EXPLORATION OF STRONTIUM UPTAKE BY BONE AND SOFT TISSUES USING STABLE ISOTOPE TECHNIQUES

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DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

The thesis has also not been submitted for any degree in any university previously.

03/04/2014

Fransiska Dewi

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SUMMARY

Due to their similar chemical properties, strontium shares similar physiological pathways as calcium. Both elements are mainly located in bones. Although skeletal deposition of radio-strontium (^{90}Sr) from nuclear accidents was the main concern in the past, interest in strontium has increased recently with the use of stable strontium as an osteoporosis drug. At high doses, strontium can positively affect bone quality and reduce fracture risk. However, concerns have been raised regarding its safety, which resulted in the European Medicines Agency's (EMA's) recommendation on its restrictions.

In the first part of the thesis, similarities and differences in bone deposition and urinary discharge between calcium and strontium were explored. In the first ever long-term study of its kind, a stable strontium isotope (^{86}Sr) has been administered together with ^{41}Ca to an adult sheep. Urinary tracer excretion was monitored over 6 months using Accelerator Mass Spectrometry (AMS) and Thermal Ionization Mass Spectrometry (TIMS). Discrimination between both tracers was strong at the beginning. Subsequently, ^{86}Sr followed ^{41}Ca excretion closely. A stronger response of the ^{86}Sr signal due to an unexpected change in bone mineral balance indicates that strontium is a more sensitive tool. Laser Ablation Mass Spectrometry (LA-ICP-MS) of bone samples revealed that tracer was deposited preferentially in areas of bone growth and high remodeling activity. Isotopic labeling of the skeleton with the long-living radiotracer ^{41}Ca has been suggested as an ultra-sensitive technique to assess changes in bone calcium balance. However, its analysis is technically demanding and limited to a dozen research laboratories worldwide. Findings suggest that strontium isotopes are a convenient alternative to ^{41}Ca for assessing changes in bone calcium balance qualitatively but not quantitatively.

In the second part, we investigated the possible risks associated with high strontium intakes, particularly in soft tissues, and their effect on bone turnover and biomechanical properties. Rats were kept on two strontium levels for 3 months. Strontium and calcium concentrations in bones and soft tissues were determined by isotope dilution mass

spectrometry (IDMS). Spatial distribution of calcium and strontium in bone was studied by Particle Induced X-Ray Emission (PIXE) analysis. An improvement in bone quality was observed in the lower dose group, which exhibited serum strontium levels comparable to patients undergoing strontium therapy. For the higher dose group, strontium had a negative impact on bone quality. A threshold level may exist beyond which strontium and possibly calcium is taken up less efficiently by bone and deposited increasingly in soft tissues. Calcification/strontification of soft tissues and blood vessels has been suggested as a mechanism leading to cardiovascular disease.

Lastly, we assessed strontium intake from diet in a group of Singaporean Chinese women. Strontium intake was found to be lower by two orders of magnitude than the therapeutic dose. Dietary strontium intake should therefore have a negligible effect on bone and soft tissues. In the same study we found that most women did not meet estimated dietary requirements for calcium and magnesium. Older women were at lower risk of insufficient intake due to a higher consumption of supplements and fortified foods.

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LIST OF SYMBOLS

A	expected value of a random variable
$\hat{A}_n$	approximation of A (expected value of a random variable)
a_{reg}	intercept of a regression line
b_{ij}	slope of a line connecting two data points
b_{reg}	slope of a regression line
Ca_f	filtered calcium
Ca_u	calcium excreted in urine
df	degree of freedom
e	base of the natural logarithm (≈2.71828)
f_1	fractional intestinal absorption of the ingested activity
k	number of samples
n	sample size
med	median of a slope
m_{ij}	mean of U statistic
MS (B) or S_b^2	mean square between group
MS (W) or S_w^2	mean square within group
N	total sample size
n_{sam}	unknown amount of the element in the sample to be determined
n_{spike}	amount of the spike added to the sample
R	sum of ranks for non-parametric statistical test
R^2	coefficient of determination
Res	residual for each data point againsts a regression line
R_{mix}	measured isotope ratio of a mixture of both sample and spike
R_{sam}	measured isotope ratio of the pure sample
R_{spike}	measured isotope ratio of the pure spike
Sr_f	filtered strontium

Sr_u	strontium excreted in urine
SS (B)	between group variation
SS (T)	total variation
SS (W)	within group variation
t	time or value from *t*-distribution for a 5% significance level
$\bar{X}_{GM}$	grand mean
$\sum R_{i,sam}$	sum of all isotope ratios of the sample
$\sum R_{i,spike}$	sum of all isotope ratios of the spike
$\sum x$	sum of all the data values
σ_{ij}	standard deviation of U statistic
σ_n	standard deviation of $\hat{A}_n$ (approximation of A)

LIST OF ABBREVIATIONS

$1,25(OH)_2D$	1,25-Dihydroxyvitamin D_3 (calcitriol)
7-DHC	7-Dehydrocholesterol
25(OH)D	25-Hydroxyvitamin D
ω3FA	Omega-3 Fatty Acids
ω6FA	Omega-6 Fatty Acids
AAS	Atomic Absorption Spectrophotometry
ALP	Alkaline Phosphatase
AMS	Accelerator Mass Spectrometry
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
AVC	Aortic Valve Calcification
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMI	Body Mass Index
BMU	Bone Multi-cellular Unit
BTM	Bone Turnover Marker
Calbindin	Calcium binding protein
Cd	Cadmium
CeLS	Centre for Life Sciences
CIBA	Centre of Ion Beam Application
Cs	Caesium
CTX	Carboxyl-terminal Cross-linking Telopeptides of Type I Collagen
DPD	Deoxypyridinoline
DXA	Dual Energy X-ray Absorptiometry
EAR	Estimated Average Requirements
EFA	Essential Fatty Acid

ELISA	Enzyme-Linked Immunosorbent Assay
EMA's CHMP	European Medicines Agency's Committee for Medicinal Products for Human Use
EPIC	European Prospective Investigation into Cancer and Nutrition study
ERa	Estrogen Receptor alpha
ERb	Estrogen Receptor beta
F-AAS	Flame Atomic Absorption Spectroscopy
FFQ	Food Frequency Questionnaire
GF-AAS	Graphite Furnace Atomic Absorption Spectroscopy
GI	Gastrointestinal
HRT	Hormone Replacement Therapy
IACUC	Institutional Animal Care and Use Committee
ICP-MS	Inductive Coupled Plasma Mass Spectrometry
IDMS	Isotope Dilution Mass Spectrometry
IRMM	Institute for Reference Materials and Measurements
LA-ICP-MS	Laser Ablation Inductive Coupled Plasma Mass Spectrometry
LBMM	Laboratory of Biomedical Mechanics and Materials
LRP5	LDL Receptor-Related Protein 5
MC-ICP-MS	Multicollector Inductive Coupled Plasma Mass Spectrometry
MS (B)	Mean Square Between groups
MS (W)	Mean Square Within groups
NTX	Amino-terminal Cross-linking Telopeptides of Type I Collagen
OPG	Osteoprotegerin
PICP	Procollagen type I C-terminal Propeptide
PINP	Procollagen type I N-terminal Propeptide
PBS	Phosphate-Buffered Saline
PIXE	Particle Induced X-Ray Emission
PBM	Peak Bone Mass

PPAR	Peroxisome Proliferator-Activated Receptors
pQCT	Peripheral Quantitative Computed Tomography
PTH	Parathyroid Hormone
PYD	Pyridinoline
QCT	Quantitative Computed Tomography
QUS	Quantitative Ultrasound
RANK	Receptor Activator of NFκB
RANKL	Receptor Activator of NFκB Ligand
RBS	Rutherford Backscattering Spectrometry
RIA	Radioimmunoassay
RIMS	Resonance Ionization Mass Spectrometry
SD	Standard Deviation
SERM	Selective Oestrogen Receptor Modulators
SOTI	Spinal Osteoporosis Therapeutic Intervention
TIMS	Thermal Ionization Mass Spectrometry
TRACP5b	Tartrate-Resistant Acid Phosphatase 5b isoform
TROPOS	Treatment of Peripheral Osteoporosis Study
UL	Tolerable Upper Level of Intake
USDA	United States Department of Agriculture
VDR	Vitamin D Receptor
VISA	Virtual Institute for the Science of Ageing
VSMC	Vascular Smooth Muscle Cell
VTE	Venous Thromboembolism
WHI CaD	Women's Health Initiative Calcium/Vitamin D
WHO	World Health Organization

Part A

Literature Review

and

Methodology

CHAPTER 1: INTRODUCTION

Mean life expectancy has increased linearly over the last two centuries and the upward trend is likely to continue (*1*). In the older population, age-related diseases, including osteoporosis, have become more common. Osteoporosis is a skeletal disease characterized by low bone mass and micro-architectural deterioration, leading to an increase in bone fragility and susceptibility to fracture (*2*). Due to the potentially devastating outcomes (*3*) and high risk of fractures (*4*), it has become an important public health issue. With continued ageing of the population, the annual number of fractures is likely to grow significantly. Assuming that age-adjusted incidence rates remain constant, the estimated hip fracture incidences will increase from 1.7 million in 1990 to 6.3 million in 2050 worldwide. Moreover, fracture rates seem to be rising. With the assumption that age-adjusted rates will rise by only 1% per annum, the hip fracture prevalence could be as high as 8.2 million by 2050 (*5*). Osteoporotic fractures also inflict a major economic burden on the health-care systems. Conservatively, the annual costs of hip fractures was estimated to be US$131.5 billion worldwide in 1997 (*6*). In 2002, the annual costs of all osteoporotic fractures were estimated to be US$20 and US$30 billion in the USA and Europe, respectively (*3*).

Hip fractures are also a major public health problem in Asia, including Singapore. It has been projected that 50% of all hip fractures in the world will occur in Asia by 2050 (*7*). In Hong Kong, the incidence of hip fracture have increased by more than two-fold in the last few decades (*8*). In 1995, six out of 1 000 women and three out of 1 000 men, who were 70–79 years-old, fractured their hip. A similar increase in hip fracture incidence has also been observed in Singapore during the same period (*9*). However, the incidence rates in Mainland China remained much lower than in Hong Kong and Singapore. Compared to the Hong Kong rates, the age-adjusted incidence in men and women in Beijing was only 50% and 25%, respectively (*10*). In Japan, the age-adjusted incidence of hip fracture was also found to be lower than in Hong Kong, 132 per 100 000 in men and 285 per 100 000 in women (*11*).

Overall, the statistic shows that suitable osteoporosis prevention and treatment strategies are clearly required. The main challenge in recognizing effective strategies to preserve bone health is the absence of techniques that are sensitive enough to evaluate the skeletal response to interventions. At the moment, dual energy X-ray absorptiometry (DXA) is the most widely used technique to assess changes in bone mineral density (BMD). However, it is not sensitive enough to evaluate subtle effects of softer interventions, such as diet and lifestyle changes, on bone health. Bone turnover can also be monitored with biochemical markers of bone metabolism. Nonetheless, biomarkers show strong intra-individual variations, which greatly limit their sensitivity (*12*, *13*).

On the other hand, isotopic labeling is an ultra-sensitive tool, which allows the assessment of short-term and soft interventions. Theoretically, both stable and radioisotopes of calcium can be used for isotopic labeling. However, due to health concerns, conventional radiotracers are rarely used. Although stable isotopes are safe, the doses required, and consequently the costs involved, would simply be high and uneconomical. Alternatively, there is a very long-living radiotracer (^{41}Ca, half-life $\approx 10^5$ years), that is also safe to use for isotopic labeling while the doses required in this application is minute. However, its technically challenging and expensive analysis has hindered its use in routine applications or clinical settings. Similar chemical behaviors between strontium and calcium have pointed to the possibility of using strontium stable isotopes as a surrogate marker for calcium in bone metabolism studies. Strontium, like calcium, is a bone seeking element as 98-99% of strontium in the human body is stored in bones. It has similar chemical properties and passively follows calcium in the body (*14*). However, its isotopic analysis requires much less sophisticated instrumentation.

Similar to calcium, strontium is also present in a regular diet, but in a much lower amount compared to calcium (*14*). In a higher amount, strontium has played a role as a therapeutic agent for osteoporosis prevention and treatment. Since 1950s, strontium has been suggested to be useful in osteoporosis treatment (*15*). A few decades later, strontium ranelate was finally approved as an osteoporotic drug. However, a recent data evaluation from clinical

studies by the European Medicines Agency's Committee for Medicinal Products for Human Use showed a higher risk of heart attack in strontium ranelate group than with placebo, with no observed increase in mortality risk (*16*). This may be consistent with the facts that strontium is similar to calcium. Excessive calcium intakes have been shown to be harmful in the long-term as they may cause soft tissue calcification, leading to kidney stones and cardiovascular diseases (*17*).

The aims of this thesis were to explore the use of stable isotope strontium (^{86}Sr) as a diagnostic tool to study bone mineral metabolism; to investigate its role as a therapeutic substance to improve bone health, together with its side effects on soft tissues; and to assess the intake of strontium, as a prospective essential nutrient present in the diet, in a group of Singaporean Chinese. Hence, this thesis would cover the roles of stable strontium isotopes as a diagnostic tool, a therapeutic agent and a potential nutrient.

CHAPTER 2: BONE

2.1. Bone Structure

Typically, a long bone consists of an epiphysis at each end, a diaphysis at the center and a metaphysis connecting them. The epiphysis is composed of a trabecular network surrounded by a thin peripheral cortical shell and a subchondral bony top. A growth plate complex exists underneath the epiphysis. After skeletal maturity, this growth plate will be replaced with a thin layer of bone. The metaphysis is composed of a sponge-like network of interconnected trabecular structures. The cortical shell is progressively thickened from the upper metaphysis toward the diaphysis, while the trabecular network becomes increasingly thin and eventually disappears. The diaphysis is composed of mainly cortical bone and contains a marrow space at the center. The skeletal joint is responsible for transferring the load from one bone to the other. In order to minimize friction between the bony ends during movement, the joint is covered by articular cartilage that is supported by subchondral bone (*18, 19*).

Cortical bone is a semi-solid shell that covers the entire bone, while trabecular or cancellous bone is a sponge-like network structure that fills the interior of the epiphysis and metaphysis. In adult, cortical bone contributes 80% of the total skeletal mass and only 30% of the total bone surface, resulting in a low turnover rate. It provides biomechanical support and protection. In contrast, trabecular bone contributes only 20% of the total bone mass and 70% of the total surface of the skeletal system, resulting in a high turnover rate. The main function of the trabecular bone is to provide biomechanical support and to fulfill homeostatic demands (*18*). Figure A-1 depicts the structure of a typical long bone.

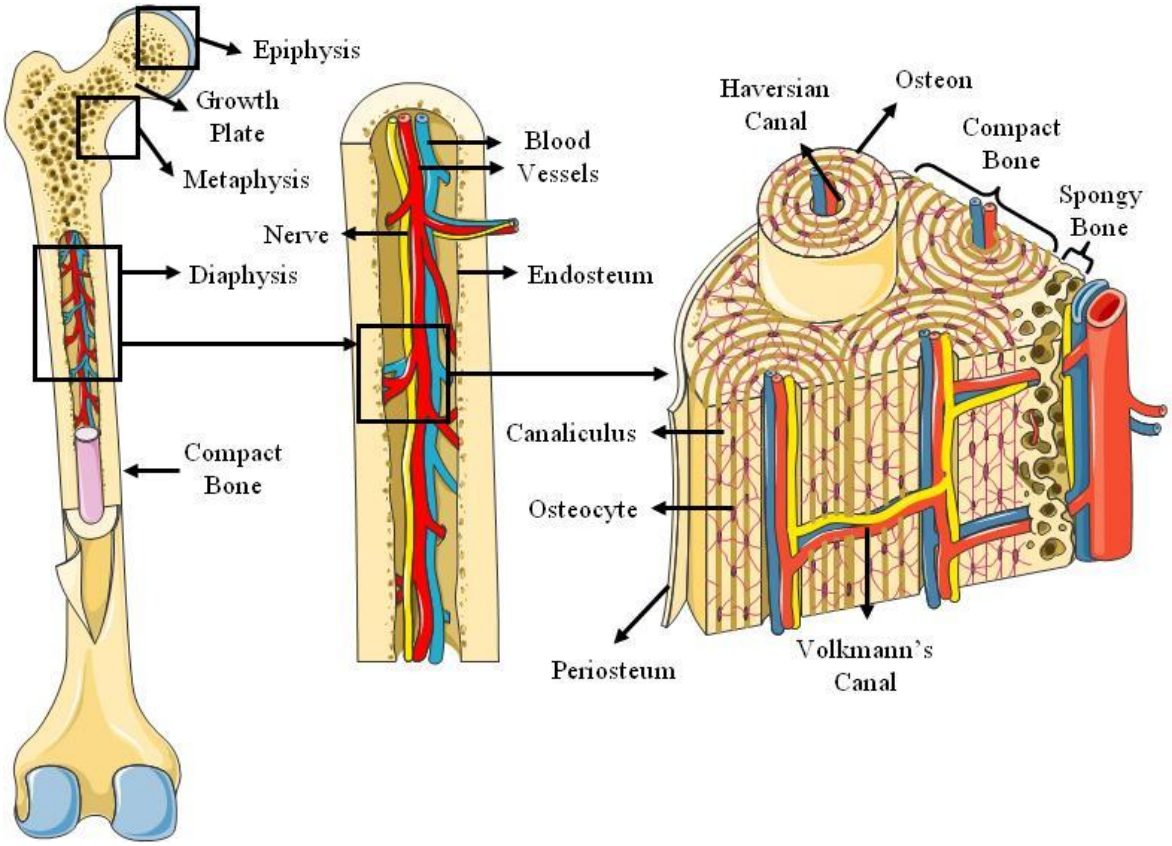

Figure A-1: (a) Schematic view of a femur showing the gross profile of a typical long bone (longitudinal cut), (b) diaphysis section and (c) schematic view of histological details of cortical bone. A typical long bone consists of an epiphysis at each end, a diaphysis at the center and a metaphysis connecting them. A growth plate complex exists underneath the epiphysis. Cortical bone contains Haversian canals, Volkmann's canals and resorption cavities, containing mainly blood vessels and nervous tissue, which allows nutrition, nerve signals and metabolites to be exchanged between cortical bone and its surrounding. Haversian canals are interconnected with Volkmann's canals, which run horizontally from periosteal to endocortical surface. As such, a network of canals exists throughout the entire cortical bone. During remodeling process, one or more existing osteons/Haversian systems are partially removed to create space for the new osteons (*18, 19*). Figures are adapted from Servier website.

2.2. Bone Composition

Bone is composed of organic matrix (20-40%), inorganic mineral (50-70%), cellular elements (5-10%) and lipids (3%). The organic bone matrix consists mainly of type I collagen (*19*). Several collagen molecules, known as tropocollagens, are bundled together forming collagen fibers. Interfibrillar cross-links are formed between the ends of tropocollagens to stabilize the matrix. Tropocollagens are aligned with numerous gap regions, into which hydroxyapatite crystals are incorporated during mineralization. A lamella sheet is made of

collagen fiber aligned parallel to each other. Hydroxyapatite, the primary molecule of bone mineral, is a naturally occurring mineral form of calcium apatite, usually denoted as $Ca_{10}(PO_4)_6(OH)_2$. It provides load bearing capacity and mechanical rigidity for the bone. It contains impurities, like magnesium, fluoride and strontium, which can be incorporated into the crystal lattice or absorbed onto the surface. Imperfect crystals are more soluble, enabling bone to be resolubilized and releasing these ions into the extracellular fluid as needed to meet homeostatic requirements of the whole body. Homeostatic needs can also be met by the minerals absorbed onto the surface. Other non-collagenous proteins, which make up 10% of the total organic matrix, play important roles in the calcification process, fixation of the hydroxyapatite crystals to the collagen, and regulation of osteoblastic and osteoclastic metabolism. Osteocalcin, osteonectin, osteopontin and bone sialoprotein are the most abundant among these proteins. During the breakdown of bone matrix, some proteins, including hydroxyproline and collagen cross-links, are released into the circulation. Therefore, evaluating the levels of these proteins in urine, plasma or serum is useful to monitor the bone turnover status as well as to diagnose, assess progression and determine the efficacy of treatments for skeletal disorders (*20, 21*).

2.3. Bone Growth, Modeling and Remodeling

The growth plate complex, which is responsible for longitudinal growth, is composed of a large number of chondrocytes and hyaline cartilage matrix. The hyaline cartilage consists of columns with multiple shelves that stack chondrocytes together. In each column, the chondrocyte develops from a flat precursor to a mature cell by proliferating to maximize its volume and producing a large numbers of cartilage matrix, which subsequently become calcified cartilage. Finally, chondrocytes experience cellular apoptosis, which gives way to the concurrent osteoclastic resorption and vascular ingrowth, which is followed by bone formation by osteoblast, resulting in a densely connected trabecular network. During bone elongation, the old cancellous bone at the diaphseal end is removed and a new metaphyseal region is added at the epiphyseal end, which consequently increases bone length. As the bone

becomes longer, radial growth proportionally enlarges its cross-sectional area. During growth, increased mechanical demands stimulate the periosteum, activating the bone modeling and eventually resulting in radial growth and enlarged cross-sectional area of the bone (*19*).

Bone modeling activities includes osteoblastic bone formation and osteoclastic bone resorption. Although these processes are triggered and carried out separately on different bone sites, they are synchronized in order to enlarge and alter the shape of the bone as needed. Throughout the growing period, these processes are going continuously. After skeletal maturity, the formation drift is limited on the periosteal surface while the resorption drift seems to cease (*22, 23*).

Bone adapts to mechanical and non-mechanical stimuli through bone remodeling. During childhood, the remodeling process is mostly responsible for substituting immature woven bone with more metabolically and biomechanically competent lamellar bone. During growth, the remodeling process enables bone elongation. During adulthood, the remodeling process replaces damaged or mechanically unfit bones in order to maintain the mechanical capacity. All cortical bone is remodeled completely every twenty years while all trabecular bone is completely remodeled every one to four years. Figure A-2 shows the remodeling process in both trabecular and cortical bones. The coupling between bone formation and resorption usually becomes unsynchronized after age 30. Consequently, a negative bone balance is created in each cycle for all remodeling bone multi-cellular units (BMUs) leading to an age-related bone loss (see Figure A-3).

2.4. Bone Mass Cycle

Bone mineral accretion continues even though growth has ceased. Depending on the skeleton site, peak bone mass (PBM) can be reached as early as the late adolescent years or as late as the mid-thirties (see Figure A-3) (*24*). Once PBM has been reached, bone mass will remain relatively constant until age-related bone loss takes place, resulting in the drastic drop in bone mass. PBM varies among individuals and is regulated mainly by genetic factors (approximately 80%) (*25*). Other factors, such as gender and environment, account for about

20%. PBM is a determining factor for osteoporotic risk fracture. Ten percent increase in PBM can result in 50% fracture risk reduction (*26*).

