporosis. However, osteoporosis is not well diagnosed (307), and consequently people are often not aware of whether they have this

condition. The CaMOS participants were therefore not likely well informed about the presence of this condition among their family members and therefore this variable did not likely accurately represent the subject's genetic predisposition. As well, this variable had frequent missing values (26.8%). Therefore this study was unable to adequately take the genetic risk of osteoporosis into consideration.

Seventh, we have assumed that the laboratory measurements of our samples were accurate. Our samples were measured in four separate laboratories (pH; sulfate; and the remaining ions were labs in both Quebec City and Calgary). It is possible that some errors were introduced by changes in calibration of the lab equipment in one or more laboratory.

6.8 Conclusion

This study provides little support for the acid-ash hypothesis. Multivariate analyses revealed no evidence of an association between urine pH and the bone outcomes. The one statistically significant finding of a negative association between urine potassium concentration and lumbar spine BMD over 5-years may indicate that there may be some importance of urine potassium, or a related parameter, for bone health. However, the acid-ash hypothesis as it is described in the literature to date is inconclusive and lacks clarity to determine which, if any, of the purported detrimental and supportive factors are important for bone health and which factors should be discarded as irrelevant or contradictory.

The evidence from this study rules out measures of acid excretion from a single measure of fasting morning urine as a source of information about the diet acid load as an important risk factor for osteoporosis. At this point it is not clear whether the lack of clear association between fasting morning urine measures of diet acid load and bone outcomes is due to the type of urine sample used, the

lack of ability of a single measure to reflect long-term diet acid load, or due to the acid-ash hypothesis not being predictive for bone health. Efforts should be made to further clarify whether the acid-ash hypothesis is worth pursuing as an important theory for understanding osteoporosis. The acid-ash hypothesis may not be true; therefore other work is needed toward other novel hypotheses and mechanisms that may improve our understanding of the prevention of osteoporosis.

The acid-ash hypothesis integrates seven nutrients into a dichotomy of acidity versus alkalinity which is appealing. However, foods are very complex in composition and the simplicity of acidity versus alkalinity may be overly simplistic for understanding the relationship between diet and the preservation of bone health.

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List of Tables

List of Figures and Illustrations

List of Symbols, Abbreviations and Nomenclature

If you do not have any symbols, abbreviations, or specific nomenclature in your thesis, you do not need to fill out this table. To add another row to the table, with your cursor in the bottom right cell, press the TAB key (beside the letter Q on your keyboard).

Symbol	Definition
BMD	Bone mineral density
Ca	Calcium
Cr	Creatinine
DEXA	Dual Energy X-ray Absorptiometry
g/cm2	Grams per square centimetre
ICC	Intraclass correlation coefficient
K	Potassium
Mg	Magnesium
mmol/l	Millimole per litre
nmol/l	Nanamole per litre
Na	Sodium
P	Phosphorus
S_b^2	between-subject variances
S_w^2	within-subject variances
SO_4	Sulfate

Appendices:

1. Baseline, 3, and 5-year CaMOS questionnaires

2. CaMOS Quality Control and Data Correction Report regarding Site Longitudinal Stability for the Quebec City DEXA machine