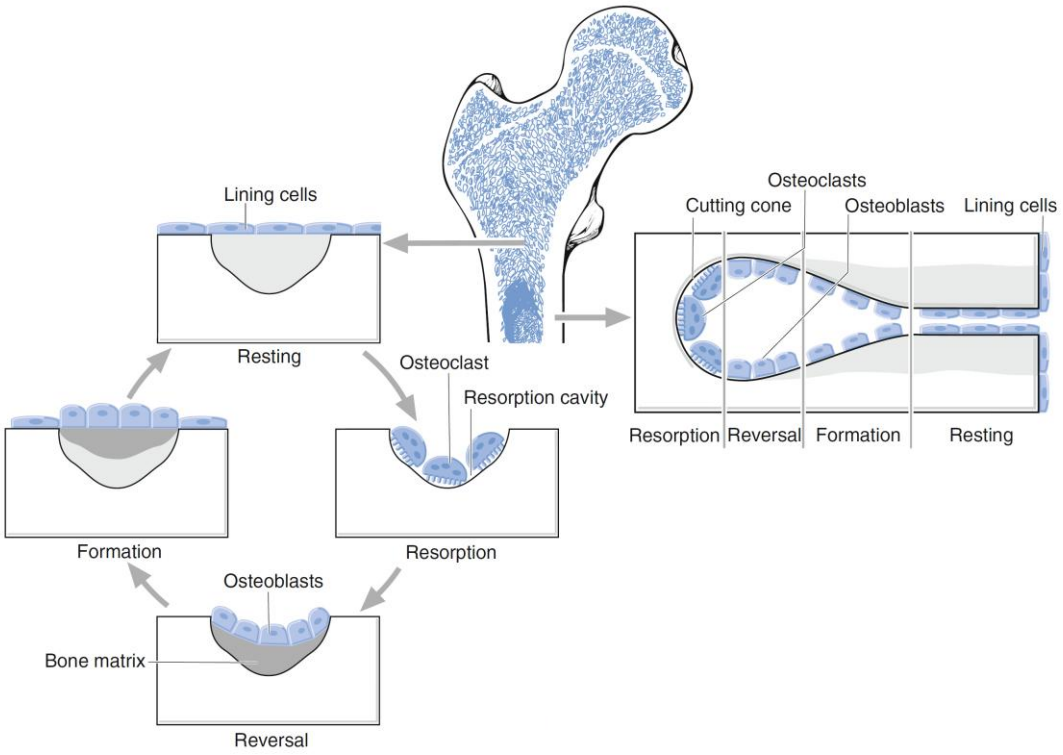

Figure A-2: Bone remodeling in trabecular and cortical bone. The remodeling cycle is composed of activation, resorption, reversal and formation. During activation, mononuclear monocyte-macrophage osteoclast precursors are recruited and activated. The lining cells were lifted off the bone surface and multiple mononuclear cells fuse to form multinucleated preosteoclasts. Osteoclast-mediated bone resorption takes only about two to four weeks. After bone resorption is completed, preosteoblasts recruited to begin new bone formation. The reversal phase is a transition from bone resorption to formation. Bone formation completes in approximately four to six month (*18*). Figure is taken from Bartl and Frisch, 2009 (*27*).

Regardless of the PBM, both males and females will eventually experience age-related changes in their bone, which result in bone mineral loss caused by increased trabecular resorption, endocortical thinning and increased cortical porosity (*28*). Both males and females experience trabecular bone loss to about the same extent. However, cortical bone loss is less in males than females, which is believed to be a result of greater periosteal bone formation in men (*28*). The gender-difference in bone strength persists during old age, due to greater overall bone loss in women than men (*29*). However, the gender-difference in bone strength is not limited to BMD, the skeletal structure (dimensions, cortical thickness), biomechanical

responses, bone turnover, as well as trabecular microstructure are also affected (*30*). The decrease in bone strength is more evident in women compared with men, particularly after menopause, due to the rapid decline in estrogen levels (*31*).

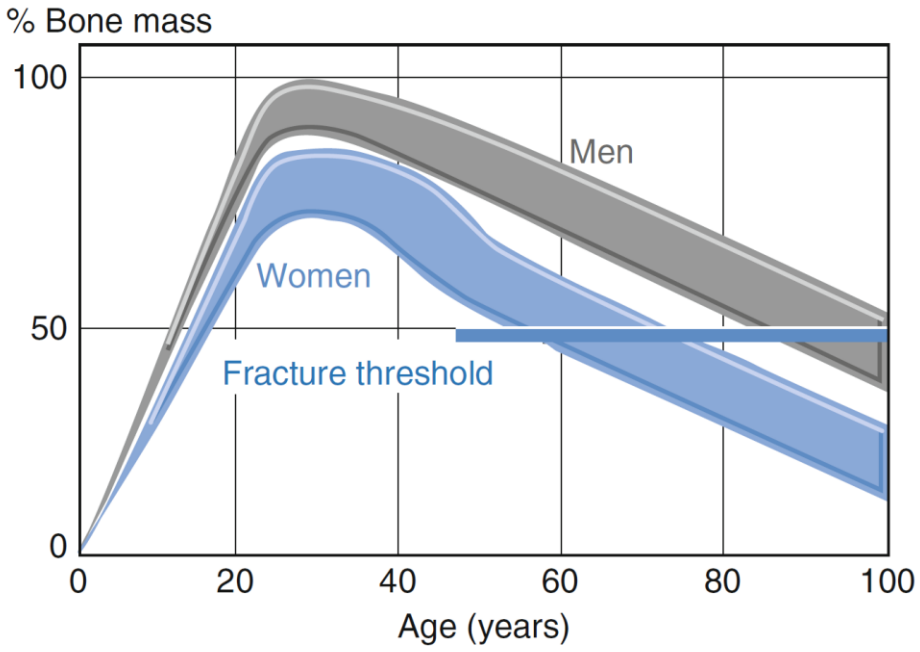

Figure A-3: Typical changes in bone mass over a lifetime. This diagram highlights the difference between the bone mass changes in men (grey) and women (blue) and in individuals who reach their full genetic potential for skeletal mass (top curve) and in those who do not (bottom curve). Once the age-related bone loss sets in, fracture risks would be higher in those individuals who do not reach their full genetic potential. The rapid decline of estrogen levels in females after menopause accelerates this bone loss process. Figure is taken from Bartl and Frisch, 2009 (*27*).

2.5. Factors Influencing Bone Mineral Deposition

2.5.1. Diet

Calcium is the most abundant cation in the human body, 99% of which exists in the skeleton as hydroxyapatite crystals, while the rest is found in the extracellular fluid, blood and soft tissues. Calcium deposition into the organic matrix of bone contributes to its strength and rigidity. At the same time, bone functions as a reservoir of calcium, making it readily available for its roles in multiple physiological and biochemical processes including neuromuscular functioning, coagulation, cell permeability, enzyme activation, hormone secretion and functioning (*32*). The calcium required for the non-osseous functions is satisfied

primarily by intracellular calcium. Since a small fraction of bone contains more calcium than the entire extracellular fluid space, the requirement of the body's cellular functions will never deplete the bone's calcium reservoir (*33*). Nevertheless, extended periods of inadequate calcium intake require enhanced resorption of bone to meet extracellular calcium needs, resulting in bone deterioration. Hence, suboptimal intakes are unlikely to lead to maximum genetically determined bone mass (*34*). On the other hand, calcium intakes above the threshold will not add appreciably to skeletal retention.

Although calcium supplements are commonly administered as a part of osteoporosis prevention and treatment, they have been associated with a higher risk of cardiovascular events (*33*). In the Women's Health Initiative Calcium/Vitamin D (WHI CaD) study, it has been observed that calcium and vitamin D supplements increased the risk of cardiovascular events (*35*). An earlier study, the Kuopio Osteoporosis Risk Factor and Prevention Study, has also observed a higher morbidity and mortality rate caused by coronary heart disease among subjects receiving calcium supplements (*36*). More recently, in the Swedish mammography cohort, intake of calcium above 1400 mg/day was associated with a higher mortality rate, including from cardiovascular events. Without supplement consumption, the increase was modest. However, the combination of high calcium intake from the diet and supplement resulted in a more significant rise in mortality (*37*). In agreement to this, Xiao *et al.* (*38*) showed that high intake of supplemental calcium, but not from dietary sources, is associated with an excess risk of death from cardiovascular disease in men but not in women. In the Heidelberg cohort of the European Prospective Investigation into Cancer and Nutrition study (EPIC-Heidelberg), Li *et al.* (*39*) also showed that while increase in dietary calcium intake might not have a substantial effect on cardiovascular health, calcium supplements should be taken with caution, since they might elevate myocardial infarction risk.

Nevertheless, some studies showed that there was no increase in adverse cardiovascular effects associated with calcium supplementation. Bhakta *et al.* (*40*) observed no significant increase in progression of aortic valve or coronary artery calcification in women taking oral calcium supplementation. Hsia *et al.* (*41*) also observed no difference in the rates of stroke or

coronary events in female participants receiving calcium supplementation or placebo. A more recent study by Lewis *et al.* (*42*) also supported these findings. Nevertheless, it needs to be highlighted that calcium intakes beyond the current recommendation has little or no value.

Besides an increase in cardiovascular events, high dietary consumption of foods rich in calcium and oxalate may cause kidney stone formation (calcium oxalate) and crystalluria (*43*). On the other hand, decreased availability of free gastrointestinal (GI) calcium to bind oxalate may also result in an increase in oxalate absorption and urinary oxalate excretion, which plays a greater role than calcium in the development of kidney stones. Thus, daily calcium intake within a certain range (1000–1500 mg/day) may help to bind oxalate, preventing its absorption and decreasing the formation of calcium oxalate stones (*43*). However, there is not enough evidence to support a correlation between renolithiasis risk and calcium intakes at the level expected to be consumed by postmenopausal women, either from diet or supplement (*44*). In response to growing concerns regarding the potential negative side effects caused by excessive calcium intakes, some adjustments had been made to the Dietary Reference Intake (DRI) for calcium in certain subject groups (*45*). As such, the Recommended Dietary Allowance (RDA) was lowered from 1,200 mg to 1,000 mg for adults 51 -70 years old and the Tolerable Upper Level of Intake (UL) was lowered from 2,500 mg to 2,000 mg per day for adults older than 51 years.

Vitamin D is produced from previtamin D_3 and partially obtained from dietary sources. Previtamin D_3 is generated from the vitamin D_3 precursor, 7-dehydrocholesterol (7-DHC), in the skin after exposure to sunlight or specifically UV B (*46-48*), and is then readily converted to vitamin D_3. In the liver, vitamin D_3 is hydroxylated to form 25-hydroxyvitamin D (25(OH)D), which will be further hydroxylated in the kidney to 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D$, calcitriol), the active form (*49*). Once formed, calcitriol will be bound to vitamin D binding protein and enters the circulation to target tissues that regulate calcium and phosphorus metabolism. In the intestine, calcitriol interacts with its vitamin D receptor (VDR) to improve the expression of an epithelial calcium channel and increases the expression of a calcium binding protein (calbindin) which results in the improved transport of calcium from

the intestinal lumen into the circulation (*50, 51*). In the skeleton, calcitriol interacts with its VDR in the osteoblast to enhance the expression of receptor activator of NFκB ligand (RANKL) (*52*). Monocytic preosteoclasts possess the receptor RANK which interacts with RANKL resulting in signal transduction leading to the formation of multi-nucleated osteoclasts to destroy the bone matrix releasing calcium into the circulation. In the kidneys, calcitriol increases tubular reabsorption of calcium. Calcitriol interacts with its VDR in the parathyroid glands and regulates parathyroid hormone (PTH) production (*50, 53*). The major roles of vitamin D are to maintain serum calcium in physiologically acceptable range to support metabolic functions and to secure the mineralization of the skeleton. Besides that, vitamin D supplementation has been shown to enhance muscle strength (*54*), which eventually reduces fall incidence (*55*), one of the major causes of fracture. As the VDR is expressed in both skeletal muscle (*56*) and myoblast cells (*57*), this association is expected. *In vitro*, vitamin D has been demonstrated to increase protein synthesis and cellular growth in muscle cells with an increase in size and number of type 2 muscle fibers (*58*) which are the first muscle fibers recruited when falling (*59*).

Phosphorus is essential to the structural integrity of the cell and for metabolites' passage. It regulates a great number of enzymes and manages the storage and transformation of the body's energy. It plays a fundamental role in providing oxygen to the tissues through 2,3-diphosphoglycerate and adenosine triphosphate (ATP) in the erythrocytes, forming part of the buffer systems of urine and blood, and its presence is critical to the body's defense against infection (*60*). In bone, calcium and phosphate metabolism work together with osteoblasts, osteocytes, and extracellular matrix proteins to mineralize osteoid as it is deposited (*61*). On the contrary, in non-skeletal tissues, a regulatory system inhibits the deposition of calcium-phosphate complexes in soft tissues (*62, 63*).

Vitamin K is necessary for the biological activity of several coagulation factors (*64*). It functions as a cofactor for the vitamin K–dependent carboxylase that assists the post-translational conversion of glutamyl to γ-carboxyglutamyl residues (*65*). Besides the hepatic tissue, γ-carboxyglutamyl–containing proteins are abundantly available in bone tissue (*66*).

Osteocalcin accounts for up to 80% of the total γ-carboxyglutamyl content of mature bone (*64*). γ-Carboxyglutamyl residues are responsible for a highly specific affinity to the calcium ion of the hydroxyapatite molecule (*67*). Although the exact role remains to be clarified, the available findings indicate a regulatory function of osteocalcin in bone mineral maturation (*68, 69*). Some *in vitro* and *in vivo* data also suggest that vitamins K and D work synergistically on bone metabolism (*70, 71*). There is also evidence that calcium balance is positively influenced by vitamin K, including increasing calcium retention (*72*).

Phytoestrogens are naturally occurring plant compounds that function like estrogen agonist–antagonists. There are three major forms of phytoestrogens: isoflavones (genistein, daidzein, glycitein), lignans (enterolactone and enterodiol) and coumestans (coumestrol). Different types of phytoestrogens have different affinities towards estrogen receptor alpha (ERa) versus beta (ERb), which alter their effects on the skeleton and other tissues (*73*). As has been reported for estrogens, phytoestrogens can modulate calcium metabolism and produce bone sparing effect (*74*). Studies have shown that phytoestrogens can: 1) directly modulate gene expression, including calciotropic receptor, cytokines, and growth factors (*75, 76*); 2) increase alkaline phosphatase activity, enhance bone mineralization (*77*) and stimulates osteoblast differentiation (*78*); 3) inhibit bone resorption by decreasing osteoclast differentiation, increasing osteoclast apoptosis or interfering with signalling pathways (*79*); 4) interact with enzymes and receptors (*80*). Through ERa-dependent mechanisms, phytoestrogens are able to increase osteoblastic osteoprotegerin production and simultaneously suppress receptor activator of NFκB-ligand gene expression associated with an osteoclastogenesis inhibition. During bone remodelling, resorption cycle is coupled to formation through the receptor activator of NFκB (*81*). Peroxisome proliferator-activated receptors (PPAR) have also been recognized as additional targets of phytoestrogen. It can trigger PPAR activation and induce divergent effects on adipogenesis and osteogenesis. ER-mediated effects can be observed at low phytoestrogen concentrations, whereas PPAR-mediated effects are only apparent at high concentrations (*82, 83*).

Typical ***magnesium*** body content is around 20 g, 50 to 60% of which is located in the bone (*84*). It is the main intracellular divalent cation and 99% of it is found in the intracellular space (*84*). It regulates the structure of ribosome, different activating effects of enzymes, transport across membranes, synthesis of proteins and nucleic acids, generation and transmission of nerve impulses, muscular and cardiac contraction, as well as in oxidative phosphorylation. Through the hormonal action of vitamin D, PTH and calcitonin, magnesium is essential to the control of calcium and phosphorus metabolism (*85*). It is also important in bone crystal growth and stabilization. A positive correlation between dietary magnesium intake and bone density and/or an increased rate of bone loss with dietary magnesium reduction has been demonstrated in several epidemiological studies. In humans, magnesium deficiency results in impaired PTH secretion and low serum $1,25(OH)_2D$ levels (*86, 87*). In several studies of postmenopausal women with osteoporosis, magnesium deficiency has been observed (*88*). Although several epidemiological studies indicate a positive association between magnesium intake and bone mass (*89-91*), other earlier studies have shown no correlation (*92, 93*).

Vitamin A is essential in bone remodeling processes. Nuclear receptors for retinoic acid exist in both osteoblasts and osteoclasts (*94, 95*). Studies have shown that both vitamin A excess (hypervitaminosis) and deficiency are harmful to bone. In animals, hypervitaminosis A causes increased bone resorption, fragility and spontaneous fractures (*96*). However, vitamin A deficiency results in lower osteoclasts number, leading to excessive skeletal deposition in the periosteum due to uncontrolled osteoblast activity (*97*). When consumed within the recommended levels, vitamin A is safe and beneficial to bone health (*98*).

Sufficient and balanced dietary intake of ***essential fatty acids*** (EFAs) has a positive effect on bone. Additionally, EFA deficiency has been known to contribute to osteoporosis (*99*). EFAs, which originate from ingested lipids, can be categorized into omega-3 fatty acids (ω3FAs) and omega-6 fatty acids (ω6FAs) (*100*). The relative concentration of ω6FAs and ω3FAs in the diet is an important determinant to the skeletal function, as it affects calcium metabolism (*101*). High intake of ω6FAs increased prostaglandin E2 and other cytokines

(*102*), which in turn activated osteoclasts and bone resorption (*103, 104*). In contrast, ω3FAs reduced the level of inflammatory cytokines (*105*). Sufficient intake of ω3FAs also facilitates calcium absorption in the intestine, reduce calcium urinary excretion, increase calcium skeletal deposition and enhance bone collagen synthesis (*101*). Overall, a low ω6/3 in the diet seems to lower osteoporosis risk and fragility fractures.

Numerous studies have confirmed a positive relationship between urinary ***sodium***, reflecting intake, and urinary calcium (*106, 107*). As sodium and calcium compete for reabsorption at the kidney (*108*), approximately 20 mg of calcium is excreted per 1 g of sodium (*108, 109*). Hence, it has been speculated that excess sodium intake might decrease BMD. So far there are only two studies reporting negative effects of sodium directly on BMD (*110, 111*). The results from other studies did not demonstrate any relationship between sodium intake/urinary excretion and BMD, but only showed higher urinary calcium with higher urinary sodium (*106, 112, 113*). The current knowledge about the role of sodium in bone health is still insufficient and conflicting (*114-116*). Heaney concluded in his review that with sufficient calcium intakes, there is no deleterious effect of prevailing sodium intakes on bone or the calcium economy (*108*). This is supported by Ilich *et al.* that determined that moderately high sodium with adequate calcium intakes do not result in decreased BMD (*117*).

2.5.2.Lifestyle

The data regarding the effects of ***activity levels*** (exercise and mechanical stimuli) on serum calcium and PTH concentrations are somewhat unclear. The effects may be determined by the intensity and duration of the exercise regimen as well as the training status of the individual. Exercise-induced acidosis and hence, exercise intensity seem to be responsible for the raises in ionized calcium during exercise (*118, 119*). Short-duration, high-intensity exercise elevates ionized calcium with a simultaneous drop in serum PTH concentrations (*120*). On the other hand, during and after prolonged endurance exercise, the higher serum ionized calcium concentrations does not always result in lower serum PTH concentrations, perhaps due to the elevation in catecholamines. Some studies determined that endurance

exercise has to last for at least 30 minutes in order to produce a raise in circulating PTH concentrations (*120-124*). Studies regarding the effect of resistance training on serum PTH concentrations are contradictory (*120, 125*). In a meta-analysis, Berard *et al.* found that physical activity have no beneficial effect on bone loss after menopause (*126*).

Chronic ***alcoholism*** can cause lower BMD and increase fracture risk due to: 1) malnourishment and malabsorption of important nutrients, especially calcium, magnesium and zinc; 2) liver disorder, abnormal vitamin D metabolites and parathyroid function; 3) toxicity to osteoblasts; and 4) increased tendency to fall leading to increase fracture risk (*127*). Hence, heavy drinkers have higher risk of bone loss (*128, 129*). However, moderate alcohol consumption seems to be beneficial for bone (*129-132*) supposedly due to the stimulation of androstenedione conversion into estrone (*133*). In postmenopausal women, the only source of their estrogen is the aromatization of androgens to estrogens. A study in postmenopausal women revealed that women with moderate alcohol consumption had higher estradiol levels (*133*).

Caffeine was initially thought to merely increase urinary calcium loss and thus considered a risk for bone loss. Later, it was found that the correlation between caffeine, calcium and bone metabolism is more complex and it probably affects intestinal calcium absorption from endogenous sources. Although, there was a negative correlation between caffeine intake and calcium intake (*134*) such that as caffeine (or coffee) consumption increased, milk consumption decreased. Caffeine hardly affected urinary calcium excretion or total calcium uptake in balance studies (*134*). However, the association between coffee consumption and bone status are inconsistent based on epidemiological data. Some showed negative associations between caffeine consumption and bone health (*135, 136*) and some did not (*137-140*). Like sodium, it seems that the harmful effect of caffeine only becomes evident when dietary calcium is insufficient (*135, 141, 142*).

CHAPTER 3: OSTEOPOROSIS

3.1. Types and Impact of Osteoporosis

Primary osteoporosis, the most common form of osteoporosis, is not a result of another specific disorder. On the other hand, secondary osteoporosis is caused by other specific diseases or medications. Primary osteoporosis, or sometimes referred as age-related osteoporosis, is mainly found in the elderly as an outcome of the accumulated impact of bone loss and structure deterioration that occurs with age (*143*). Postmenopausal women are usually at greater risk, while younger individuals are rarely inflicted by primary osteoporosis. Although primary osteoporosis can occur in both sexes, it is two to three times less common in men, owing to the fact that women experience two phases of age-related bone loss: 1) a rapid phase that begins at menopause and lasts between four to eight years, and 2) a slower continuous phase that continues throughout the rest of life (*144*). On the other hand, men go through only the second phase (slow and continuous). Hence, women typically lose more bone than men.

Young adults and even older individuals may suffer from secondary osteoporosis as a side effect of another condition or medication. They experience greater levels of bone loss than a healthy individual of the same age, gender, and race. The development of osteoporosis can be caused or contributed by a wide variety of diseases, medications and toxic agents (*145, 146*). Several genetic disorders have been associated with secondary osteoporosis. Disorders such as diopathic hypercalciuria and cystic fibrosis were found to be the most common causes. In other cases, estrogen or testosterone deficiency caused by Turner's, Kallman's or Klinefelter's syndrome, anorexia nervosa, athletic amenorrhea, cancer, or any chronic illness that interferes with the onset of puberty may also lead to secondary osteoporosis (*144*). Osteoporosis can also be a side effect of particular medications, including glucocorticoid, cyclosporine A, tacrolimus and anticonvulsants (*145, 146*).

Bone disease brings a devastating impact on patients and their families. For most individuals a fracture may result in a significant drop in physical health and even death

resulting from fracture complications (*147*). Fractures may cause pain, height loss as well as inability to perform basic daily activities. Besides increasing risk of health complications, such as pressure sores, pneumonia and urinary tract infections, fractures may also affect patients psychologically by lowering their self-esteem and mood. A fear of suffering another fracture from falling may immobilize some patients permanently. However, osteoporosis and fractures should no longer be thought of as an unavoidable part of aging. By concentrating on fracture prevention and lifestyle changes, such as nutrition and increased physical activity discussed in Part A, Section 2.5, combined with early diagnosis and proper treatment that will be discussed in the following sections, the damaging impact of osteoporosis can be mitigated, if not avoided.

3.2. Pathophysiology

Osteoporotic fractures are a result of both reduced bone strength and increased rate of falls. Although BMD is still the best available non-invasive assessment of bone strength in routine clinical practice, there are other skeletal characteristics, such as bone macro- and micro-architecture, matrix and mineral composition, degree of mineralization, micro-damage accumulation and the rate of bone turnover, that also contribute (*148*). The bone mass of an older individual is a result of the PBM accumulated during early age and also the subsequent bone loss. Although genetic factors greatly contribute to PBM, environmental factors in intrauterine life, childhood and adolescence also control the genetically determined pattern of skeletal growth (*25*).

At the cellular level, an imbalance between the activities of osteoclasts and osteoblasts results in bone loss. In adults, the skeleton is constantly remodeled in a synchronized sequence of bone resorption and formation. When the processes of bone resorption and formation are not synchronized, remodeling imbalance will occur. This imbalance can be exacerbated by an increase in the initiation rate of new bone remodeling cycles (*27*).

In postmenopausal women, bone loss may result from estrogen deficiency as well as via estrogen-independent age-related mechanisms, such as reduced mechanical loading and secondary hyperparathyroidism. Estrogen plays an important role in normal physiological remodeling and estrogen deficiency following menopause causes commonly an imbalance in remodeling with a considerable increase in bone turnover. This causes a progressive loss of trabecular bone, partially due to an increase in osteoclastogenesis. Higher formation of osteoclasts is found to be a consequence of increased elaboration of osteoclastogenic proinflammatory cytokines, including interleukin-1 and tumour necrosis factor, which are negatively controlled by estrogen (*149-151*). Increased production of transforming growth factor β is another direct effect of estrogen in accelerating osteoclast apoptosis (*152*).

Osteoclastic bone resorption *in vitro* and *in vivo* is regulated mainly by the receptor activator of NFκB (RANK), its ligand (RANKL) and the decoy receptor osteoprotegerin (*153*). RANKL is expressed by osteoblasts constitutively on their cell surface. It interacts with RANK, which is expressed on osteoclast precursors and promotes its differentiation. On mature osteoclasts, this interaction leads to their activation and prolonged survival. Osteoprotegerin, primarily secreted by osteoblasts and stromal cells, blocks the interaction of RANKL with RANK *in vivo* and consequently acts as a regulator of bone turnover. Estrogen stimulates osteoprotegerin expression in osteoblasts and thus exert its antiresorptive effects on bone (*154*).

Key novel genes and pathways for osteoblast function and differentiation have been discovered. LDL receptor-related protein 5 (LRP5) modulates osteoblast function, and thus bone formation. It is expressed on the membrane of the osteoblast between Frizzled and Kremen (receptors). Frizzled and LRP5 bind to Wnt, thus activating bone formation. Inhibition of bone formation will occur when Wnt inhibitors bind to Kremen and LRP5, unabling LRP5 to bind Wnt. Differences in bone density and fracture risk have been linked with polymorphisms of LRP5 (*155, 156*).

3.3. Osteoporosis Treatments

3.3.1. Antiresorptive Agents

Bisphosphonates inhibit bone resorption leading to reduction of fracture risk. They include alendronate, risedronate, ibandronate and zoledronic acid. The direct intracellular target of bisphosphonates is the enzyme farnesyl diphosphate synthase in the HMG-CoA reductase biosynthetic pathway. Its inhibition reduces protein prenylation, causing disturbance of signaling pathways required for bone resorption by osteoclast. Due to its affinity to calcium, bisphosphonate molecules accumulate in bones and are taken up by osteoclasts. In postmenopausal women with osteoporosis, risedronate has increased BMD and reduced vertebral and non-vertebral fracture risks by 41% and 49%, respectively (*157*). It also reduced hip fracture rate by 30% in older women receiving osteoporosis treatment (*158*). In postmenopausal women with low BMD, alendronate has reduced hip fractures by 50% and vertebral fractures by 44–50% (*159, 160*). Ibandronate was found to reduce hip and vertebral fracture risk (*161, 162*). Zoledronic acid reduced vertebral and hip fracture by 70% and 41%, respectively (*163*). When given two weeks after hip fracture, zoledronic acid offers 35% relative decrease in recurrent fracture and all-cause mortality (*164, 165*). In rare occasions, bisphosphonates may cause adverse skeletal events, including atypical fragility fractures and osteonecrosis, which might be a result of bone remodelling suppression (*166, 167*). To reduce the risk of adverse effects, patients receiving long-term biphosphonate therapy are usually given a drug holiday. Since bisphosphonates are incorporated into the bone, they continue to exert an antiresorptive effect for a period of time after the discontinuation of the therapy (*168*).

Selective oestrogen receptor modulators (SERMs) include raloxifene, lasofoxifene and bazedoxifene. In a study on postmenopausal women, raloxifene has decreased vertebral fracture risk (*169*). Analyses of a subgroup of women with severe vertebral fractures showed a decrease in both new vertebral and non-vertebral fracture risk (*170*). Bazedoxifene and lasofoxifene significantly decreased new vertebral fracture risk in postmenopausal women with osteoporosis and non-vertebral fracture risk in subjects with higher fracture risk (*171,*

172). The main concern with SERMs treatment is the increased risk of venous thromboembolism (VTE).

Denosumab is a human monoclonal IgG2 antibody directed against RANKL or usually called ***RANKL inhibitor*** (*173*). RANKL is a member of the tumour necrosis factor family, that is expressed by osteoblasts and its precursors and functions to stimulate recruitment and differentiation of osteoclast precursors (*174*). The effect of RANKL on osteoclastogenesis can be inhibited by osteoprotegerin (OPG) that is produced and secreted by osteoblasts and its precursors. Denosumab functions like OPG, limiting RANKL activity and thus reducing osteoclast numbers and activity. Clinical trials have shown that denosumab increased BMD, reduced bone turnover markers and fracture risks in postmenopausal women (*175-178*). Increased risk of nausea, abdominal pain and flatulence have been reported, but no increased risk of infections, neoplasms, jaw osteonecrosis or serious atrial fibrillation has been observed (*178*).

Since bone loss happens rapidly at the start of postmenopausal period as a result of oestrogen deficiency, ***hormone replacement therapy*** (HRT) seems to be a logical means to delay this bone loss. In postmenopausal women, it has been observed that the overall risk of osteoporotic fractures dropped by 24% and the risk of both hip and spinal fractures dropped by 34% each (*179*). The BMD of the lumbar spine also increased by 5.1% and bone turnover rate decreased. However, the benefits of HRT disappear after withdrawal of treatment (*180*). In addition to that, UK Committee on the Safety of Medicine recommends that HRT should not be used as the first-line treatment of osteoporosis, due to the adverse effects such as increased risk of stroke, VTE as well as breast cancer (*181*).

3.1.2. Anabolic Agents

Recombinant PTH 1-34 and 1-84 enhance bone remodelling by stimulating pre-osteoblasts maturation into osteoblasts. A clinical trial on postmenopausal women with previous vertebral fracture showed a rise in BMD at the lumbar spine and femoral neck in the treatment groups. The increase in BMD is quick and of greater magnitude compared to other

treatments. It is particularly useful to treat patients with high fracture risk or pre-existing fragility fractures (*182*). The main concern is the development of osteosarcoma with long-term treatment, as seen in rat models receiving high-dose treatment (*183*). However, this has not been reported in human. Due to safety concern and plateau in bone formation after 18 months of treatment, PTH treatment is limited to a low dose for at most 2 years. Nevertheless, owing to its cost, complexity of administration and the lack of data on non-vertebral fracture reduction, PTH is not considered as first-line therapy.

3.1.3. Double-Acting Agents

Strontium ranelate dissociates bone remodeling by reducing bone resorption without affecting bone formation. Strontium was found to reduce relative risk of vertebral fractures by 49% and 41% after 1 year and 3 years, respectively (*184*). In 3 years, it increased BMD at the lumbar spine and the femoral neck by 14.4% and 8.3%, respectively. BMD of the lumbar spine, femoral neck and total hip continued to increase up to 8 years (*185*). In postmenopausal women, the strontium group experienced reduction in relative risk of major fragility fractures by 19% (*186*). A subgroup analysis on women aged ≥ 80 years who are at an elevated risk of fractures showed an early and sustained reduction of both non-vertebral and vertebral fractures following strontium treatment (*187*). As the change in BMD value may partially be caused by accumulation of strontium that attenuates X-ray more strongly than calcium, BMD values must be interpreted with caution.

Side effects of strontium ranelate include transient nausea, diarrhoea and headache. A slight rise of VTE has been noted, but no increased incidence in patients treated with strontium compared with alendronate (*188*). Nevertheless, a recent data evaluation from clinical studies in postmenopausal women published by the European Medicines Agency's (EMA's) Committee for Medicinal Products for Human Use (CHMP) has revealed a higher risk of heart attack with strontium ranelate than with placebo. However, there is no observed increase in mortality risk. Considering the other side effects including blood clots and serious skin reactions, EMA suggested to restrict the use of strontium ranelate only for treatment of

severe osteoporosis with high risk of fracture and on patients without any cardiovascular problems (*16*).

CHAPTER 4: METHODS TO STUDY BONE METABOLISM AND BONE HEALTH

4.1. Clinical Techniques

As mentioned earlier, although BMD assessment has been used as the benchmark for diagnosis of osteoporosis, low BMD alone does not necessarily lead to a fracture. Even though many clinical trials have shown the usefulness of the diagnostic threshold of a T-score < –2.5 as a cut-off for osteoporosis, the clinical use of the term osteopenia (–1 > T-score > –2.5) is less clear. It is well established that fracture risk increased sharply with decreasing BMD, but assessment of fracture risk should take into account all aspects of risk and intervention should not be deduced only from BMD. Based on the data gathered by World Health Organization (WHO), several osteoporosis risk factors have been identified, including fracture history, glucocorticoid use, family fracture history, cigarette smoking, alcoholism and low bodyweight (*189*). Multivariate models combining these risk factors with age and BMD allow the prediction of 10-year fracture probability. This will enhance the ability to design more targeted and cost effective treatments.

Biochemical markers of bone turnover in serum and urine have been established and used as diagnostic tools. They are non-invasive and relatively inexpensive techniques to assess *in vivo* bone formation and resorption rates. Serum bone-specific alkaline phosphatase and the amino-terminal propeptide of type 1 procollagen (bone formation markers) and urine or serum telopeptides of collagen crosslinks (bone resorption markers) are widely used. However, day-to-day and circadian variability have limited their values. Independent of BMD, a correlation between osteoporotic fracture and bone turnover indices in postmenopausal women and in elderly women has been shown (*190*). Elderly women whose bone resorption markers exceed the reference range showed an increase in fracture risk by about two-fold, after adjustment for BMD. Hence, a combined approach using BMD, clinical risk factors and bone turnover markers could enhance fracture prediction (*191*).

4.1.1. Bone Mineral Density

In clinical settings, BMD measurement by using DXA has widely been used as a tool to diagnose osteoporosis, assess fracture risk and therapeutic effects (*192*). Nevertheless, it involves relatively high cost and its availability is limited, particularly in less developed countries. Furthermore, the correlations between bone strength and BMD, measured by DXA, are found to be between 0.51 and 0.80 (*193-196*), which means that it accounts for only 50 to 80% of bone strength. Another technique, quantitative computed tomography (QCT) can measure volumetric BMD (mg/cm3). Cancellous bone can be separately measured without the interference from the cortical bone and aortic calcification. Lately, quantitative ultrasound (QUS) has also emerged as a cheaper and more accessible alternative for identifying osteoporosis, evaluating fracture risk and initiating osteoporosis treatment.

DXA can simultaneously measure the transmission of gamma-rays of two different energies which enables it to accommodate the different thicknesses of soft tissue, allowing this technique to be used on any part of the body. The sites that are commonly measured include lumbar spine, hip, forearm, whole body and skeletal segments. DXA is easy to use, yet it has high precision *in vivo* (*197*). A value for BMD by DXA 2.5 standard deviation (SD) or more below the mean for young adult women (T-score = -2.5) has been defined as a cut-off for osteoporosis. Using this criterion, 30% of all postmenopausal women is identified as osteoporotic and more than 50% would have suffered from a prior fracture (*197*). During osteoporosis treatment, accurate detection of BMD changes can only be achieved when the change is larger than the precision error of the measurement. In practice, changes of this magnitude are not likely to be achieved within one year and would require at least three years of treatment (*182, 184, 198*). Besides that, only a small fraction of fracture reduction can be explained by BMD changes (*199, 200*).

In ***QCT***, a thin transverse slice is scanned through the body. The image is used to quantify the volumetric BMD (mg/cm^3). The correlations between QCT and vertebral body compressive strength are approximately 0.72 to 0.74 (*201, 202*). Besides BMD, QCT can also analyze bone geometry (*203, 204*). The biggest source of measurement error is fat within the

bone marrow. Fortunately, the accuracy can be improved by using dual energy techniques, which carry out scans at two different potentials. Peripheral QCT (pQCT) can be used in a peripheral part of the body. It is precise, inexpensive and involving low radiation doses (*205*). In experimental and clinical studies done *ex vivo*, pQCT variables were shown to correlate with biomechanical predictors of fractures and fragility. Hence, it is useful in identifying women who are prone to osteoporosis (*206, 207*). MicroCT has a spatial resolution of 1–100 μm and can be used to replace the tedious histomorphometry technique. It can also be coupled with sophisticated software to render 3D analysis of bone structure (*208*). Nevertheless, CT has some limitations, including the large extent of operator dependence, limited access to scanners and lack of a technique to evaluate the proximal femur. These limit its usage in clinical practice (*208*).

QUS is a recent alternative method for bone mass assessment, measuring bone strength, density and elasticity or fragility (*209*). Being low cost, portable and radiation-free, have promoted its usage for osteoporosis assessment and bone fracture risk evaluation. QUS devices can be categorized into three groups: 1) trabecular sound transmission which is best for measuring the heel and most commonly used in clinical settings; 2) cortical transverse transmission which is currently used only in phalanx contact devices; and 3) cortical axial transmission which is used in phalanges, the radius and the tibia (*197*). QUS can differentiate patients with osteoporotic fractures from healthy people with the same age (Durosier *et al.*, 2006; Krieg *et al.*, 2008; Hans and Krieg, 2008). However, there are a few factors that may contribute to *in vivo* measurement errors, which include foot or leg positioning; surrounding soft tissue and its thickness, temperature and composition; the properties of the coupling medium; and the quality of sound transmission from the coupling medium to the skin. A standard or phantom can be used to examine instrument performance and to perform the necessary calibrations to improve accuracy (*210*).

4.1.2. Biochemical Markers

Bone remodeling rate is an important determinant of bone strength that is not measured by BMD tests. Increased bone remodeling rates have been associated with severe osteoporosis (*211, 212*). In fact, high levels of bone turnover markers (BTMs) are correlated with an increase in fracture risk (*212*), while lower bone turnover is associated with therapeutic efficacy of anti-resorptive agents. Hence, reliable bone remodeling assessment could be useful for predicting fracture risk. Even though the analytical precision for measurement of several BTMs has improved with the development of automated assays, their large biological variability still complicates their use in the clinical setting (*213, 214*). The biological variability can be categorized into modifiable factors, such as circadian variability (*215, 216*) and food intake (*217, 218*), and non-modifiable factors, such as disease states, medication affecting the skeleton and a recent fracture (*219*). Collection of paired fasting samples at a standard time on different days helps to reduce the variability, but it is impractical for everyday clinical practice.

BTMs can be classified as markers of bone resorption or bone formation, although the two processes are typically coupled. During bone resorption, products of type I collagen breakdown are generated (amino- or carboxyl-terminal cross-linking telopeptides, pyridinium cross-links). They are currently the most widely used bone resorption markers. Matrix proteins (osteocalcin), products of post-translational processing of type I collagen molecules (procollagen type I N- or C-terminal propeptides) and enzymes (alkaline phosphatase) released from osteoblasts during bone matrix synthesis can be used as bone formation markers (*220*).

4.1.2.1. Bone Formation Markers

Osteocalcin is a bone matrix protein that is synthesized by mature osteoblasts and constitutes about 15% of the non-collagenous bone matrix proteins (*221*). It has calcium-binding properties that are facilitated by three vitamin K-dependent γ-carboxy glutamic acid residues (*222*). Even though osteocalcin participated in the mineralization process, its exact

function is still unknown (*223*). Most of the synthesized osteocalcin is incorporated into the skeleton, but a small portion gets into the circulation (*224*). It is rapidly degraded in serum, resulting in detectable osteocalcin fragments together with the full-length molecule (*224, 225*). Although osteocalcin is a very good bone formation marker, it has a high circadian and biological variability (*223*) which negatively affects the measurement reproducibility. Osteocalcin can be detected by using enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) or a chemiluminesence immunoassay (*220*).

Alkaline phosphatase (ALP) is a glycosyl–phosphatidyl–inositol attached to ecto-enzyme existing on the osteoblast membrane (*226*). Its exact function is not confirmed, but its presence is necessary for bone mineralization (*227*). Typically, bone and liver isoforms contributed to about equal amounts of ALP in serum (*228*), which can be distinguished by heat inactivation (*220*). However, immunoassays for the bone isoform can provide a better assessment of bone-specific ALP, which has greater specificity for osteoblast function (*229*). Nevertheless, in patients with normal liver function, assessment of total ALP assays is good enough.

Procollagen type I N-terminal propeptide (PINP) and ***procollagen type I C-terminal propeptide*** (PICP) are derived from post-translational cleavage of type I procollagen molecules preceding the assembly into fibrils. They get into the circulation and can be used as bone formation markers (*230*). Although skin, tendon, dentin, and cartilage also contribute to a small amount of PINP and PICP, they originate mainly from bone (*223, 230, 231*). This is due to the higher turnover rate of skeletal tissues compared to non-skeletal tissues (*223*). RIA or ELISA, and electrochemiluminescence immunoassay can be used to determine serum PINP and PICP (*232*). However, unlike PINP, PICP is broken down by the mannose receptor, which is controlled by growth and thyroid hormones. This makes the data interpretation in patients with pituitary or thyroid dysfunction complicated (*231, 233, 234*). Hence, PINP is usually preferred over PICP.

4.1.2.2. Bone Resorption Markers

Amino-terminal cross-linking telopeptides of type I collagen (NTX) is a type I collagen breakdown product that results from osteoclastic bone resorption. It can be measured in either serum or urine. Although serum NTX drops significantly following antiresorptive therapies, it is not as sensitive as urinary NTX (*235*). However, it is still unclear if collagen from dietary sources can affect serum NTX levels. On the other hand, measurement of NTX in 24-hour urine is less affected by dietary collagen intake and it is able to overcome the circadian variability in bone turnover (*220, 236*).

Carboxyl-terminal cross-linking telopeptides of type I collagen (CTX) can also be determined using ELISA, RIA, and an electrochemiluminescence assay in both serum and urine (*220, 237*). With time, C-terminal telopeptide α1 chain of type I collagen is subjected to β-isomerization and racemization (*238*). Consequently, the ratio of α-CTX to β-CTX, representing the breakdown product of newly synthesized and aged collagen, has been proposed to assess rapid bone turnover. In patients with Paget's disease, the α-CTX/β-CTX ratio increases as a result of the breakdown of newly synthesized woven bone and decreases significantly following bisphosphonate therapy (*239*). Similarly, in postmenopausal women, higher ratio of α-CTX/β-CTX is positively correlated to fracture risk (*240*). However, inter-individual variability of the α-CTX/β-CTX ratio is high (*220*), Hence, further studies are required to determine whether this ratio can be used as a better measure than the total CTX.

Pyridinoline (PYD) and ***deoxypyridinoline*** (DPD) are covalent pyridinium cross-links resulting from collagen breakdown. They are produced during bone resorption, released into the circulation and passed into the urine (*241, 242*). They can be detected by RIA and ELISA (*220, 223, 242*). In addition to bone, collagen cross-links are also present in cartilage, vessels, and ligaments. Due to higher turnover rate in bone, most of the circulating and urinary PYD comes from bone (*223*). Unlike PYD, DPD originates almost exclusively from bone and dentin (*223*). Overall, both of them can be used to reliably assess bone resorption.

Tartrate-resistant acid phosphatase (TRACP5b) is the 5b isoform of acid phosphatases. It is expressed in the osteoclast and is the only marker of osteoclast activity. In

some conditions causing high bone turnover, such as Paget's disease, bone metastases, multiple myeloma and ovariectomization, the level of TRACP5b is normally elevated (*243*). Nevertheless, due to its poor sensitivity in reporting changes in bone turnover resulting from anti-resorptive therapy, it is not widely accepted (*243, 244*).

4.2. Tracer Techniques

4.2.1. Stable and Radioisotopes to Study Mineral and Trace Element Metabolism

Both stable and radioactive isotopes can be used to trace mineral metabolism in the body. For this purpose, they have to be administered in the forms that are distinguishable from their naturally-existing forms. The isotopes, usually called 'tracer', can be administered either orally or intravenously. For oral administration, the tracer can be incorporated into foods either intrinsically (incorporated biosynthetically into a plant or animal) or extrinsically (added to the food before ingestion). After administration, tracer appearance in plasma, urine and feces can be monitored over several days, weeks or even years depending on its characteristics (*245*). This isotopic technique can be used to assess mineral absorption, retention, excretion or even compartment sizes and transfer rates between the different mineral compartments in the body. Calcium and strontium isotopes are listed in Table A-1. However, not all of them are suitable to be used as a tracer in metabolic studies.

There are a few advantages in using radioisotope tracers for studying mineral metabolism. They can be measured in blood, urine or feces samples by scintillation counting, while whole body counting can be used to assess body retention directly by measuring the total amount of absorbed tracer in the body. Besides a small natural background radiation, the radiotracer is usually the only source of radioactivity in the sample. Hence, a proper correction for background activity on the decay counting technique is sufficient. High precision in

Table A-1: List of calcium and strontium isotopes with their atomic masses, natural abundances and half-lives.

Isotope	Atomic Mass[a, b]	Mole Fraction[a,b]	Half-life[b]
Calcium			
^{34}Ca	34.014		< 0.035 µs
^{35}Ca	35.005		25.7 ms
^{36}Ca	35.993		0.100 s
^{37}Ca	36.986		0.182 s
^{38}Ca	37.976		444 ms
^{39}Ca	38.971		0.861 s
^{40}Ca	39.962	0.969 41 (156)	5.92 x 10^{21} a
^{41}Ca	40.962		1.02 x 10^{5} a
^{42}Ca	41.958	0.006 47 (23)	
^{43}Ca	42.958	0.001 35 (10)	
^{44}Ca	43.955	0.020 86 (110)	
^{45}Ca	44.956		162.7 d
^{46}Ca	45.953	0.000 04 (3)	> 0.4 x 10^{16} a
^{47}Ca	46.955		4.536 d
^{48}Ca	47.952	0.001 87 (21)	4.4 x 10^{19} a
^{49}Ca	48.956		8.72 m
^{50}Ca	49.958		14 s
^{51}Ca	50.962		10 s
^{52}Ca	51.965		4.6 s
^{53}Ca	52.970		0.4 s
^{54}Ca	53.974		0.1 s
^{55}Ca	54.981		22 ms
^{56}Ca	55.986		11 ms

[a] Taken from a table in IUPAC Technical Report (*246*)

[b] Taken from a table in CRC Handbook of Chemistry and Physics, 94th Edition (*247*)

Isotope	Atomic Mass[a, b]	Mole Fraction[a,b]	Half-life[b]
Strontium			
^{73}Sr	72.966		> 25 ms
^{74}Sr	73.956		> 1.5 µs
^{75}Sr	74.949		88 ms
^{76}Sr	75.942		7.9 s
^{77}Sr	76.938		9.0 s
^{78}Sr	77.932		2.7 m
^{79}Sr	78.930		2.1 m
^{80}Sr	79.925		1.77 h
^{81}Sr	80.923		22.3 m
^{82}Sr	81.918		25.36 d
^{83}Sr	82.918		1.350 d
^{84}Sr	83.913	0.005 6 (1)	
^{85}Sr	84.913		64.85 d
^{86}Sr	85.909	0.098 6 (1)	
^{87}Sr	86.908	0.070 0 (1)	
^{88}Sr	87.905	0.825 8 (1)	
^{89}Sr	88.907		50.6 d
^{90}Sr	89.910		28.9 a
^{91}Sr	90.910		9.5 h
^{92}Sr	91.911		2.64 h
^{93}Sr	92.914		7.4 m
^{94}Sr	93.915		1.25 m
^{95}Sr	94.919		25.1 s
^{96}Sr	95.922		1.06 s
^{97}Sr	96.926		0.42 s
^{98}Sr	97.928		0.65 s
^{99}Sr	98.933		0.27 s
^{100}Sr	99.935		0.201 s
^{101}Sr	100.941		0.115 s
^{102}Sr	101.943		68 ms
^{103}Sr	102.949		> 0.3 µs
^{104}Sr	103.952		> 0.3 µs
^{105}Sr	104.959		> 0.3 µs

measuring counting rates can be achieved, resulting in high precision of radioisotope analysis (*248*). Thus, minute amount of tracer, that is unlikely to perturb the system being studied, is usually sufficient (*245*). In the case when two or more radioactive tracers are present in the same sample at the same time, contributions of the different isotopes can be distinguished separately by repeated counting measurements or by spectrometric measurements. Nevertheless, the concern about exposure to ionizing radiation together with the advancement in mass spectrometric techniques for analysis of stable isotopes has promoted the use of stable-isotope tracers (*245*). In most cases, stable isotopes can replace radioisotopes in research, medicine, and industry (*249*).

In nature, there are six naturally occurring calcium stable isotopes. ^{40}Ca (96.94%) is the most abundant isotope and hence cannot be used as a tracer due to the large quantity of ^{40}Ca that will be required. ^{44}Ca (2.08%) is the second most abundant isotope of calcium and it has been widely used in human studies. However, due to its relatively high natural abundance, relatively high doses are required. The high doses of tracer may increase the total element intake and perturb the system under investigation. Tracers with very low natural abundances, such as ^{46}Ca (0.003%), are ideal tracers. However, the high costs of these isotopes as well as the challenging measurement can be drawbacks. Other isotopes with relatively low abundances, such as ^{42}Ca (0.65%), ^{43}Ca (0.14%) and ^{48}Ca (0.19%), are better alternatives as they are less expensive, less challenging to be measured, and the doses can be kept relatively low. Similar principles are used in determining strontium tracers to be used. There are four naturally occurring stable strontium isotopes. The most abundant isotope, ^{88}Sr (82.58%), cannot be used as a tracer due to the large quantity of tracer that will be required. ^{87}Sr is a radiogenic nuclide produced by a radioactive decay of ^{87}Rb. Other options would be ^{86}Sr (9.86%) and ^{84}Sr (5.6%). The least abundant isotope, ^{84}Sr, would be an ideal choice in most cases even though it is relatively more expensive. Thermal ionization mass spectrometry (TIMS) and inductive coupled plasma mass spectrometry (ICP-MS) are commonly used to measure isotope ratios for stable isotope analysis. These techniques will be discussed in Part A, Section 7.1.1 and 7.1.2.

4.2.2. Short-term Kinetics

After tracer administration, its appearance in plasma or serum, urine and feces can be monitored and assessment of mineral absorption, retention, excretion or even compartment sizes and transfer rates between the different mineral pools in the body can be performed.

4.2.2.1. Plasma or Serum Isotope Appearance

The appearance of oral doses of tracer in plasma or serum can be used to estimate its bioavailability. It is usually less precise, because of the extra load required to produce an increase in the serum level of the element and might lead to major perturbation in its homeostasis regulation (*250*). However, isotopic methods require a much smaller rise in total serum content of the element in order to produce detectable changes in serum tracer concentration. Hence, the disturbance in the homeostasis regulation can be greatly reduced. In order to calculate fractional absorption from the orally administered dose, the dual isotope tracer method involving one orally and one intravenously administered isotope, has to be used. These isotopes must follow similar kinetics irrespective of route of entry (*251*). Although this method has normally been used with radioisotopes, it could be used with stable isotopes as well. This approach allows assessing absorption of minerals, but it is invasive as it requires continuous blood sampling and is therefore not preferred.

4.2.2.2. Fecal Excretion

Absorption of the isotope is calculated as the difference between intake of the isotope dose and fecal excretion. This method can measure the luminal disappearance of the isotope without correction for endogenous losses, which means, it can only measure apparent absorption. Determining the optimum time period for fecal collection is crucial to ensure that all non-absorbed isotope is excreted with little or no isotope that has been absorbed and re-excreted into the gut. The transit time of the tracer varies considerably with diet composition, genetic and physiological differences. The colonic passage accounts for the major part of the time as intestinal transit is usually less than 24 hour. After several days in the colon, it is

therefore highly likely that fecal samples which contain most of the non-absorbed isotopes will also contain endogenously excreted isotopes (*252*). In order to overcome this source of error, which leads to an underestimation of the absorption, endogenous losses of the element have to be measured (*251*).

The administration of one isotope allows only the calculation of apparent absorption which results in an underestimation of true absorption. True absorption can only be determined by intravenous administration of a second isotope. Both tracers have to be metabolized in the same way and the same fraction is excreted in the feces. True calcium absorption can be calculated from apparent absorption added with the amount of absorbed and re-excreted label that is estimated based on the fraction of intravenous label in the feces.

4.2.2.3. Urinary Excretion

The most widely used method to assess calcium absorption is the dual isotope tracer method with urinary monitoring (*253*). One oral and one intravenous isotopic tracer are administered and calcium absorption is assessed based on the relative recovery of the oral and intravenous isotopes in urine. Samples of 24-hour urine are collected and the relative fractions of the two isotopes are measured. The ratio corresponds to the fraction of absorbed calcium in the diet. The molar ratio of the two isotope labels in urine can be calculated from the measured isotope ratios based on isotope dilution principles. Calculation techniques are described in Part A, Section 7.1.4. True fractional calcium absorption can be calculated from the molar ratio of the isotopic labels after correction for dose (*254*).

Several variations of this method have been used. They differ in the timing of the urine collection and types of isotopes used (stable (*255*) or radioisotopes (*256*) or a combination of both (*257*). A collection of urine for at least 24 hour after dosing was described in Degrazia *et al.* (*258*). This technique was later adopted by Hillman *et al.* (*255*), Eastell *et al.* (*259*), etc. Later on, it was proposed that a spot urine sample that was taken at any time between 24 hour and 48 hour after dosing was sufficient to assess calcium absorption. However, calcium absorption might be underestimated if the measurement is

based on samples collected during the first hours after isotope administration when intestinal absorption of the oral isotope is incomplete (*254*).

In general, the most widely used method to assess true fractional calcium absorption is the dual isotope method with 24-hour urine collection. The method has been validated against other techniques to assess calcium absorption. It has been demonstrated that the assessment of true fractional calcium absorption using a complete 24- hour urine collection is well correlated with the data from fecal balance (*259*), which has been discussed in Section 4.2.2.2. Abrams *et al.* (*260*) also reported that the dual isotope tracer method shows less variability than the fecal balance method. The dual isotope tracer method has been shown to correlate well with results for the serum isotope appearance technique (*261*) and whole body counting (*257*).

4.2.2.4. Compartmental Analysis

Compartmental modeling is the most common approach for mathematical evaluation of calcium kinetics data. Physiological processes such as intestinal calcium absorption, endogenous calcium losses into feces, renal handling of calcium, bone calcium deposition rate, bone calcium resorption rate, body pool sizes and turnover rates of exchangeable calcium can be described. The number of samples and the length of the study period influence the choice of the compartmental model; i.e. with more samples available and with longer monitoring period, a more complex model can be applied to describe the data.

When using compartmental modeling, a few assumptions have to be made: 1) the system is in steady state (pool sizes do not change during the study period); 2) the tracers do not perturb the system; 3) rapid and slow exchangeable pools are covered in the sampling period; and 4) when the system has been perturbed, e.g. by an intervention, a new steady state has to be reached before calcium kinetics can be studied (*248*).

To describe tracer kinetics, a single compartment system is the simplest model that can be used (see Figure A-4a). The tracer is introduced once and leaves the system with the carrier material as a constant fraction of the total amount in the system. Its concentration will

decrease with time. Two compartment models are more complicated (see Figure A-4b). The tracer enters compartment 1, from which it might leave the system or get transferred to compartment 2. After the tracer mixes with material in compartment 2, some of it will return to compartment 1. At the start, the tracer concentration drops as a non-linear function of time. At equilibrium, the concentration follows a straight line in both compartments in a semi logarithmic plot (*262*). In multi-compartment models, tracer concentration will never be the same in all compartments (see Figure A-4c). A compartment is in equilibrium with other compartments, when the rate of change in tracer concentration becomes the same as for the other pools (*262*).

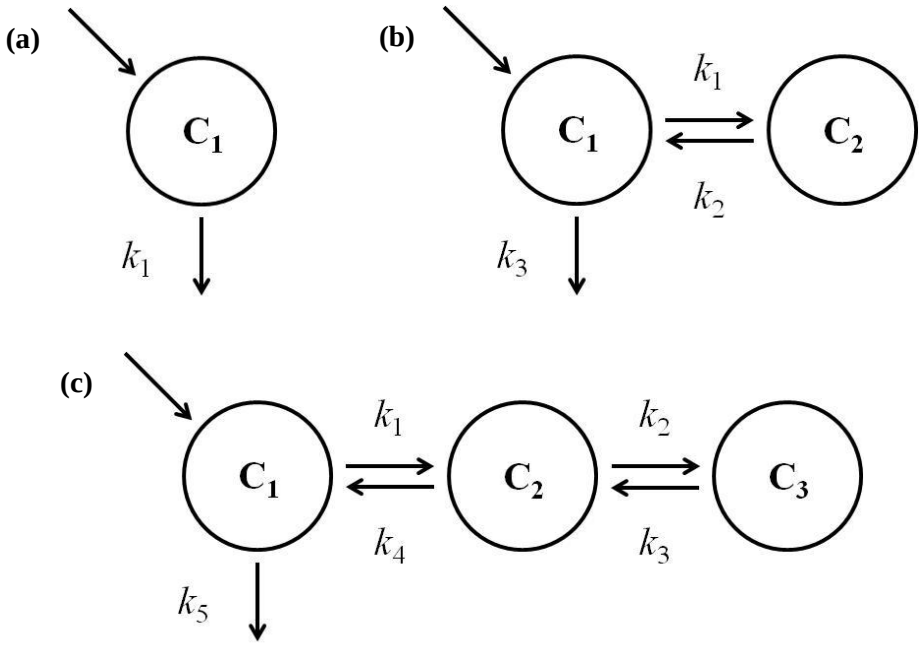

Figure A-4: Schematic illustration of compartment models with their transfer routes (*k*). (a) A single compartment model, (b) a two compartment model, and (c) a three compartment model.

A number of compartmental models to describe calcium metabolism have been proposed. There are several one compartment models (*263, 264*), which are no longer used. Thereafter, additional compartments have been introduced by Heaney (*265*), Cohn *et al.* (*266*), Aubert and Milhaud (*267*) and Neer *et al.* (*268*). Despite varying numbers of compartments and individual compartmental sizes, there are no significant differences in total pool size and bone mineralization rate between the different models. All models, except

Neer's, pointed that time needed for compartmental equilibration is five days or less. However, Neer's model includes compartments that need 10-20 days to equilibrate, which leads to larger total pool size and lower accretion values.

Compartmental models have also been used to describe long-term calcium metabolism. Three compartmental models, which include a central dosing compartment, bone surface compartment and deep bone compartment, have been employed by Johnson *et al.* (*269*), Hillegonds *et al.* (*270*), Denk *et al.* (*271*), and Sharma *et al.* (*272*). Four compartmental models, which separate bone compartment into cortical and trabecular bones, have been used by Staub *et al.* (*273*) and Lee *et al.* (*274*). Whereas Hui *et al.* (*275*) employed a more complicated five compartmental model by splitting the bone compartments into slow, medium and fast exchanging compartments.

4.2.3. Long-term Kinetics

Since long-term kinetic studies using ^{41}Ca are of great relevance to the technique used in this thesis, this section will be elaborated with greater details to observe how this method has evolved and improved throughout the years.

4.2.3.1. ^{41}Ca Studies

In vivo, bone calcium can be labeled by administration of calcium isotope tracers. After administration, tracer that is not incorporated into the skeleton will be slowly excreted through urine. After excess tracer has been eliminated from the dosing compartment, tracer signal in urine will originate mainly from tracer that has been incorporated into the skeleton. Hence, changes in urinary tracer excretion can be used to assess the changes in bone metabolism (*276*). The cost of using calcium stable isotope tracers, would be very high, whereas the use of conventional calcium radiotracers is not possible due to ethical considerations. A compelling reasonable alternative is ^{41}Ca, a very long-living radioisotope which requires the use of ultra-trace levels of ^{41}Ca using accelerator mass spectrometry (AMS) (*277*) or resonance ionization mass spectrometry (RIMS) (*278*). The sensitivity of

conventional mass spectrometric techniques including TIMS or ICP-MS is not high enough. Even with the use of high-resolution instruments, they are not capable to suppress the neighboring peaks interference on the ^{41}Ca signal effectively. To overcome this limitation, the dose would have to be increased substantially, which would defeat the purpose of using ^{41}Ca as a tracer. Although ^{41}Ca is a radioisotope, when it is used in combination with AMS or RIMS for the analysis, only very small amounts of the tracer have to be administered (*276*). Thus, the health risk is negligible. For comparison, the effective radiation dose that comes from the dosing material is about 1/1000 of the dose received from a single chest X-ray scan. It is also comparable to the amount of natural radioactivity of 5 kilograms potatoes.

Due to bone formation and resorption processes, ^{41}Ca tracer will be incorporated into bone matrix. Tracer that is not incorporated will be gradually excreted in urine. After the majority of the tracer left in the body is incorporated in bone matrix, the ^{41}Ca signal can be used to follow the effect of interventions on bone. Eventually, ^{41}Ca will be resorbed from the bone matrix and subsequently transferred to plasma. Some of it will be excreted in urine, while the rest will be re-incorporated into bone matrix through bone accretion processes. These processes determine the changes in the plasma ^{41}Ca, which can be monitored through the changes in ^{41}Ca/Ca isotopic ratio in urine (see Figure A-5).

To assess changes in bone calcium metabolism as a result of an intervention, the kinetics of urinary tracer excretion must be established. Compartmental modeling techniques to describe the observed urinary ^{41}Ca excretion pattern are widely used for data evaluation. The effect of an intervention on bone metabolism can be recognized as changes to the transfer rates between different compartments. This can be done by fitting a compartmental model to experimental data by using nonlinear mixed effects regression analysis. Data analysis using compartmental modeling has been elaborated in Part A, Section 4.2.2.4. and non-compartmental modeling will be discussed in Part A, Section 4.3.2.

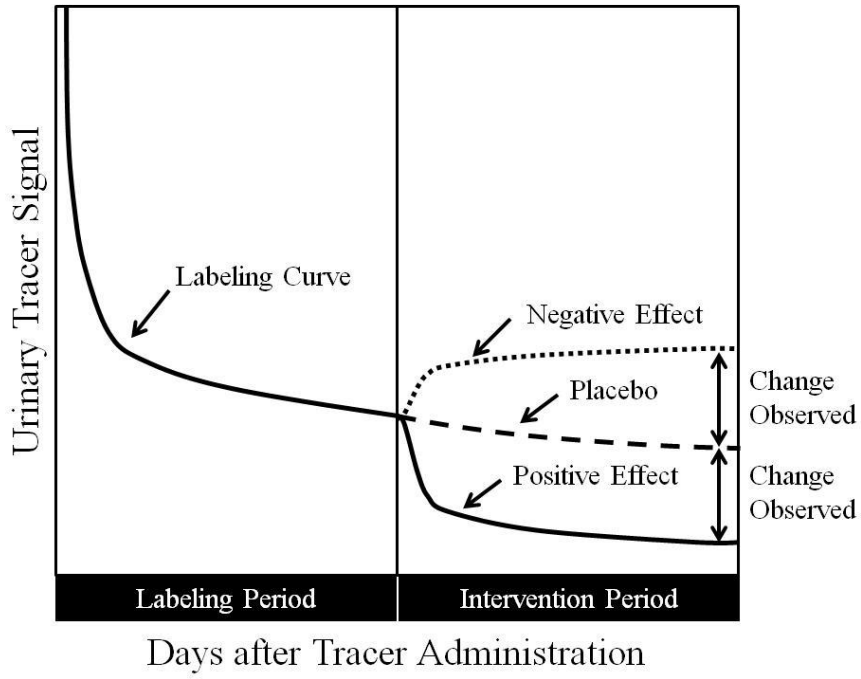

Figure A-5: Basic principle in monitoring changes in bone metabolism using ^{41}Ca. Following a single isotope administration, the tracer urinary excretion pattern is monitored throughout the labeling period. Once tracer that is not incorporated into bone has been washed out from the body, all urinary ^{41}Ca can be assumed to originate from bone and the labeling curve can be used as an index against which changes resulting from an intervention can be measured. Figure is taken from Denk *et al.*, 2006 (*271*) with modifications.

Previous studies have shown that bone calcium can be labeled using ^{41}Ca, allowing changes in bone calcium metabolism to be assessed at much higher sensitivity compared to other established techniques. The first study using ^{41}Ca as a long term tracer for monitoring bone resorption in dogs was published by Elmore *et al.*(*277*). ^{41}Ca and ^{45}Ca were given intravenously to mature dogs. Serum levels of the isotopes were measured by AMS. Twenty-five weeks after dosing, a subgroup of the labeled dogs was given cadmium (Cd) in their drinking water to study the effect of Cd exposure in bone metabolism. ^{41}Ca and ^{45}Ca were reported to be resorbed from bones at similar rates.

Following this study, Johnson *et al.* (*269*) conducted another study using ^{41}Ca in humans. They labeled the skeleton of a premenopausal woman with an intravenous dose of ^{41}Ca and monitored its urinary excretion for more than one year. A compartmental modeling approach was used to describe the urinary excretion pattern of ^{41}Ca. The applied model was a

sequential three compartmental model consisting of dosing compartment that was proposed to describe extracellular fluid, and two bone compartments (exchangeable bone and deep bone). The transfer rates between the different calcium pools and the pool sizes were calculated based on the urinary excretion data of ^{41}Ca over 1000 days. Based on the results of the ^{41}Ca study, it was found that the two bone compartments consist of about 1.1 kg calcium and the extracellular fluid compartment of 1.2 g calcium. This was in good agreement with the literature (*279*) indicating that ^{41}Ca is a useful tool to describe calcium kinetics in human. During the monitoring period, the ^{41}Ca excretion pattern is described very well with the proposed model even in a non-controlled study (*269*). Variations in the ^{41}Ca signal around the fitted curve might be explained with menstrual cycle phases, changes in diet or exercise.

In a subsequent study, a continuous feeding protocol was carried out in a postmenopausal woman (*280*). The study involved the administration of ^{41}Ca, which was divided between three meals a day for two weeks, with spot urine and feces sample collection. At day 10 of the feeding period, a standard dual isotope kinetic study using stable isotopes was performed which included intravenous ^{42}Ca and oral ^{44}Ca administration, followed by 24-hour urine sampling. The results from both protocols were compared and found to be in good agreement.

The next study was conducted in 25 subjects, who received ^{41}Ca orally and their urinary excretion was followed over 500-600 days (*281*). It was suggested that the ^{41}Ca tracer excretion pattern could be best described with a combined power and exponential law, as proposed by Norris *et al.* (*282*). The mathematical excretion pattern of ^{41}Ca was proposed as baseline from which perturbations (e.g. due to an intervention) might be measured. In the same study, one subject received two therapeutic agents for osteoporosis treatment after which ^{41}Ca excretion was followed for 400 days. The effectiveness of an alendronate intervention in reducing urinary ^{41}Ca excretion was compared to raloxifene. Results demonstrated that only alendronate and not raloxifene induced a drop in the ^{41}Ca signal. In this study, bone resorption was also studied in parallel during intervention phase using urinary NTX, a conventional biomarker for bone resorption. It was shown that the results from

biomarkers are much more variable than that of the ^{41}Ca signal. Hence, ^{41}Ca could be used to study individual's response to therapy and provide an advantage over conventional biomarkers technique.

In a more recent study, ^{41}Ca was administered orally to a postmenopausal woman followed by collection of urine, blood and saliva samples at different time points over a period of two months after dosing (*283*). It was demonstrated that ^{41}Ca could be successfully determined in urine, serum and saliva although ratios in the saliva were not similar to blood and urine. They also demonstrated that the ^{41}Ca/Ca ratio is similar in urine and serum and there is similarity between isotopic concentrations in urine and serum. This finding avoids the need for blood sampling. Hence, urine sampling is sufficient for future studies of ^{41}Ca in humans.

Hillegonds *et al.* (*270*) developed a kinetic model of human calcium balance using ^{41}Ca. It was predicted that this method was able to detect a 10% change in bone turnover rate in the subjects, which would allow close monitoring of patients with altered bone turnover associated with various diseases, including osteoporosis, cancer, kidney disorders, arthritis and many others. In a subsequent study, the serum ^{41}Ca/Ca signal in labeled subjects was found to be significantly different between healthy individuals and patients with renal failure (*284*).

Denk *et al.* (*271*) conducted a study using ^{41}Ca in postmenopausal women. It was found that following isotope administration, it requires 150 to 250 days until tracer incorporation into bone matrix reaches the maximum amount. In this study, the urinary ^{41}Ca excretion pattern was described by a three-compartmental model. Besides describing the urinary ^{41}Ca excretion pattern, the model can also identify changes in transfer rates between the different compartments which allow the effect of an intervention to be evaluated. It was concluded that this technique could potentially assess the influence of diet and lifestyle on bone calcium metabolism, which is useful in the development of better strategies for osteoporosis prevention. They continued to explore the potential of this technique by assessing the effect of bisphosphonate interventions on bone health (*285*). The correlation

between changes in the ^{41}Ca signal during the intervention with changes of PINP as a biomarker of bone formation and serum CTX as a resorption marker was observed. This study showed that once the skeleton was labeled, ^{41}Ca can be used to directly detect changes in bone calcium metabolism in response to controlled interventions. As this method is sensitive, short-term interventions might be sufficient, ensuring better compliance.

A study on perimenopausal women was conducted by Hui *et al.* (*275*) using ^{41}Ca tracer. They monitored long-term dynamic bone remodeling balance during the transition between premenopausal and menopausal stage, which is useful to achieve early prediction of fracture risk for osteoporosis prevention or treatment. Besides providing predictive bone state for osteoporosis prevention, dynamic tracking may also be useful for monitoring treatment outcomes.

More recently Lee *et al.* (*274*) developed a four-compartmental model for ^{41}Ca kinetics by fitting urinary ^{41}Ca and total bone calcium data in post-menopausal women. The estimated bone resorption is in agreement with earlier studies using ^{45}Ca. Hence, they concluded that if bone loss is constant, the model could be used to estimate bone resorption using urinary ^{41}Ca signal. The model simulations also verified that changes in urinary ^{41}Ca/Ca ratio could be used to estimate changes in bone metabolism as a proxy for bone balance.

Also using a four-compartment model to study ^{41}Ca kinetics, Sharma *et al.* (*272*) evaluated the impact of bisphosphonate intervention and the effect of metabolic bone disease such as osteoporosis and on bone remodeling. Moreover, the inclusion of cellular accommodation might allow this model to offer a more realistic prediction of disease progression.

Kejun *et al.* (*286*) used ^{41}Ca to quantitatively analyze and monitor the whole process of drug-induced osteoclast skeletal resorption *in vitro*. The behavior of osteoclast with the administration of strontium ranelate, an osteoporosis drug, was studied by using ^{41}Ca labeled hydroxyapatite to simulate the bone.

4.2.3.2. Non-compartmental Analysis

Besides the compartmental evaluation technique, non-compartmental approaches have also been used for long-term kinetics. These include a combination of power and exponential function to describe tracer retention or excretion from body. Norris *et al.* (*282*) was the first to describe such a model, followed by Burkinshaw *et al.* (*287*). In general, non-compartmental models involve an expanding pool, which is described by a power function and an exponential function describing bone accretion and resorption. The combination of these functions is shown by Eqn. 1.

$$x(t) = A \cdot t^{-b} \cdot e^{-kt} \quad (1)$$

where A is a factor empirically fitted to the data. The power term (t^{-b}) describes the decline of the pool specific tracer concentration because of the progressive dilution of tracer by increasing quantities of body calcium. The exponential term (e^{-kt}) represents the decline of the pool specific tracer concentration due to a first order, non-exchanging loss of tracer from the pool.

A range of different non-compartmental and compartmental models exist to describe calcium metabolism. Since all models are approximations, the model chosen is the one that best describes the data. An important measure of the goodness of the description of the data by a model is the coefficient of determination, R^2, that is calculated from the sum of the squares of the distances of the points from the best-fit curve determined by non-linear regression. Several other mathematical tools are available to assess the goodness of a model used to describe data, which will not be discussed in detail in this overview. The non-compartmental and compartmental models were originally established with short-term tracer studies, conducted over 5-20 days. Important features of bone metabolism such as bone remodeling were thus not fully covered as a remodeling cycle takes about 120 days. Even so, several more recent studies have shown that these models are also applicable for long-term studies.

CHAPTER 5: STRONTIUM METABOLISM

Strontium is a trace element found in ocean water and calcareous rocks (*288, 289*). It is inherently found in present-day diets (*290*). Since it is a bone-seeking element., 98% of strontium in the human body can be found in the skeleton (*291*). Strontium metabolism is closely related to that of calcium due to their chemical similarities. Both strontium and calcium ions have a valency of 2+, similar ionic radii, complexing and chelating abilities, resulting in products with various solubilities and binding strengths. Their relative binding affinities differ among anionic compounds. Generally, in most biological systems, preference is given to calcium over strontium.

The formation of strontium radionuclides as byproducts of nuclear fission and atomic bomb detonation has discouraged the study of strontium as a calcium analog in clinical research. Earlier investigations on strontium have mainly focused on finding measures to reduce the body burden of radiostrontium caused by contamination of the environment and food supply. Hence, little has been done to study the metabolism of strontium as well as its functional role in the human body. However, animal studies have shown that in many physiological processes, such as in muscular contraction and blood clotting, strontium can be a substitute for calcium. These processes are triggered by calcium as well as by strontium, but to a lesser degree. Hence, strontium metabolism will be discussed here in comparison to calcium.

5.1. Physiological Functions

Calcium is the most abundant mineral element in the body. The majority (~99%) of calcium is found in the skeleton and teeth. The remaining calcium exists in soft tissues and body fluids, accounting for <1% of total calcium in the body. It is an essential nutrient, not only for bone and teeth mineralization, but also for regulating intracellular events in the body. Even though strontium is also mainly deposited in bone, the ratio of strontium to calcium amount in the skeleton is only 0.00045 (*292*). In hydroxyapatite, strontium can substitute

calcium positions (*293*). It has been suggested that in addition to sharing some chemical and physical characteristics, they also exhibit similar contributions in a number of biological process (*14*). Although the response to stimulation seems to be weaker, strontium often mimics the actions of calcium (*294, 295*).

Calcium provides ***skeletal rigidity*** by forming hydroxyapatite [$Ca_{10}(OH)_2(PO_4)_6$], which is deposited in collagen fibrils (*296*). In return, bone mineral serves as a reservoir for calcium (*297*). Hence, calcium may enter the extracellular fluid either from the intestine or bone. However, calcium is needed not only to maintain skeletal structures but also to control the cells' and tissues' behaviour in respond to environmental changes, including a diet, a stimulus or a pathogen (*298*). Non-skeletal functions of calcium can be categorized into structural, such as maintaining intracellular structures, preserving the integrity and permeability of biological membranes, defining a few structural features of chromosomes; and regulatory, such as regulating enzymes biochemically. Calcium is not only stored in the skeleton, but mobilisable calcium is also stored in specialized compartments (endoplasmic reticulum) within cells (*299*). Release of intracellular calcium into the cell cytosol triggers cellular events and responses.

Depending on the cell type, the concentration of ***intracellular*** calcium may vary from 0.02 to 15 mmol/L cell water (*298*). Most of cellular calcium (>99.9%) is bound within organelles and the nucleus. In a resting cell, the concentration of free calcium in the cytosol is about 10 000-fold compared to concentration in plasma, causing an electrochemical gradient across the cell membrane (*299*). Even a small increase in the cell membrane permeability or a small release of calcium from an internal store can cause a very significant change in cytosolic calcium concentration. This will 'switch on' metabolic pathways within the cells and may even damage it. This electrochemical gradient is maintained by a pump that is responsible for counterbalancing the small passive leakage of calcium into the cell.

Calcium has the ability to trigger events within the cell, which are initiated by a primary stimulus, which may be physical (touch or an action potential) or chemical (hormone or neurotransmitter). An increase in cytosolic calcium is responsible for muscle contraction

and vesicular secretion, several cell aggregation (e.g. blood clotting), cell transformation, cell division and activation of intermediary metabolism (*299*). To maintain those vital activities, plasma calcium concentrations must be tightly regulated.

Calcium plays a crucial role in ***blood coagulation*** (clotting) (*298*). The process of clot formation involves a cascade of proteolytic reactions that require a series of inactive enzymes or clotting factors to be activated, which will subsequently activate another inactive factor. The response amplifies each time, which eventually results in the conversion of prothrombin to produce thrombin and the production of a fibrin clot.

Calcium is also essential for the activity of several ***extracellular digestive enzymes***, including proteases, phospholipases and nucleases (*298*). A calcium ion sensing receptor is expressed in the gastrointestinal tract, which is believed to play a role in gastric acid secretion.

Calcium plays an essential role in triggering the release of neurotransmitter from nerve cells and in muscle contraction (*298*). For ***neurological function***, calcium selective ion channels in the cell membranes open when the membranes are depolarised, leading to an influx of calcium and eventually a rise in cytosolic calcium concentration. This results in the release of neurotransmitters (e.g. acetylcholine) in nerves.

For ***muscular function***, calcium regulates the interaction of thick (mainly myosin) and thin (mainly actin) filaments in muscle fibers and it plays a vital role in skeletal, heart and smooth muscle contraction (*298*). When the muscle fibre is at rest, a regulatory protein complex (troponin and tropomyosin) inhibits the formation of cross-bridge by blocking the access to actin cross-bridge binding sites. Calcium ions released from the sarcoplasmic reticulum can bind to calcium binding sites on the troponin-binding molecule, resulting in conformational change, which eventually allows cross-bridges to form. Such binding results in muscular contraction. As calcium is pumped back into the sarcoplasmic reticulum, cytosolic calcium concentrations decrease and muscle relaxation occurs following the detachment of cross-bridges. As the thin filaments of smooth muscle do not contain troponin, the binding of calcium to calmodulin, which has a similar to structure to troponin, happens as

the intracellular calcium ion concentration increases. This complex results in the phosphorylation of myosin, causing myosin cross-bridges to form with actin, which results in the muscle contraction. Conversely, dephosphorylation results in muscle relaxation.

Under normal circumstances, strontium level in the body is much lower than calcium. Therefore, it does not interfere in any biological process that involves calcium. However, when there is excess of strontium, it may interfere in these processes. Because strontium is similar to calcium, it can replace calcium imperfectly in the skeleton as well as other cellular compartments. Although many calcium-dependent enzymes, secondary cell messenger systems and transporter systems will still function in the presence of strontium in place of calcium, changes in the kinetic parameters may take place. Thus, neurotoxic and neuromuscular disturbance may be observed as a result of high strontium uptake (*300*).

5.2. Skeletal Deposition and Release

In normal individuals, the total body calcium increases from about 20 gram at birth (*301*) to about 1200 g once they reach maturity (*127*). All the calcium accumulated in the process is obtained from dietary sources and deposited in the skeleton in the form of hydroxyapatite. Strontium can be incorporated into bone via two mechanisms: surface exchange or ionic substitution. However, even when large doses of strontium is administered to experimental animals over a long period of time, the total bone strontium is still much lower compared to calcium. Theoretically, only one out of ten calcium atoms can be substituted by strontium (*302*). There are three important mechanisms determining the uptake and release of bone seeking elements: 1) apposition, resulting in an increase in mineral volume; 2) resorption, contributing to the modeling and remodeling of the skeleton together with apposition; and 3) surface and diffuse exchange, that occur at all bone surfaces in contact with blood and allow bone-seeking elements to enter the entire bone volume.

On the first day of $SrCl_2$ administration through drinking water, MacDonald *et al.* noticed a rapid drop in the calcium to strontium ratio in rat and mouse femur, which was followed by a much slower decrease during the following 6 to 8 weeks (*303*). It was

suggested that strontium is incorporated into the skeleton by two mechanisms: 1) an initial rapid mode which relies on osteoblastic activity and ionic exchange at the crystal surface in new bone; and 2) a second slower mechanism that involves the incorporation of strontium into the crystal lattice in old bone (*304*). It has been demonstrated that incorporation of strontium into the bone of strontium-treated monkeys occurred mainly by ionic exchange at crystal surfaces, and to a smaller extent by heteroionic substitution into the crystal lattice (*302*). Strontium was dose-dependently taken up by the bone mineral. It was distributed heterogeneously in cortical and trabecular bone and in new and old cortical bone, with approximately three- to four-fold more strontium in new than in old compact bone, and 2.5-fold more strontium in new than in old trabecular bone. Strontium incorporation did not significantly alter the crystal lattice parameters and the cohesion properties of the mineral crystals, which displayed the properties of a "young" bone.

As reviewed by Comar and Wasserman, calcium was more effectively transported than strontium across a biological membrane (*305*). A few soft tissues from the rat also revealed higher uptake of ^{45}Ca than of ^{85}Sr *in vitro*, but the discrimination was much less pronounced in bone (*306, 307*). Following repeated injections, similar ^{89}Sr/^{45}Ca ratios were reported in rat bone (*308*). Some discrepancies between results regarding strontium vs. calcium discrimination could be due to: 1) differences in strontium and calcium transport from blood into bones; 2) differences in the composition of bone interstitial fluid and the experimental solution used *in vitro*; and 3) stronger binding of plasma protein to calcium than strontium. In the kidneys and intestine, the preference of biological membranes towards calcium has been demonstrated clearly (*305*), but it is less clear for the transport from blood into bone.

Five different factors affecting the strontium incorporation into the skeleton have been identified from animal studies: dose, plasma strontium level, gender, duration of treatment and skeletal site. Dose-dependent increase in bone strontium have been verified (*309*). The bone strontium content increased proportionally to the administered dose after four and eight weeks of strontium ranelate treatment (35–140 mg Sr^{2+}/kg per day). However, the

strontium content in bone reached a plateau level at higher dose levels (*310*). The most likely explanation for the non-proportional correlation between strontium dose and bone strontium level appears to be the saturation of the gastrointestinal absorption mechanisms (*308*).

Intestinal absorption discriminates calcium and strontium, by favoring calcium (*311*). It has been shown in humans that strontium gastrointestinal absorption takes place partially via an active transport mechanism that involves a calcium-binding protein (*308*). Although a nonlinear relationship has been shown between the plasma strontium levels and the administered dose, there was a linear correlation between plasma concentration and strontium incorporation into the bone after oral strontium treatment (34 to 255 mg/kg/day of strontium) for 26 weeks (*312-314*).

No gender discrimination in strontium concentrations in bone or plasma was shown in rats (*315*). However, after oral strontium ranelate administration, female rats acquired less bone strontium than the male rats. However, this difference was less evident in monkeys (*313, 316*). Apparently, the higher strontium concentrations found in male rats are caused by higher strontium gastrointestinal absorption. So far, no evidence of such gender discrimination has been found in humans.

Incorporation of strontium into bones is also dependent on the length of treatment (*309, 310*). Although from 10 to 25 days of strontium ranelate treatment the strontium content in rat femur doubled (*310*), the increase was less significant from days 28–56. In rat, bone strontium content might have approached a plateau level after four weeks of treatment. On the other hand, the plasma strontium levels were almost constant after 10 and 29 days of treatment (*310*), which indicates that plasma level had plateaued after 10 days.

Strontium incorporation into bone also depends upon the skeletal site. The diaphysis of the femur always contained less strontium than the lumbar vertebra, which contained less strontium than the iliac crest in animal experiments (*312-314*). Bone mineralization is mainly determined by the bone turnover rate (*317*). The distribution of strontium depends on the relative proportions of cortical and trabecular bone in the skeleton. Since bone turnover is higher in trabecular than in cortical bone, newly formed bone is more abundant in trabecular

than in cortical bone (*302*). In strontium-treated monkeys, the bone strontium content was lower in the lumbar vertebra than in the iliac crest (*312-314*), which can be explained by the lower bone turnover and regional blood flow in the lumbar vertebra compared to the iliac crest.

5.3. Absorption

In many organs, common transport paths for calcium and strontium have been described. They compete for intestinal absorption, renal tubular reabsorption, etc. Under normal physiological circumstances, discrimination occurs between calcium and strontium for the following functions: gastrointestinal absorption, renal excretion, placental transfer, and mammary secretion (*318*). Calcium is transported more easily than strontium whenever there is an active transport across those biological membranes (*14*).

In the body, the gastrointestinal tract is the main route of entry of strontium. Papworth *et al.* proposed two routes of entry for strontium: carrier- and diffusion-mediated (*319*). The way strontium and calcium are absorbed from the ingesta can partly explain the preferential intestinal absorption of calcium over strontium. Two factors in the transcellular path of absorption that are involved in calcium transport, the high-affinity calcium-binding protein and the plasma membrane ATP-dependent calcium-pump, are parts of the discriminatory process (*320*). Binding of calcium to each of these proteins is considerably greater than that of strontium (*321, 322*). In addition to that, calcium is also transferred across the intestinal membrane by a paracellular, diffusional-type process that also appears to give preference to calcium over strontium (*320*). However, some studies showed that strontium is absorbed entirely via passive diffusion or paracellular transport (*323, 324*). In general, strontium is not as well absorbed as calcium, the strontium/calcium absorption ratio being roughly 0.50 (*325*). The preferential absorption of calcium might partially be attributed to the relatively smaller size of the calcium atom.

Strontium absorption is greatly dependant on the food matrix. Since the focus of earlier research works on strontium was reduction of consumption of radioactive strontium,

food items are categorized based on the fractional intestinal absorption of the ingested activity (f_1 value). Hollriegl *et al.* conducted a study to assess if the f_1 value corresponds with the actual strontium absorption from food items by using dual-isotope technique (*326*). They found f_1 values that ranged between 0.29-0.59 in various food and drink items. This finding was independent of age and gender. Strontium intestinal absorption increases under fasting conditions and is reduced by the presence of high dietary calcium and phosphate, or chelating agents (*327, 328*). Strontium intestinal absorption also gradually increases during pregnancy and lactation. At the end of the lactation period, it reaches a maximal fractional absorption (*329*). The strontium gastrointestinal absorption depends largely on age. It varies between 10% (in the elderly) to 90% of the dietary intake (in infants) (*330*). However, within the same age group, inter-individual variations have also been observed, which are attributed to the actual functional state of the gastrointestinal tract (*331*). In addition to the gastrointestinal pathway, strontium can also be absorbed via the lung and skin (*330*).

5.3.1. Dietary Factors Enhancing Absorption

A study in a rat model found a decrease in the quantity of calcium and strontium in the feces and an increase in the urinary excretion of these minerals in ***vitamin D***-treated animals (*332*). In this study, it was estimated that in the rachitic rats dosed with calcium and strontium orally about 40% of the calcium and 45% of strontium passed through the gastrointestinal tract unabsorbed, while in the vitamin D-treated animals only about 15% of the calcium and 30% of the strontium passed through unabsorbed. The bones of the vitamin D-treated animals also retained a larger proportion of the metal ions than those of the untreated rats. If the only action of vitamin D was to promote absorption of calcium from the digestive tract, the rachitic animals should retain as much of the injected calcium and strontium in the bone as do the vitamin D-treated rats. However, this was not the case; the injected animals in a state of healing accumulated nearly twice as much calcium and strontium in the bone. In the vitamin D-deficient animals more of the injected calcium and strontium were excreted in the urine. This finding has shown that vitamin D has a direct

action on the mineralization of bone in rachitic animals, besides promoting healing indirectly by increasing the absorption of these minerals from the digestive tract.

Lactose has been reported to improve calcium and strontium absorption in the intestine (*333-338*). However, a few studies have shown little statistically significant differences in the absorption and utilization of calcium from lactose-containing dairy products and from calcium supplements (*339-342*). Differences in the physiological state of animals or human subjects, including the level of calcium intake, age or maturity, the level of intestinal lactase, might have led to controversies (*343-346*). Although much attention had been given to the interrelationship between lactose and calcium metabolism, the mechanism is still not confirmed. A popular explanation is based upon the fermentation of the poorly absorbed lactose by intestinal bacteria and the subsequent favorable effect of a lowered pH on calcium absorption (*347*). However, many of the sugars that were attacked with difficulty by intestinal bacteria also increased calcium retention (*347-349*). Another theory is that the increased glucose and galactose resulting from the hydrolysis of lactose may improve dissolution of calcium and eventually enhanced its absorption in the intestine (*350*). Due to similarities between strontium and calcium, the effects of lactose are expected to be similar on these two compounds.

Dietary protein has been shown to increase calcium absorption in the intestine (*351*). One possible mechanism is by improving the secretion of gastric acid, which is needed to liberate calcium from the food matrix and allow its absorption (*352*). Besides that, some protein digestion products, like casein, have been shown to directly enhance calcium absorption (*353, 354*). However, the findings on the effect of dietary protein on calcium absorption are still contradicting. A study on healthy young women showed reduced intestinal calcium absorption in the low-protein-diet group and normal intestinal calcium absorption in the high-protein-diet group (*355*). This was in agreement with earlier studies (*356-358*). In contrast, some studies found no differences in calcium absorption between high- or low-protein-diet groups (*359, 360*). These contradictions might be caused by differences in the level of dietary calcium intake (*361*).

5.3.2. Dietary Factors Inhibiting Absorption

The results from the studies of the effects of dietary ***calcium*** on the absorption of ingested radioactive strontium show that calcium significantly affects strontium metabolism (*362, 363*). Higher dietary level of calcium causes a reduction in strontium absorption (Thompson and Palmer, 1960). However, Wasserman *et al.* pointed out that the increased dietary calcium levels caused a decrease in the total radiostrontium retention because calcium effective diluted strontium (*363*).

Gruden *et al.* suggested that ***phosphate*** acts at two sites in the body; the gastrointestinal tract – reducing absorption, and in the kidney – diminishing the strontium and calcium excretion in urine (*364*). They also suggested that it is very likely that the complexing of calcium and strontium by phosphates is responsible for this action of phosphates. High phosphate diets or phosphate infusions cause a rise in the concentration of inorganic phosphates in serum (*365, 366*) and a fall in plasma total and ultrafiltrable calcium (*365, 367*). This is followed by a simultaneous fall in urinary calcium elimination (*365, 367, 368*). Results from an earlier study show a difference in the phosphate action on calcium and strontium metabolism (*369*). Vojvodic showed that phosphates selectively affect strontium absorption, due to the formation of strontium phosphate which is less soluble than those of calcium (*370*). Hence, phosphate seems to discriminate calcium and strontium in favour of strontium in the absorption process.

5.4. Excretion

Strontium is mainly excreted in urine and, to a smaller extent, in feces and sweat. Typically, the total clearance in adult varies between 9.4 and 11.7 mL/minute, while the renal clearance varies between 4.0 and 5.4 mL/minute (*308*). Data from a “reference” man have described strontium balance as follows: dietary intake (1.9 mg/day), urinary loss (0.34 mg/day), loss in faeces (1.5 mg/day), loss in sweat (0.02 mg/day) and loss in hair (0.0002 mg/day) (*300*). In subjects receiving an intravenous injection of $SrCl_2$, the urinary to faecal

excretion ratio was observed to be three (*331, 371-374*). Thus, urine seems to be the major excretion route of absorbed strontium. Although strontium and calcium share a common tubular transport path in the renal tubules (*375*), the renal clearance of strontium is around three times that of calcium. This is perhaps due to smaller tubular reabsorption, as a result of larger size of the strontium atom than that of calcium (*376*).

Faecal excretion of strontium indicates the presence of a mechanism to transfer absorbed strontium into the gastrointestinal tract, either from the bile or directly from the plasma. Singer *et al.* compared the biliary excretion of calcium and strontium and their excretion into different parts of the gastrointestinal tract in a dog model (*377*). Calcium and strontium were excreted into all parts of the tract and roughly 20% more strontium was found than calcium. Approximately 75% of excreted strontium and 50% of excreted calcium was found in the small intestine. While for the large intestine, the values were about 20% for strontium and 40 % for calcium. About 6-9% of the calcium and strontium excreted into the tract was assumed to originate from the bile.

An increase of dietary phosphorus reduced the urinary excretion of calcium and strontium. However, it seems to increase the faecal excretion of both elements. The kidney is believed to play an important part in the overall discrimination between calcium and strontium, and that the discrimination between calcium and strontium in the kidneys may be associated with the dietary phosphorus level (*369*).

5.5. Homeostasis

Calcium concentration in plasma is tightly regulated between 1.1 and 1.3 mmol/L (*378*). Calcium homeostasis is maintained at three sites: the kidneys, bone and gastrointestinal tract (*296*). Homeostasis is regulated directly or indirectly by PTH, which is one of three major calciotropic hormones that are involved in calcium homeostasis besides calcitonin and calcitriol (1,25-dihydroxyvitamin D_3). Its production and secretion is regulated by plasma calcium concentration. A drop in calcium concentration results in PTH secretion to restore plasma calcium concentrations. On the other hand, a raise in plasma calcium concentration

inhibits PTH secretion. Calcitonin is a hormone that acts to reduce plasma calcium, opposing the effects of PTH. Calcitriol is an active form of vitamin D. It controls the levels of PTH and calcium in the body to prevent low plasma calcium. Hence, high level of vitamin D in the diet or supplements might lead to increase plasma calcium.

Low plasma calcium concentrations will increase neuromuscular excitability (*298*). If it becomes too low, hypocalcaemic tetany may occur. This will cause paraesthesia (itchy, tingly skin), laryngeal spasms, respiratory arrest and cardiac arrhythmia, which can be fatal. On the other hand, excessive amounts of calcium in plasma will result in hypercalcaemia, which can be characterized by anorexia, nausea, vomiting, muscle weakness, itchiness, extreme thirst and liquid excretion (*298*). If not treated, kidney failure may also occur. Since the focus of this thesis is on calcium and strontium supplementation and not on deficiency, only hypercalcemia will be discussed in this section in more detail,

Aortic valve calcification (AVC) formation and progression may be affected by several factors, including endothelial disease, inflammation, and atherosclerosis (*40*). In addition, calcific valvular and vascular diseases have been suggested to share similar characteristics to bone remodelling (*379*). Hence, it became a concern if supplemental calcium intended to improve bone density may result in enhanced aortic valve and vascular calcification.

The pathophysiology of AVC can be separated into two phases (*380*). The initial phase is characterized by inflammation, deposition of oxidized lipoprotein, and co-localization with deposition of calcium (*381*). It is similar to the early aortic valve lesion pathology and it is also a characteristic of atherosclerosis. The advanced phase is characterized primarily by calcium ossification (*382*), which happens at a rate that is dependent on calcification load (*383*). Calcium accumulates faster as the calcium load goes higher (*380*). Thus, any intervention that influences aortic calcium load may have significant clinical implications.

Vascular calcification may be initiated at two different sites: atherosclerosis at intimal location and vascular ossification at medial location (see Figure A-6). Atherosclerosis is characterized by a series of events, which include endothelial dysfunction, intimal edema, foam cell formation and migration of leukocytes and macrophages culminating in the rupture

of a plaque followed by thrombus formation (*384*). Atherosclerotic lesions are normally formed in curved arteries and areas closed to 'junctions', such as in the opening of carotid arteries, lumbar aorta and coronary arteries, in which the blood flow may be hemodynamics. Arterial calcification can take place without (medial calcification) or within (neo-intimal calcification) atherosclerotic lesions. Medial calcification is most common in the femoral arteries (*385*).

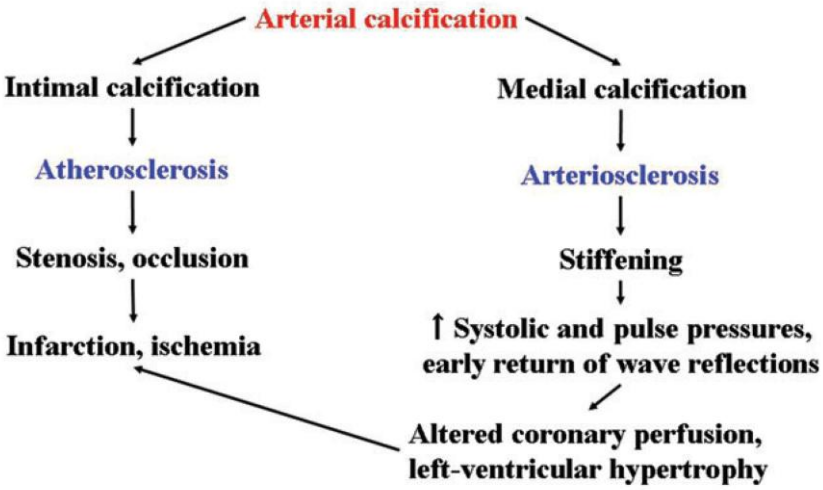

Figure A-6: Clinical effects of arterial calcification. Figure is taken from Nitta, 2011 (*386*).

Generally, biomineralization only occurs when type I collagen and alkaline phosphatase are present in the same tissues. Co-expression of these two proteins is adequate to cause ectopic calcification (*387*). In calcific atherosclerosis, type I collagen and alkaline phosphatase co-localize with mineral deposits (*388*), and are produced by vascular cells *in vitro* (*389, 390*) whereas in normal artery wall, only type I collagen, but not alkaline phosphatase, is present. Crystallization may initiate within extracellular membrane vesicles, such as matrix vesicles or apoptotic bodies, which provide a micro environment rich in calcium and phosphate and transfer membrane-bound alkaline phosphatase (*391*). Usually, such crystalline matrix vesicles are adjacent to apoptotic cells within atherosclerotic plaques (*392*) and are able to concentrate calcium and phosphate and start new crystal formation (*393*). Nevertheless, medial calcification is often related to elastin, a major component of the vascular wall, instead of type I collagen (*394*). Elastin is present in atherosclerotic plaques

(*395, 396*). Elastin fibers or their degradation products may promote early crystal initiation (*395, 396*). Thus, although still related with chondro-osseous conversion of vascular cells and the expression of regulatory proteins like osteopontin (*397*), vascular elastin calcification might involve distinctive regulatory pathways not present in bone.

There are two different hypotheses regarding arterial calcification process (*398*). The presence of osteoblast- and osteoclast-like cells as well as the secretion of several bone-related proteins in lesions affected by arterial calcification support the hypothesis that arterial calcification is an active process (*385*). Both calcification of complex atherosclerotic plaques and medial arterial calcification include many complex bone-synthetic pathways (which are similar to skeletal osteogenesis) in the vessel wall, which involves a range of genes and proteins that involve in mineral metabolism. Various bone-related proteins, including osteonectin, osteopontin, PTH, PTH-related peptide, osteoprotegerin, and bone morphogenic protein, are present in atherosclerotic plaques and in sites of medial arterial calcification (*399*). The passive hypothesis is based on the concept that when the threshold for physiological calcium phosphate solubility is exceeded, they will physiochemically precipitate in areas of advanced tissue degeneration or necrosis within the vascular wall (*400*).

In normal condition, mesenchymal stem cells differentiate to adipocytes, osteoblasts, chondrocytes, and vascular smooth muscle cells (VSMC). A few conditions, including hyperphosphatemia, uremia and hyperglycemia can initiate the transformation of VSMC to a chondrocyte or osteoblast-like cells. These osteo/chondrocytic-like VSMC can be calcified and collagen and non-collagenous proteins can be laid down in the blood vessels. This process is enhanced in a setting of high calcium and phosphorus, and abnormal bone remodeling, increasing the risk of vascular calcification. Eventually, calcium and phosphorus will be incorporated into matrix vesicles, which will initiate mineralization. This site can be further mineralized into hydroxyapatite. However, the presence of the inhibitors present in the circulation (fetuin-A) and in the arteries (such as pyrophosphate, matrix Gla protein and osteopontin) will decide whether artery calcification occurs or not in the end (*17*).

As observed in some studies, an increased risk for cardiovascular events was only correlated with the use of calcium supplements, but not consumption of dietary calcium (*37-39*). This could be due to the sharp increase in serum calcium which has been detected after consumption of calcium supplements but not after consumption of calcium-rich foods (*401*). Some studies have shown a positive correlation between serum calcium levels and vascular calcification (*402, 403*). High serum calcium levels might cause this pathological change by affecting calcification modulators such as pyrophosphate and binding to the calcium-sensing receptors on VSMC (*404*). Other studies have reported positive correlations between serum calcium levels and some predictive biomarkers of cardiovascular disease, including fasting insulin and lipid measures (*405, 406*) and with myocardial infarction risk (*407-409*). A close relationship was also observed between PTH, a key regulator of calcium metabolism, and cardiovascular risk (*410, 411*).

Conversely to calcium, strontium is not under homeostatic control, meaning its total amount in the body and its level in biological fluids is not maintained by a feedback mechanism (*14*). Nevertheless, this does not eliminate the possibility that strontium levels can be influenced by calcium and hormones. In mammals, extracellular fluids have only micromolar concentrations of strontium but they have millimolar concentrations of calcium. Hence, strontium is unable to compete with calcium, and there is little need for strontium specific mechanisms (*300*). Strontium also appears to be less biologically active than calcium, and its toxicity is not pronounced. It is possible that intracellular strontium is more strictly regulated than extracellular strontium, but little is known about this.

CHAPTER 6: STRONTIUM AND BONE HEALTH

6.1. Diagnostic Applications

6.1.1. Use of Strontium as a Surrogate Marker or Tracer for Calcium

As there is no long-living gamma-emitting radioisotope of calcium, a substitute is required for long-term studies. Because of the chemical similarity of calcium and strontium, radioactive strontium has been used earlier as a tracer in calcium metabolic studies. However, with the advancement in the area of mass spectrometry, stable strontium isotopes have been used in place of radioactive strontium. In general, earlier studies have observed preferential absorption of calcium by the intestine as well as re-absorption of calcium by the kidneys. However, the preferential absorption and re-absorption are regulated and can be defined by certain factors (*325, 375, 412, 413*). In the skeleton, the discrimination between calcium and strontium was negligible and non-measurable (*304, 414, 415*). Nevertheless, it has to be noted that this was derived from short-term studies, which might overlook the discrimination between the two elements in the long-term.

Cohn *et al.* conducted a short-term study using ^{85}Sr and ^{47}Ca on male subjects and developed a three-compartmental model (*416*). This study has demonstrated that the differences in the parameters for both strontium and calcium were small and strontium-derived values did reflect calcium metabolism quite well during the study period. A series of older studies (*417-419*) and another more recent short-term study using stable isotope (*420*) have confirmed this finding. However, the long-term data required to assess the slow-exchange rate from bone as well as the difference in the turnover and resorption of strontium and calcium were not available.

Another study using ^{85}Sr and ^{45}Ca administered intravenously to cancer patients found that the isotopes were almost equally distributed between bone and soft tissue for the first few days after the tracer administration. After four months, the majority of the isotopes (99.5%) which were retained by the body were found in bone (*421*). The net retention of the isotopes appears to level off at about 60% for ^{45}Ca, and 25% for ^{85}Sr, with a steady increase in

the $^{45}Ca/^{85}Sr$ ratio in bone. In a more recent study, Rokita *et al.* showed that strontium passively followed the route of calcium in bone minerals (*422*). They concluded that strontium may be applied as an adequate tracer for studying the mechanism of calcium uptake by the skeleton. At selected parts of the skeleton, the *in vivo* measurements of strontium content may be used to determine the rate of the formation of bone minerals.

A study by Reeve *et al.* suggested that if there is any differences in the *in vivo* uptake and release by the skeleton of strontium and calcium, they are small and indistinguishable without more advanced techniques for their measurement (*414, 423*). Reeve and Hesp have suggested that both calcium and strontium tracers are released from the skeleton by resorption and diminution (*414*). It seems unlikely that osteoclasts discriminate for or against bone which they are resorbing because it contains strontium. In addition to that, an *in vivo* study by Marshall *et al.* suggested that diminution of the two tracers were similar (*415*). Lastly, if the two tracers are resorbed at the same rate, and their rates of accretion are also the same within measurable limits, their rate of removal by other processes such as solid state diffusion should also be identical. It therefore appears that strontium is an adequate and suitable tracer for the study of long term skeleton kinetics provided appropriate measures are taken to allow for the differences in the handling of calcium and strontium by tissues other than bone. This has been confirmed by a later study by Reeve *et al.* (*304*).

Walser and Robinson (*375*) examined Sr/Ca relationships in the kidneys and developed a mathematical model to establish a correlation between the clearance of these cations. In their approach, the rate of reabsorption of calcium and strontium from tubular fluid in the nephrons is assumed to be directly correlated to their concentration in that fluid. On the basis of that assumption, they came up with the equation $(Sr_u/Sr_f) = (Ca_u/Ca_f)^{0.7}$, where Sr_u and Ca_u represent strontium and calcium excreted in urine, and Sr_f and Ca_f represent filtered strontium and calcium, respectively. The concentration of filterable cations in plasma and the glomerular filtration rate are needed for this model. Based on the equation, the reabsorption rate of strontium is 0.7 of that of calcium.

Staub *et al.* have described a nonlinear compartmental model for strontium based on plasma strontium concentration data from postmenopausal women receiving oral doses of strontium ranelate twice-daily and after discontinuation of the treatment (*424*). The model was in a good agreement with experimental data at the initial stage, including calcium and strontium discrimination, but the experimental evidence was limited. Blumshon *et al.* also indicated that strontium is a useful surrogate for calcium in clinical assessment of the absorbability of calcium from dietary sources (*412*). The preferential absorption of calcium over strontium was reported to be around 1.9. Similarly, Milsom *et al.* determined that the strontium absorption test is useful to assess calcium absorption in patients and a preferential absorption of calcium over strontium was observed to be a factor of ~1.9 (*325*).

Sips *et al.* has also designed a study to determine calcium intestinal absorption using stable strontium (^{88}Sr) (*425*). They administered calcium (enriched with ^{45}Ca) and ^{88}Sr concurrently and assessed the correlation between the intestinal absorption of these two tracers. A good correlation between both elements was observed. They also conducted a preclinical screening on this technique by testing how vitamin D administration, a potent enhancer for calcium intestinal absorption, may affect the absorption of calcium and strontium. Based on the results, it was concluded that the ratio between true absorption of calcium to strontium remain constant (1.7:1) before and after the vitamin D treatment, which validate further the usefulness of this technique under various conditions. The discrimination factor, 1.7, was also in agreement to what has been found earlier by Milsom *et al.* (*325*) and Blumsohn *et al.* (*412*). The same technique has been utilized by Zittermann *et al.* to investigate the difference in the calcium intestinal absorption and urinary excretion between exercise-trained young men and sex- and age-matched men with sedentary lifestyle (*426*).

6.1.2. Use of Strontium to Study Bone Metabolism

Although short-term studies have shown that strontium can be used as a surrogate marker for calcium, its use in the assessment of long-term bone metabolism is still limited to qualitative assessment rather than quantitative. Isotope analysis alone is not able to provide a

true estimate of bone mineralization rate (*427*). However, it is sufficient for clinical applications (*304*).

Using radioactive strontium, Bishop *et al.* found that the cumulative excretion of strontium could be categorized into three stages. In stage 1, a fraction containing approximately 70% of the administered dose was excreted within the first 20 to 30 days. During this stage, kidneys were the major excretion pathway, contributing to about 70% of the total excretion. In stage 2, a fraction containing about 15% of the dose was excreted with a half-life of approximately 50 days. The tracer was excreted almost equally in both urine and faeces. In the last stage, the excretion of the remaining tracer (approximately 15% of the dose) was negligible (*371*). They suggested that these three excretion processes were closely associated with the degree of binding between strontium and the calcified tissue. Hence, the high excretion rate at the beginning might be caused by the relatively high mobility of loosely-bound strontium newly placed in the bone. By monitoring the concentration of the radioactive strontium in plasma and urine for the first two days after tracer administration, it was observed that about 65% of the administered dose was deposited in the skeleton during the first 10 to 12 hours. However, the rate of removal was also fast. At this point, the bone strontium might be freely exchangeable with the calcium of the extracellular fluid. Hence, a substantial amount of strontium was removed, mainly through the kidneys. The next stage represented a tighter binding between strontium and the apatite crystals. Although exchange between strontium and calcium still proceeded, it happened at a much lower rate. The last stage was attributed to a much tighter binding, most likely resulted from penetration of strontium into the crystal lattice, which signified a more ‘permanent’ retention when strontium could only be released through bone resorption.

Strontium isotopes have been used to measure rates of bone deposition in man (*417, 418, 428-431*). Eisenberg and Gordan conducted a study using strontium gluconate on non-patients and patients with various bone health conditions (*419*). The results of their study indicated that nonradioactive strontium can be used to detect alterations of skeletal metabolism in various defined physiological and pathological states.

Palmer and Karagianes administered ^{85}Sr intravenously to beagle dogs to study bone mineral replacement after immobilization (*432*). Radiotracer remaining in the bone was measured and the ^{85}Sr uptake in the bone of the immobilized leg was shown to be up to 400% higher than in the control leg. They also suggested that changes of less than 1% in bone mineral could be detected and located by using this technique. They concluded that the sensitivity, simplicity and minimal radiation dose associated with this technique points towards potential application for use in human.

McCarthy has performed experiments to examine if the short-term exchange of bone mineral could be influenced by perturbation of the energy metabolism of bone cells (*433*). Three radioactive tracers, ^{125}I-albumin, ^{85}Sr, and ^{86}Rb, were simultaneously injected into the nutrient artery of the tibia. Following which, a measurement of the tracers' concentration in the venous outflow from the tibia was carried out for five minutes. Such experiment was carried before and after the administration of potassium cyanide into the bone. Based on the measurements, extraction ratios for ^{85}Sr and ^{86}Rb with respect to ^{125}I-albumin could be calculated. It was observed that net extraction after five minutes of ^{85}Sr was significantly higher. This result has shown that the metabolic activity of bone cells could significantly affect the efflux of ions from exchangeable mineral.

Shimmins and Smith did a long-term study of ^{85}Sr retention in patients with osteoporosis and Paget's disease for periods of up to 160 days (*427*). Together with the data obtained from autoradiography, this finding strongly suggested that the administered tracer was exchanging back from both diffusely-labeled fully mineralized bone as well as the incompletely-mineralized sites of active bone formation at comparable rates. They suggested that during active bone formation, no tracer was permanently deposited on one specific site. Additionally, because tracer was deposited in both the fully mineralized and mineralizing bone, the relative proportions of isotope in these different bone compartments could not be calculated. Hence, the bone mineralization rate could not be estimated by isotope tracer studies alone. Nonetheless, these estimates might be useful in clinical practice.

In the 1970's a technique for correction of long-term exchange processes in the kinetic estimation of whole body bone formation rates using strontium radioisotope was developed (*423*), but it was not adopted elsewhere until late the 1980's. The researchers did a study to evaluate the difference between the whole body bone formation and resorption rates measured by kinetic methods and histological indices of bone formation and resorption based on *in vivo* tetracycline labeling of iliac trabecular bone (*434*). They found slight correlations between the results from these two measurements, which could not be explained by the methodological uncertainties alone (*435*). Their other works suggested that a more accurate determination of bone formation rates might be achieved through corrections for long-term exchange and non- or slow mineralizing osteoid surfaces by kinetic (*436*) and histological (*437*) methods, respectively.

To obtain good correlations with histomorphometric data, more recently Eastell (*438*) used ^{85}Sr as a surrogate marker for estimating whole body bone formation and to correct for long-term exchange processes (*434*). Since calcium does not have any gamma-emitting radioisotope with a half-life that is sufficiently long for this purpose, ^{85}Sr seems to be adequate to study the long-term handling of calcium by the skeleton (*304*). The purpose of this study was to validate the use of ^{85}Sr in osteoporosis studies. They observed a strong correlation between the bone-specific marker (Dpd) and long-term exchange parameter. The weakest correlation was found with hydroxyproline (a nonspecific marker of bone resorption).

6.2. Therapeutic Applications

In 1952, Shorr and Carter showed after administering a moderate dose of strontium lactate that the deposition of calcium in bone was greater than total calcium storage when calcium was given without strontium (*15*). This finding was the first one suggesting that strontium could be useful in osteoporosis treatment. In the later years, different types of strontium salts have been explored and strontium ranelate became the most heavily studied. It was finally approved to be used as an osteoporotic drug. It must be noted, however, the

bioactive ingredient that plays a role in modifying bone properties is strontium, regardless of the form of the salts used in these studies,

6.2.1. Strontium Ranelate

Several studies have indicated that strontium ranelate has both anti-resorbing as well as bone-forming capability. *In vitro*, strontium ranelate inhibited bone resorption in mouse calvaria cultures (*439*), reduced osteoclast activity in isolated rat cells (*440*) and suppressed the bone-resorbing activity of mouse osteoclasts (*439*). The amount of osteoclasts in chicken bone-marrow cultures treated with calcitriol was also decreased by strontium ranelate (*439*). In short, inhibition of bone resorption *in vitro* by strontium might partially be a result of inhibition of osteoclast activity, but it is not completely stopped. Strontium ranelate also has positively affected bone formation *in vitro*. In an osteogenic model of differentiating mouse osteoblastic cells, strontium ranelate increased alkaline phosphatase activity, osteoblast differentiation marker and increased collagen synthesis (*441*). In rat calvaria cell and organ cultures, strontium ranelate enhanced the pre-osteoblastic cell replication leading to enhanced activity of functional cells and synthesis of bone matrix (*442*). These effects were not reproduced with calcium ranelate and were only specific for strontium ranelate. Several mechanisms of action have been suggested for the cellular actions of strontium that are not fully established (*443*). Strontium triggered the calcium-sensing receptor in some cells, causing the activation of inositol trisphosphate production and mitogen-activated protein kinase signaling (*444*, *445*). In bone cells, other receptors sensitive to strontium might also be effective (*446*). Through activation of the extracellular signal-regulated kinase pathway in osteoblasts, strontium also induced cyclooxygenase-2 expression and prostaglandin E2 production (*447*). This might be a part of the bone-forming effect of strontium ranelate.

The anti-resorbing and bone-forming effects of strontium ranelate have also been observed *in vivo*. Strontium improved bone mass in the lumbar vertebra and femur in adult rats by increasing trabecular thickness and number and decreasing trabecular separation in the metaphysis (*448*). These indicated that strontium ranelate enhanced bone strength, as shown

by the mechanical properties of lumbar vertebrae, midshaft humerus and femur, by improving bone microarchitecture (*448*). Plasma alkaline phosphatase activity was also higher in strontium-treated rats, pointing to the bone-forming activity of strontium ranelate (*448*). In normal adult mice, strontium ranelate also improved vertebral bone formation, reduced bone resorption and subsequently increased bone mass (*449*). Similarly, in normal adult monkeys, strontium ranelate was found to reduce bone resorption and to increase mineralizing surfaces (*450*). The available findings support strontium ranelate's anti-resorbing and bone-forming effects *in vivo* result in improved bone mass and strength.

Based on the findings from *in vivo* studies, strontium ranelate was expected to be effective in preventing bone loss in osteoporosis animal models. In ovariectomized rats, strontium ranelate prevented trabecular bone loss by decreasing bone resorption, while maintaining bone formation (*451*). In a model of immobilization-induced bone loss, strontium ranelate prevented the increase in bone resorption and trabecular bone loss (*452*). Strontium ranelate also increased bone-specific alkaline phosphatase levels in plasma and decreased urinary hydroxyproline excretion, pointing to the fact that it has opposite effects on bone resorption and bone formation *in vivo* (*452*). These findings led to the possible anti-osteoporotic effects of strontium ranelate in humans.

The efficacy of strontium ranelate in postmenopausal women with osteoporosis has been assessed in several clinical trials. In a double-blind randomized controlled trial, osteoporotic women received either strontium ranelate (500 mg, 1 g or 2 g) or placebo together with calcium (500 mg) and vitamin D. An annual increase of 7.3% in lumbar vertebrae BMD was observed in women treated with 2 g of strontium ranelate (*453*). Although, overestimation of BMD was expected as a result of increased attenuation of X-ray by strontium (*454*), new vertebral fracture prevalence in the second year was reduced by 44% in patients treated with 2 g of strontium ranelate compared with the placebo group (*453*). This showed the anti-osteoporotic effects of strontium ranelate. The efficacy of strontium ranelate was subsequently assessed in the Spinal Osteoporosis Therapeutic Intervention (SOTI) trial and the Treatment of Peripheral Osteoporosis Study (TROPOS). In the SOTI trial, strontium

ranelate treatment significantly decreased new vertebral and clinical vertebral fracture incidences by 40–50% after one and three years (*184*). Reduced back pain and height loss were also observed. Bone alkaline phosphatase levels (bone formation marker) increased by 8.1%, while telopeptide crosslink levels (bone resorption marker) decreased by 12.2% in strontium-treated patients compared with the placebo group, showing the opposite and concomitant changes in bone turnover markers. After strontium ranelate treatment for three years, no delayed mineralization was observed, which emphasized the safety of strontium ranelate on bone mineralization in osteoporotic subjects (*184*). In the TROPOS trial, the risk of non-vertebral fractures was reduced by 16% in the strontium-treated patients throughout the three year study (*455*). The relative risk for major fragility fractures was also reduced by 19%. In a subgroup of patients with high hip fracture risk, strontium ranelate reduced the risk by 36% over a period of three years (*455*). In general, strontium ranelate was well tolerated. The most common adverse effects, such as nausea and diarrhea, disappeared within the first three months (*184*, *455*). These trials concluded that strontium ranelate has substantial clinical benefits for treating postmenopausal osteoporosis. It is now available in European countries as well as some Asian countries, including Singapore, for osteoporosis treatment.

6.2.2. Other Types of Strontium

6.2.2.1. Citrate

Strontium ranelate is not indicated for use in Canada or the United States. However, other strontium sources, available in pharmacies as nutritional supplements, are promoted to improve bone health (*456*). Daily doses are recommended such that the elemental strontium intake is similar to strontium ranelate. Due to the scarcity of data comparing the bioavailability of strontium ranelate to other salts, the uptake level of these alternative salts remains unknown. Strontium citrate is widely available as an alternative strontium source. However, levels of bone strontium achieved after consumption of this salt are not known and no direct comparison with strontium ranelate has been conducted in humans. An animal study to compare bone strontium levels in rats receiving equivalent strontium amounts in the form

of strontium citrate and strontium ranelate has been conducted (*456*). It was found that both salts were equally effective to deliver strontium to the bone. These data suggest similar pharmacokinetics between strontium ranelate and strontium citrate in the rats.

6.2.2.2. Chloride

Comparison between strontium ranelate and strontium chloride has been carried out in three-month-old ovariectomized rats (*457*). The study showed that strontium uptake in bone of the strontium chloride-treated animals was generally lower than that of strontium ranelate. In a rat study, strontium chloride has been shown to decrease the number of active osteoclasts while increasing osteoid formation slightly (*458*). Strontium chloride has also been shown not to produce deleterious effects on growth and mineral homeostasis. Strontium chloride has been shown to increase bone formation, without causing apparent change in bone resorption, and eventually bring about a 10% increase in trabecular bone volume.

A study by Fisch evaluated the digestive tolerance of strontium ranelate and strontium chloride on cynomolgus monkeys (*459*). Endoscopic examination of the oesophagus, the stomach and the first part of the duodenum was done after fasting. Strontium ranelate did not induce any toxic effect on the oesophagus, the gastric mucosa and the first part of the duodenum. In contrast, acute and superficial damages were observed on all animals receiving strontium chloride, including haemorrhagic and erosive lesions. It was concluded that oral administration of strontium ranelate is safe for the gastric mucosa while strontium chloride induced superficial and reversible lesions.

6.2.2.3. Gluconate

Skoryna conducted clinical studies to determine strontium and calcium levels in 100 subjects who were receiving a regular diet that supplied approximately 1 g of calcium to 2 mg of strontium a day (*460*). Strontium gluconate was administered for periods of at least 3 months to 50 of these patients with various conditions that might be affected by stable strontium: metastatic bone carcinoma, cachexia, postmenopausal osteoporosis and hepatic

cholestasis. No toxic reactions were observed in patients receiving supplemental strontium. On the contrary, subjective improvement was reported. Strontification around metastatic lesions appeared to be related to the inhibition of bone resorption in areas of new bone formation.

6.2.2.4. Lactate

McCaslin and Janes conducted a long-term study in patients with postmenopausal osteoporosis (*461*). A significant clinical improvement was observed in 84% of the patients receiving strontium lactate treatment orally for periods ranging from 3 months to 3 years. A study by Warren and Spencer (1971) also provided data on strontium metabolism using strontium lactate. Daily oral doses of strontium lactate for periods ranging from 24 to 36 days resulted in a positive strontium balance in all subjects. Following a daily intravenous infusion of stable strontium (administered as strontium gluconate) for 6 days, patients kept on a low-calcium diet excreted each day an average of 235 mg of strontium in the urine and 60.2 mg in the feces. This study showed that humans can take in considerable amounts of strontium without any toxic effects and that not all strontium is tightly bound to the bone crystal lattice, as indicated by the high rates of strontium excretion. Once strontium administration is stopped, it is replaced by calcium.

6.3. Toxicity

If calcium intake is low, high amounts of dietary strontium can induce bone changes similar to rachitic lesions in experimental animals (*462-464*). This seems to be caused by impaired intestinal absorption of calcium as well as reduced renal production of 1,25-dihydroxy cholecalciferol. The effects of strontium on intestinal calcium absorption might be caused by the common absorption pathway shared by them, which favors calcium. It has been proven that calcium-binding protein binds strontium to a lesser degree than calcium (*465*), and that strontium inhibits the kidney 1-hydroxylase, impairing the production of 1,25-$(OH)_2$ vitamin D_3 (*466, 467*).

In a study carried out in a region in Turkey, a relationship between strontium concentrations in soil and rickets in childhood was observed (*468*). There was a high prevalence of childhood rickets in that region (32% compared with 4.4% nationally among children up to 5 years of age). In this region, the children's diet is based largely on grains from strontium-rich soils, which were categorized based on their strontium concentration (Group 1 >350 mg/kg; Group 2 <350 mg/kg). The proportion of children with at least one rachitic sign and the severity of disease were higher in Group 1.

Due to the chemical similarity of strontium to calcium, strontium uptake is inevitable. Due to the high calcium requirement during growth period, the absorption and retention of strontium are significantly higher in children. As a result, children experience a greater potential systemic dose from strontium exposures compared to adults (*300*). Numerous laboratory animal studies have demonstrated abnormal skeletal development in young animals subjected to high levels of dietary strontium (*469-472*).

Abnormal skeletal development caused by high exposure to strontium has not been established in adults. In contrast children as well as young animals show more effective strontium absorption from the intestine, which may explain partly why they are more sensitive to excessive strontium intake. Rat studies showed that strontium absorption was four to eight times higher in weanlings than in adults. It is assumed that the absorbed dose may be eight times higher in infants than in adults (*473, 474*). This age-dependent difference in absorption could be partly explained by the duodenal level of vitamin D–dependent calcium-binding protein, which is much lower in mature rats than in young rats (*475*).

Due to their immature skeleton with high bone remodeling rate and strontium's potential to negatively affect bone development, children are particularly vulnerable to excess strontium. Besides, impairing intestinal absorption of calcium as well as renal production of 1,25-dihydroxy cholecalciferol, strontium also binds to hydroxyapatite crystals and possibly interfere with the normal crystalline structure of bone in rats (*469*). In rats, excess strontium may also inhibit the normal maturation of chondrocytes in the epiphyseal plates of long bones (*471*). Excess strontium seemingly interferes with the mineralization of complexed acidic

phospholipids, which is believed to assist in the formation of hydroxyapatite crystals in developing bone (*476*). Consequently, bone will contain an excess of complexed acidic phospholipid and it will have a significantly lower ash weight. This finding is in agreement with the reduced rate of matrix vesicle degradation found in rachitic cartilage of strontium-treated rats (*477*).

Nevertheless, strontium has been used to treat patients with osteoporosis or other skeletal disorders. Typically, the treatments involve administration of "low doses" of strontium, in the form of lactate, gluconate, carbonate or ranelate over several years (*300*). Until recently, no adverse effects were reported in studies on osteoporosis patients. However, based on an evaluation from clinical studies in postmenopausal women, EMA's CHMP has indicated that there is a higher risk of heart attack with strontium ranelate than with placebo. However, there is no observed increase in mortality risk. Thus, it was recommended to limit the use of strontium ranelate (Protelos®, Osseor®) only for treating patients with severe osteoporosis and high fracture risk (*16*) without any cardiovascular problems. Further assessment on this matter has been initiated.

The finding of cardiovascular events with strontium ranelate treatment is not surprising considering the similarities between calcium and strontium. Like calcium, high strontium intakes may increase the risks of soft tissue mineralization by formation of poorly soluble salts. For therapeutic purpose, strontium ranelate is administered at a dose level (500 mg/day of strontium) that is comparable to regular calcium supplementation (500-1,000 mg/day of calcium) which has been suggested to increase the risks of soft tissues calcification (*35*, *38*). In addition to that, strontium ranelate is usually taken together with up to 1,000 mg of calcium supplement and up to 800 IU of vitamin D (*478*). Therefore, taking into account the calcium intake from dietary sources, the overall strontium and calcium intake may approach the UL of calcium (2,000 mg per day) (*479*).

6.4. Strontium as an Essential Element?

Strontium is ubiquitous in nature. The relative abundance of strontium to calcium in the earth's crust is estimated to be 8/1000 (w/w), with some regional variations (*14*). The strontium to calcium ratio in plants could vary between 1.4 and 5.7/1000 (w/w) in England, similar to that of soil (*480*).

Strontium is not an essential element, but it may promote growth in some plants (*481*). A study showed that strontium levels in water, plaque, and enamel is inversely related to caries prevalence in humans (*482*). In rats, moderate doses of strontium also prevented caries (*483*). In experimental animals and osteoporotic patients, strontium has been given to improve bone properties and reduces fracture risk. A certain degree of strontium deficiency might be related to osteoporosis, but the data comparing bone strontium in osteoporotic and normal subjects are very limited. However, iliac crest biopsies of an osteoporotic rabbit model did not show a decrease in bone strontium levels (*484*). Nevertheless, among the trace metals present in human bone, strontium was the only one correlated with improved bone compression strength (*485*).

Generally, the amount of strontium in regular diets is negligible compared to calcium. Based on literature, a regular diet may contain from 0.20 mg/day of strontium to 2.36 mg/day of strontium. In Asia, average daily strontium intakes have been assessed in Vietnam (1.3 mg/day), Philippine (1.1 mg/day), China (2.9 mg/day), Japan (2.3 mg/day), India (1.5 mg/day), Bangladesh (1.8 mg/day) and Pakistan (2.6 mg/day) (*486, 487*). In UK and Finland, average daily intakes of strontium were estimated to be 1.3 mg/day and 1.9 mg/day (*486, 488*), which were comparable with that of Asia. In Germany and Switzerland, the intakes were estimated to be between 1.1 to 4.5 mg/day (*489, 490*). The intake might be boosted by the consumption of strontium-rich water.

Major dietary sources of strontium include cereal (2440 µg/kg), vegetables (530 – 940 µg/kg), meat and poultry (450 ug/kg), seafood (274 µg/kg), full cream milk (180 to 2800 µg/L) and water (20 – 60 µg/L) (*491, 492*). Higher strontium contents in drinking water have been reported in several countries in Europe (10.6 – 12,200 µg/L) (*493*), Riyadh (25.4 – 406.4

µg/L) (*494*) and Japan (1.96-4539 µg/L) (*495*). In Singapore, water is not a relevant strontium source due to intensive water processing and infrequent consumption of bottled mineral-rich water

Overall, the strontium dietary intake is lower by two orders of magnitude than the therapeutic dose of strontium used in osteoporosis therapy (approximately 500 mg/day of strontium in the form of strontium ranelate). Hence, dietary strontium intake is likely to have a negligible effect on bone health.

CHAPTER 7: METHODOLOGY AND STATISTICS

7.1. Mass Spectrometry

Calcium absorption and kinetic studies are evaluated based on the determination of isotope ratios, usually performed by mass spectrometry, which is based on the principle that the different isotopes of an element are physically separated and determined quantitatively (*496*). The element is ionized in the ion source. These ions are then separated by their mass to charge ratio either in a magnetic field (magnetic sector mass spectrometry) or by the application of radiofrequency and constant voltages applied to four parallel rods (quadrupole mass analyzer). Using magnetic sector field technology, ions are accelerated linearly into the sector field and deflected due to their mass to charge ratio, which allows simultaneous ion beam detection. There are two different types of sector field instruments: single- and double-focusing mass spectrometers (see Figure A-7). In a single focusing mass spectrometer, an initially divergent ion beam with a given value of mass/charge ratio is brought to focus. Double focusing mass spectrometers with high resolving power is used for analyses which require high resolution measurements. Their principles are identical to those of single focusing machines, but they include an electric focusing sector field before separation by the magnetic field. In quadrupole mass analyzer, the quadrupole is switched back and forth to select ions with a distinct mass to charge ratio (peak hopping). Using this principle, ions are filtered and not separated, which does not allow for simultaneous ion intensity measurements. In general, quadrupole instruments have lower resolutions and precision compared to magnetic sector instruments and are less expensive (*497*).

After separation, ions can be detected by different systems. For high ion currents, detection with a Faraday cup gives the highest precision. A simultaneously detection of separated ion beams in magnetic sector field instruments is possible by using a multi-collector array of Faraday cups. Secondary ion multipliers such as dynode multipliers or Daly detectors can provide higher sensitivity. Faraday cups and secondary electron multipliers can be used in parallel when extremely low isotope ratios have to be measured.

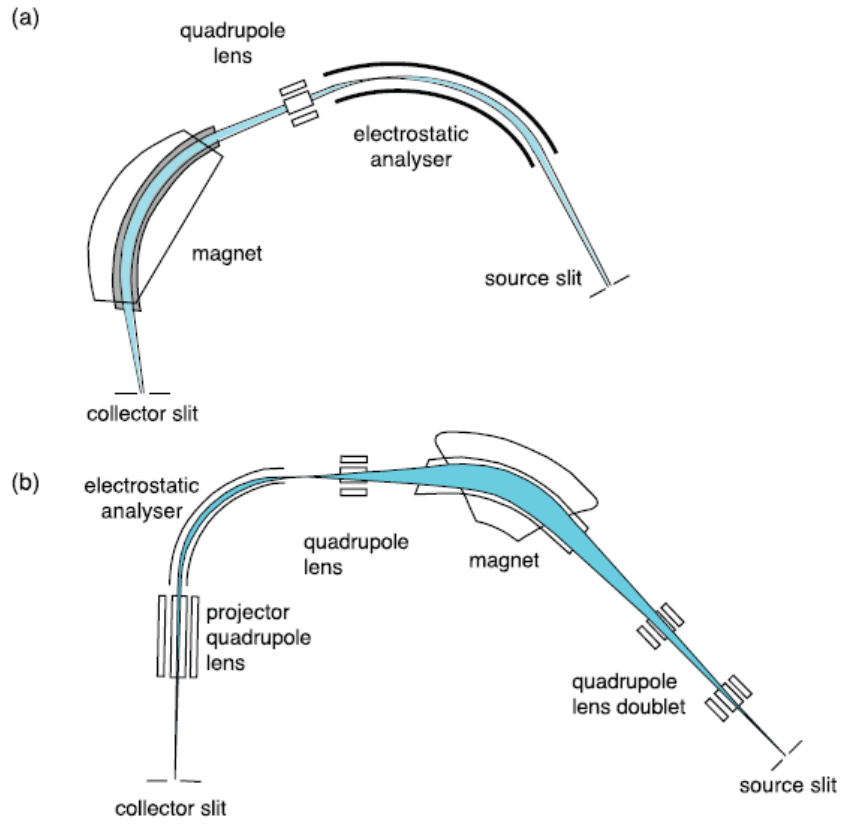

Figure A-7: Double-focusing sector mass spectrometers with different set ups. (a) A forward geometry mass analyzer with electrostatic sector before the magnet and (b) a reverse geometry mass analyzer where the magnet precedes the electrostatic sector. Figures are taken from Ireland, 2013 (*496*).

There are many different types of mass spectrometry. However, we will only focus on the techniques used in this thesis, namely TIMS, ICP-MS and AMS.

7.1.1. Thermal Ionization Mass Spectrometry (TIMS)

In TIMS, atomic or molecular ions are formed on the hot surface of a filament. Owing to its high precision, accuracy and sensitivity, TIMS has become a reference technique for isotope ratio measurements and has been used in geochemistry, nuclear chemistry, in the determination of atomic weights and in nutrition sciences to determine isotope ratios in enriched biological samples.

Before the analysis, biological samples have to be purified and the element has to be separated from organic matrix. Small amounts (usually in ng to µg) of the isolated sample are placed on the evaporation filament consisting of a metal strip made from tantalum or rhenium. The ionization process is usually endothermic and thus ionization efficiency increases at higher filament temperatures. For most elements, the optimum range for filament temperature lies between 800-2000°C (*498*). During the ionization process, only singly charged ions are formed because of the high ionization temperature. Single-, double- or triple-filament technique can be used for the evaporation and ionization of samples. If the single-filament technique is employed, evaporation and ionization take place on the same filament. For elements, which evaporate at relatively low temperatures the double- or triple filament technique is preferred as it allows to create an adequate ion beam at higher temperatures, allowing the creation of a stable and adequate ion beam (*499*). At the moment, single- or double filaments are more commonly used (see Figure A-8).

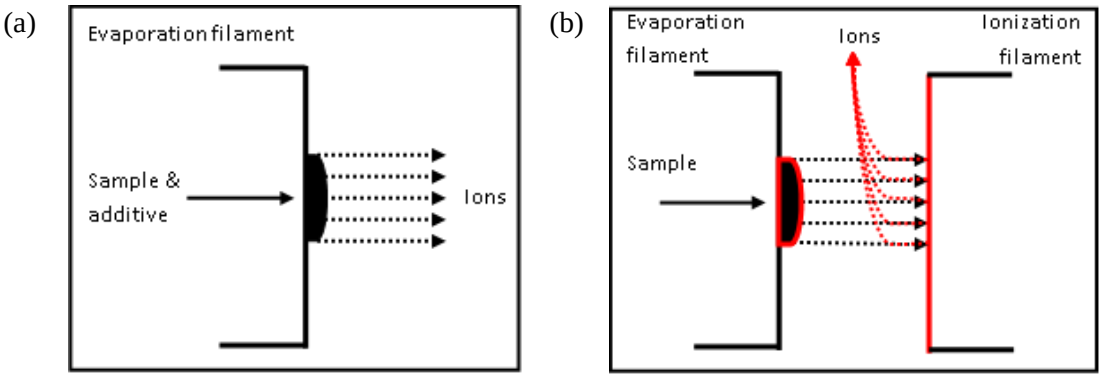

Figure A-8: Commonly used filament assemblies in TIMS. (a) Single-filament, where evaporation and ionization take place on the same filament and (b) double-filament, where evaporation and ionization take place on two separate filaments.

As electron transfer can occur between the analyte and the filament in both directions, both positive and negative ions can be generated. Although positively charged ions are the most common, negative ions can be obtained from high electron affinity atoms or molecules under thermo-ionization conditions (*500, 501*). After leaving the ion-source, ions are focused with several lenses systems, pass the aperture slit and enter the magnetic sector field, where

they are separated by their mass to charge ratio. The selected ions are detected by Faraday-cup or secondary electron multipliers (see Figure A-9). Using a single Faraday-cup, a series of different masses can be measured in sequence using the "peak jumping method". The disadvantages of this method include being time consuming, having small sample throughput, and a reduced internal statistical precision (*276*). Multi-collector systems consisting of several Faraday-cups (up to 9) require wide beam dispersion, but it can improve the precision and shorten analysis time compared to single collectors.

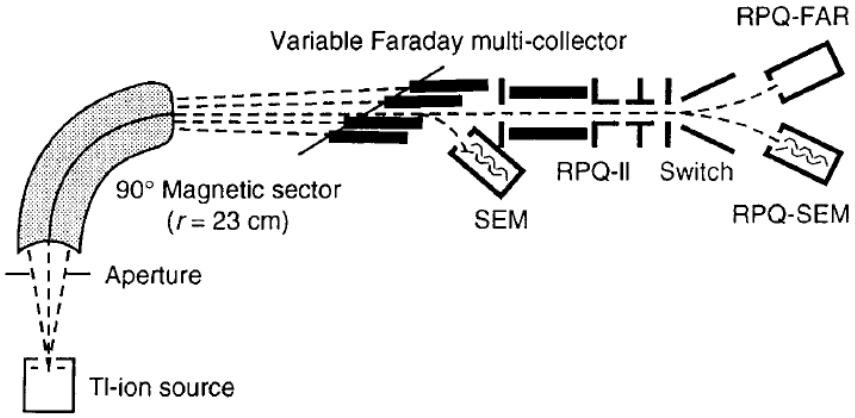

Figure A-9: Schematic diagram of a TIMS instrument with enhanced abundance sensitivity. Ions are form in the ion-source and then focused with several lenses systems, pass the aperture slit and enter the magnetic sector field, where they are separated by their mass to charge ratio. The selected ions are detected by Faraday cups or secondary electron multipliers. Figure is taken from Heumann *et al.*, 1995 (*502*).

For calcium analysis, a double filament technique is usually used. Usually 5-10 μg of a isolated and purified calcium sample is loaded on the evaporation filament as $CaNO_3$ or $CaCl_2$. Typical evaporation temperatures are 1200–1500°C. The exact conditions for analysis depend on various parameters, such as sample loading technique or filament material. The long-term external reproducibility of calcium isotope ratio analysis by TIMS was reported to fall between 0.12‰ and 0.4‰ (*503*). The average precision for $^{44}Ca/^{40}Ca$ ratio analysis in standard reference material (SRM) 915a was reported to be 0.09‰ (*504*). The precision of measurements involving less abundant isotopes, such as ^{48}Ca, can be improved by avoiding

the most abundant isotope, ^{40}Ca. Gopalan *et al.* reported a precision of 0.18‰ for determination of $^{48}Ca/^{42}Ca$ ratios by multi-collector TIMS (*505*).

7.1.2. Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Since ICP-MS became commercially available, it has been used in a large number of human studies (*506*). ICP-MS uses an ion source, which operates at high temperatures (3727-7454˚C) and achieves extremely high ionization efficiency for almost all elements. Compared to other sample introduction techniques, liquid solution nebulization is the most frequently used and most economical. Typically samples in liquid form are introduced by pneumatic or ultrasonic nebulization into the high-energy argon plasma. For direct introduction of analytes in solids, laser ablation with a spatial resolution on the micrometer scale (~ 1 μm) can be used. It is ideal for micro-sampling on surfaces and in-depth profile analysis. It is very useful in some cases, such as in geology for the analysis of rock inclusions (*507*), in analysis of tree rings and biological tissue sections (*508, 509*) or for other microsurface area samplings in material and forensic sciences (*510*) as well as on valuable archaeological objects. There are other types of sample introduction methods (see Figure A-10) that will not be discussed in detail. The sample introduction system, such as laser ablation chamber, is coupled directly via a connection tube to the ICP torch. In the plasma, the samples are ionized (see Figure A-11). The ions are then accelerated into a high vacuum and focused by electrostatic lenses into the mass analyzer. For detection, ions have to be transferred from 5000-7000 K to room temperature and from atmospheric pressure at sample introduction to high vacuum.

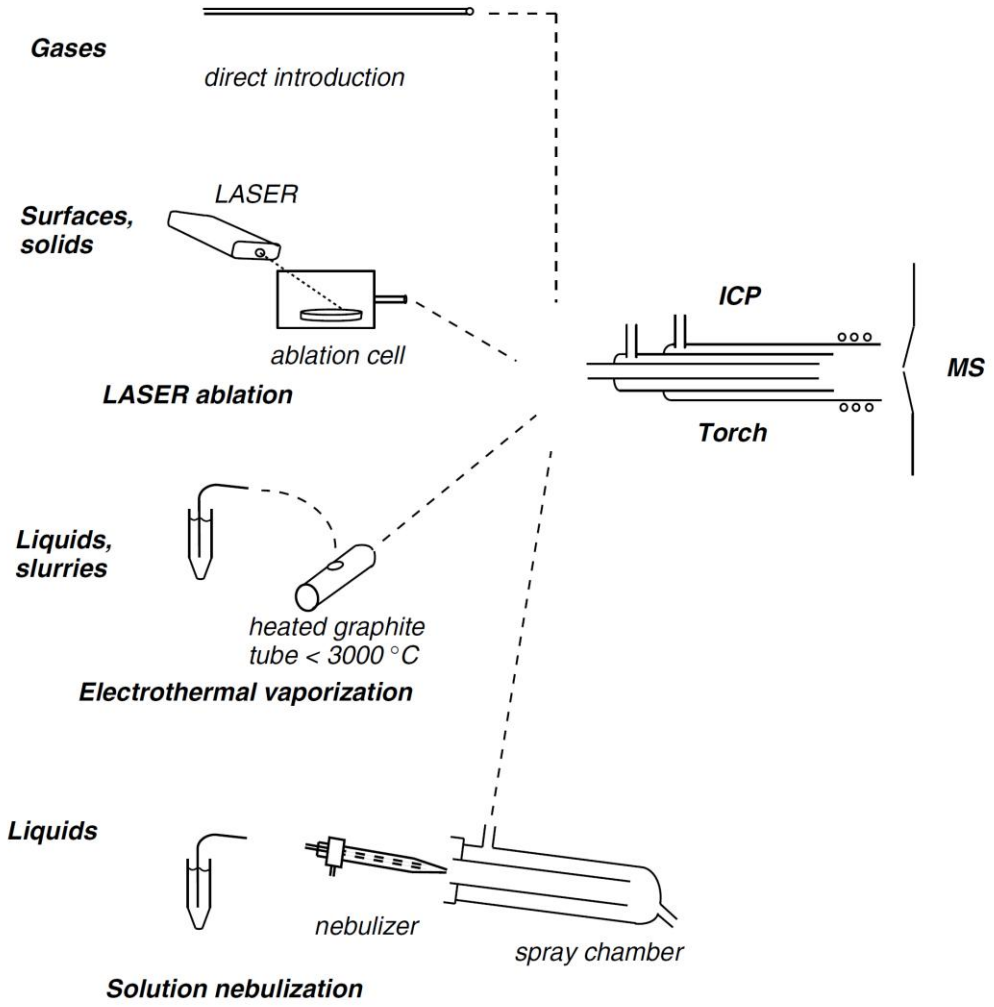

Figure A-10: Principles of sample introduction in ICP-MS. Liquid solution nebulization is the most economical and commonly used technique. However, sample digestion and dissolution is required. Laser ablation allows direct access to analytes in solid samples and on surfaces. It also provides high spatial resolution. Electrothermal vaporization enables *in situ* sample preparation as well as pre-concentration. Direct introduction of samples in gas form is only applicable for volatile compounds. Figure is taken from Ammann, 2007 (*511*).

In quadrupole instruments (see Figure A-12), photons as well as ions are produced in the plasma and have to be removed before entering into the detector. Photons are removed with a photon-stop, which is a small metal plate in the center of the ion beam that reflects the photons. Positively charged ions are guided around this stop by positively charged cylinder lenses and can enter the quadrupole, where they are separated by their mass-to-charge-ratio. Due to isobaric interferences, especially when the analyte is present in a complex matrix, the

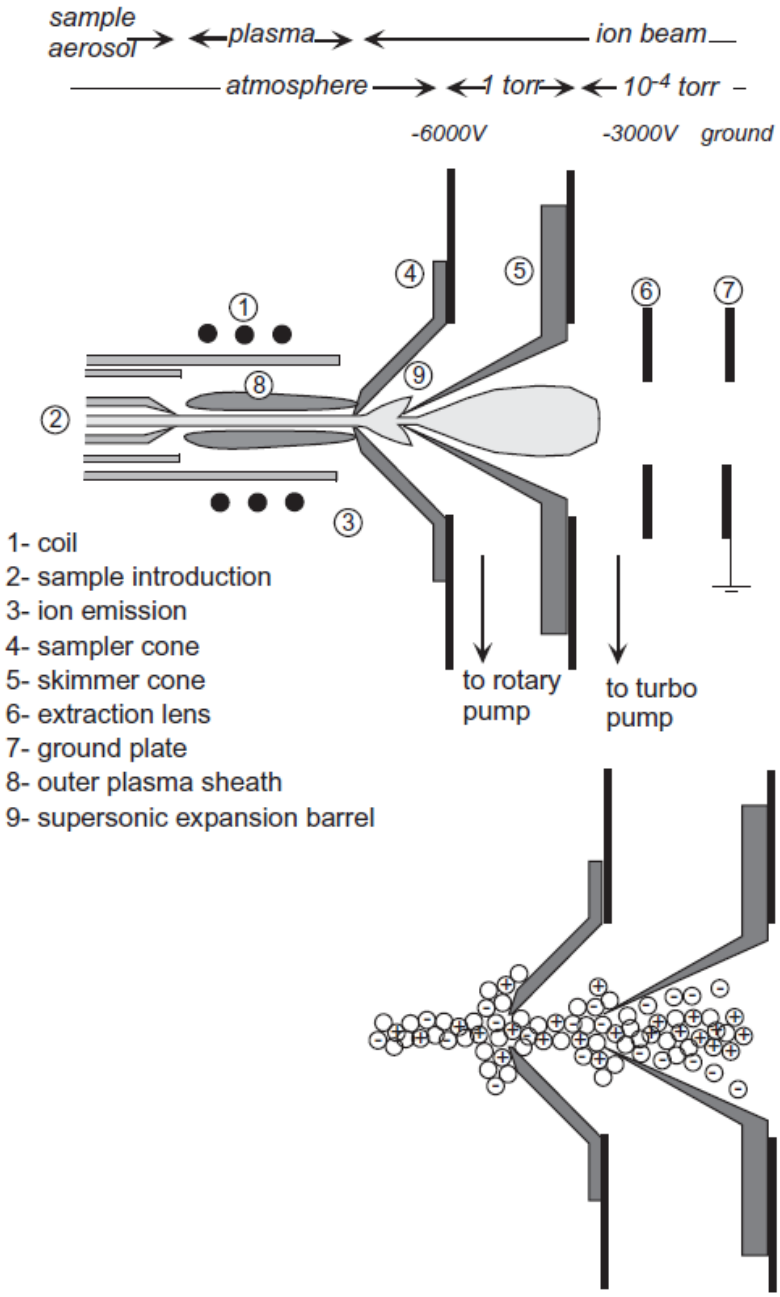

Figure A-11: The inductively-coupled plasma source. The figure shows the electrical potentials, the vacuum cascade and the distribution of ions and neutral in an ICP-MS. In the plasma, the samples are ionized. The ions are then accelerated into a high vacuum and focused by electrostatic lenses into the mass analyzer. Figure is taken from Albarede and Beard, 2004 (*512*).

achievable accuracy and precision for isotopic analysis using a quadrupole separation is limited. However, due to their relative low costs quadrupole instruments are most commonly used. Double-focusing mass analyzes can be used for high precision isotopic analysis as isobaric interferences can be efficiently reduced by this technique.

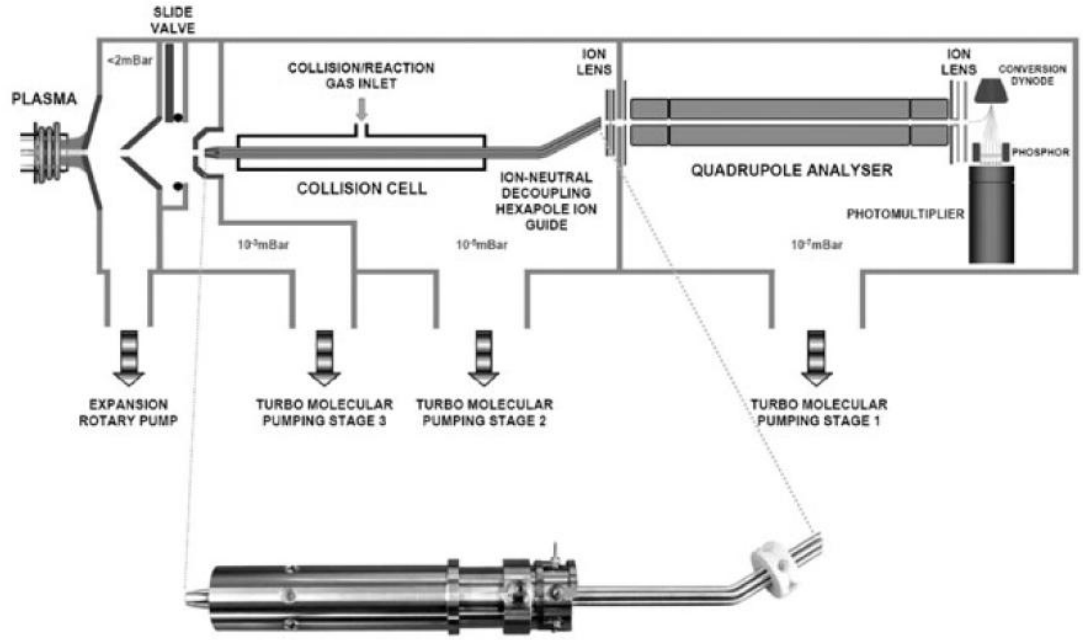

Figure A-12: Experimental arrangement of quadrupole-based ICP-MS. Photons and ions are produced in the plasma. Photons are removed with a photon-stop. Positively charged ions are guided by positively charged cylinder lenses and enter the quadrupole, where they are separated by their mass-to-charge-ratio. Figure is taken from Becker, 2005 (*513*).

Mass bias, which greatly influences the measurement accuracy, can be corrected by measuring a SRM. However, not all elements have such SRM. When laser ablation sample introduction is used, measuring an appropriate SRM that match the sample matrix becomes even more crucial. Other factors that may limit the measurement precision of laser ablation ICP-MS (LA-ICP-MS) include detector dead time, signal stability (plasma instabilities, fluctuation in the laser ablation process and inhomogeneous sample), instrumental background, sample contamination, mass drift, counting statistics, and isotopic interferences (*514, 515*).

The development of multicollector (MC) – ICP-MS has offered new perspectives (*516*). In this technique, the ICP ion source is combined with a sector field mass spectrometer and an array of Faraday detectors. MC-ICP-MS allows simultaneous detection of isotopes and, hence, cancels out any signal variation due to plasma instabilities as well as heterogeneities in the sample. The detection of calcium isotopes by ICP-MS is difficult due to

interference of an intense argon peak with ^{40}Ca at mass-to-charge-ratio of 40. MC-ICP-MS instruments cost more than conventional ICP-MS and comparable to TIMS (*506*). For calcium analysis, a measurement repeatability of 0.07% RSD with MC-ICP-MS has been reported (*517*) whereas with the conventional quadrupole ICP-MS a precision of 0.25% RSD could be achieved (*518*).

7.1.3. Accelerator Mass Spectrometry (AMS)

AMS is one of the most sensitive measurement techniques, especially for some radionuclides: ^{3}H, ^{10}Be, ^{14}C, ^{26}Al, ^{36}Cl, ^{41}Ca, ^{59}Ni, ^{99}Tc and ^{129}I. With the exception of ^{3}H (half-life: 12.25 years), the half-lives of these radionuclides are in the range of 10^{3}–10^{7} years and they are very rare in nature (*519, 520*). The main purpose of AMS was to overcome the basic limitations of decay-counting and conventional mass spectrometry. In principle, it determines the ratio of the rare isotope to an abundant isotope of the same element, e.g. $^{41}Ca/^{40}Ca$. This is accomplished by accelerating ions of these isotopes. After which, the rare isotope is counted individually, while the abundant isotope is measured as an electrical current in a Faraday cup (see Figure A-13).

The measurement of isotope ratios in the range of 10^{-10}-10^{-12} is only possible with an efficient suppression of molecular and isobaric interferences. This is performed by acceleration of the negative secondary ions with a tandem accelerator after making a first mass analysis. Within the mass analysis, the main isotope is separated from the rare isotope. After that, the remaining negative ions are accelerated to the positive high-voltage terminal of the tandem accelerator where they encounter either a thin carbon foil or low-pressure gas. In the accelerator terminal, the ions are stripped of electrons and converted to positive ions, and further accelerated to ground potential. During this conversion, molecular ions, which would be a major source of interference, are destroyed. The higher energy which was gained by accelerating the ions is used to suppress isobaric interferences using detection systems, such as gas ionization chamber, gas-filled magnet, etc. Another advantage of the higher beam

energy is that cross sections of scattering processes of residual gas atoms, which can cause a background in the detector, are smaller (*521*).

The potential of AMS to become a routine research tool is limited due to the small number of physics laboratories that are able to perform AMS measurements at ultra-trace levels.

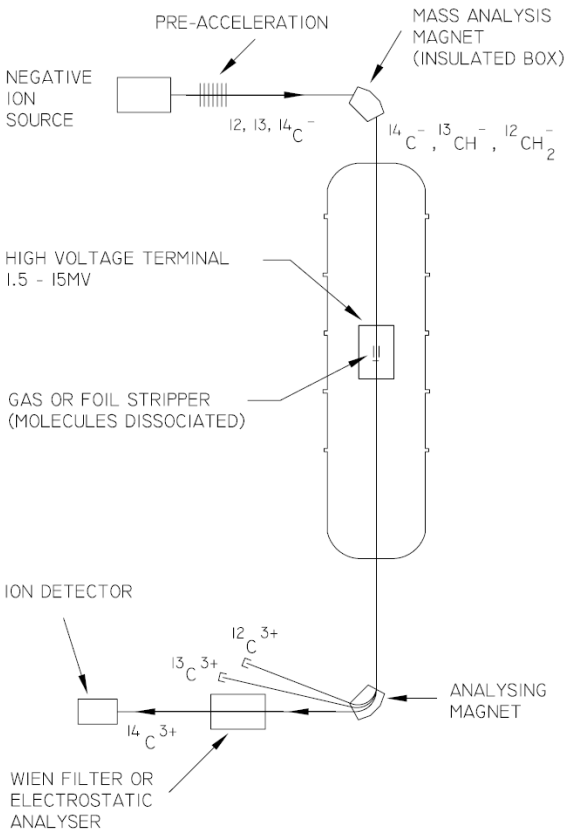

Figure A-13: The essential features of an AMS system. Carbon isotopes are used for illustration. Ions of these isotopes are accelerated. The rare isotope is then counted individually, while the abundant isotope is measured as an electrical current in a Faraday cup. Figure is taken from Fifield, 1999 (*521*).

7.1.4. Isotope Dilution Mass Spectrometry (IDMS)

IDMS is a microanalytical quantitative technique that can be used to determine the concentration of a trace element with high accuracy and precision. This technique requires a known quantity of spike, an isotopically enriched solution of an element, to determine the unknown amount of the same element in the sample (*249*). After adding a known amount of

spike into the sample and equilibrating the spike with the sample, the amount of the element of interest in the sample can be calculated using this IDMS equation (see Eqn. 2).

$$n_{sam} = n_{spike} \left(\frac{R_{spike} - R_{mix}}{R_{mix} - R_{sam}} \right) \left(\frac{1 + \sum R_{i,sam}}{1 + \sum R_{i,spike}} \right) \quad (2)$$

In Eqn. (2), n_{sam} is the unknown amount of the element in the sample to be determined and n_{spike} is the amount of the spike added to the sample. To calculate the value of n_{sam}, isotope ratio measurements of the pure sample, pure spike and a mixture of both, represented by R_{sam}, R_{spike} and R_{mix} respectively, are required. These ratios have a common reference isotope in the denominator and the enriched isotope is the numerator. $\sum R_{i,sam}$ and $\sum R_{i,spike}$ are the sum of all isotope ratios of the sample and spike, respectively.

In certain cases, for example to study formation, degradation and inter-conversion rates of elemental species, multiple spike technique can be used (*522*). The multiple spike contains a mixture of the species of interest, each one enriched with a different isotope. A known amount of spike can be added into the sample and equilibrated with the sample. After which, the isotope ratio variation with time can be measured.

IDMS can provide high acccuracy and high analytical precision, which requires a mass spectrometer that is able to measure isotope ratios with high accuracy and precision. Once the sample and spike have been equilibrated, quantitative sample handling is not required. If there is sample loss during the preparation steps, a proportional amount of spike will be lost as well. Hence, the ratio of the element of interest in spike to sample remains unchanged. In addition to that, high sensitivity and low detection limits can also be achieved (*276*).

7.2. Statistical Approaches

7.2.1. One-Way Analysis of Variance (ANOVA)

A One-Way ANOVA is a statistical technique to test the equality of means of three or more groups at one time by using variances (*523*). There are a few assumptions that have to

be fulfilled, including: 1) normally or approximately normally distributed populations; 2) independent samples; and 3) equal variances of the populations. The null hypothesis is that all population means are equal, while the alternative hypothesis is that at least one mean is different.

There are a few elements that need to be calculated. Firstly, the grand mean of a set of samples is calculated from the sum of all the data values divided by the total sample size (see Eqn. 3).

$$\bar{X}_{GM} = \frac{\sum x}{N} \tag{3}$$

However, when the individual data is not available, ANOVA can still be performed if the number of samples, the sample means, the sample variances, and the sample sizes are known.

Secondly, the total variation, which is the sum of the squares of the differences of each mean with the grand mean, should be calculated (see Eqn. 4). The total variation comprises of between group variation (see Eqn. 5) and within group variation (see Eqn. 6).

$$SS\ (T) = \sum (x - \bar{X}_{GM})^2 \tag{4}$$

$$SS\ (B) = \sum n\ (\bar{x} - \bar{X}_{GM})^2 \tag{5}$$

$$SS\ (W) = \sum df.s^2 \tag{6}$$

In principle, ANOVA compares the ratio of between group variation to within group variation. When between group variation is much larger than the variation within each group, it indicates that the means are not the same. If the sample means are close to each other, and hence the grand mean, between group variation will be small. There are *k* samples involved with one mean for each sample, thus there are *k-1* degrees of freedom. The variance due to the interaction between the samples is denoted MS (B), Mean Square Between groups, or S_b^2, which is the between group variation divided by its degrees of freedom. Within group variation is the variation caused by differences within individual samples. Each sample is independent; there is no interaction between samples. Each sample has degrees of freedom equal to one less than their sample sizes, and there are *k* samples. Thus, the total degrees of

freedom is *k* less than the total sample size ($df = N - k$). The variance due to the differences within individual samples is denoted MS (W), Mean Square Within groups, or S_w^2, which is the within group variation divided by its degrees of freedom.

The F test statistic is estimated by dividing the between group variance by the within group variance (see Eqn. 7).

$$F = \frac{{S_b}^2}{{S_w}^2} \tag{7}$$

The degrees of freedom for the numerator are the between group degrees of freedom (*k-1*) and the degrees of freedom for the denominator are the within group degrees of freedom (*N-k*).

If the test statistic is greater than the F critical value with *k-1* as the numerator and *N-k* denominator degrees of freedom, the null hypothesis will be rejected, meaning that at least one of the means is different (*524*). Nevertheless, ANOVA does not show where the difference lies. Another test, usually called posthoc test, such as the Scheffe' or Tukey test, is needed for this purpose.

7.2.2. Mann-Whitney U-test and Kruskal-Wallis-H test

The Mann-Whitney test is a non-parametric test, which is usually applied as an alternative to a Student's t-test if the data are not normally distributed (*525*). The null hypothesis is that two populations' medians are the same, while the alternative hypothesis is that they are different.

There are a few assumptions that have to be fulfilled (*525*), including: 1) the observations from the two groups are independent of each other; 2) the responses are ordinal; 3) the distributions of both groups are equal under the null hypothesis; 4) under the alternative hypothesis, the probability of an observation from one population is above an observation from another population is not equal to 0.5.

The test involves the calculation of a statistic (*U*) with a known distribution under the null hypothesis. The first step is to sum up the ranks for the observations which come from

group 1. Where there are tied groups, the rank given is equal to the midpoint of the group. The sum of ranks in sample 2 can then be determined, since the sum of all the ranks add up to $N(N + 1)/2$ where N is the total number of observations. U is then calculated by using Eqn. 8 or Eqn. 9.

$$U_1 = R_1 - \frac{n_1(n_1+1)}{2} \tag{8}$$

$$U_2 = R_2 - \frac{n_2(n_2+1)}{2} \tag{9}$$

where n_1 and n_2 are the sample sizes for group 1 and 2, and R_1 and R_2 are the sums of the ranks in group 1 and 2. It does not matter which of the two groups is considered group 1. When consulting significance tables, the smallest value of U_1 and U_2 is chosen.

For large samples, U is approximately normally distributed and the standardized value z can be calculated using Eqn. 10.

$$z = \frac{U - m_U}{\sigma_U} \tag{10}$$

where m_U and σ_U are the mean and standard deviation of U, is approximately a standard normal deviate whose significance can be checked in tables of the normal distribution. m_U and σ_U are estimated by Eqn. 11 and Eqn. 12, respectively.

$$m_U = \frac{n_1 n_2}{2} \tag{11}$$

$$\sigma_U = \sqrt{\frac{n_1 n_2 (n_1+n_2+1)}{12}} \tag{12}$$

Compared to the Student's t-test, the Mann-Whitney test is more widely applicable. It is especially preferred when the data are ordinal, but the spacing between adjacent values is not constant. In addition to that, in the presence of outliers, the Mann–Whitney test is more robust as it compares the sums of ranks.

The Kruskal–Wallis test is the non-parametric alternative to one-way ANOVA (*526*). The null hypothesis is that the medians of the population are equal, while the alternative is that there is a difference between at least two of them. In one-way ANOVA, the test statistic is calculated as the ratio of the treatment sum of squares to the residual sum of squares. The

Kruskal–Wallis test uses the same approach but instead of the raw data, it uses the ranks of the data (*526*). The test statistic is given in Eqn. 13.

$$T = \frac{12}{N(N+1)} \sum_{j=1}^{k} \frac{R_j^2}{n_j} - 3(N+1) \tag{13}$$

where R_j is the total of the ranks for the j[th] sample, n_j is the sample size for the j[th] sample, k is the number of samples, and N is the total sample size, given in Eqn. 14.

$$\sum_{j=1}^{k} n_j \tag{14}$$

This is approximately distributed as a χ^2 distribution with (k – 1) degrees of freedom. If there are ties, the adjusted test statistic is calculated as given in Eqn. 15.

$$T = \frac{1}{S^2} \sum_{j=1}^{k} \frac{R_j^2}{n_j} - \frac{N(N+1)^2}{4} \tag{15}$$

where r_{ij} is the rank for the i[th] observation in the j[th] sample, n_j is the number of observations in the j[th] sample, and S^2 is calculated by Eqn. 16.

$$S^2 = \frac{1}{N-1} \left(\sum_{j=1}^{k} \sum_{i=1}^{n_j} r_{ij}^2 - \frac{N(N+1)^2}{4} \right) \tag{16}$$

If the null hypothesis is rejected, a least significant difference can be calculated to identify which pairs of treatments differ. If the difference between their mean ranks is bigger than the least significant difference (if the inequality in Eqn. 17 is true), treatments i and j are significantly different at the 5% significance level.

$$\left| \frac{R_i}{n_i} - \frac{R_j}{n_j} \right| > t \times \sqrt{S^2 \left(\frac{N-1-T}{N-k} \right) \left(\frac{1}{n_i} + \frac{1}{n_j} \right)} \tag{17}$$

where t is the value from the t distribution for a 5% significance level and $N - k$ degrees of freedom.

7.2.3. Monte-Carlo Simulation for Estimation of Measurement Uncertainty

Monte Carlo uses random numbers as a mean to calculate something from variables that are not fully random but follow a given probability density function. For instance, let X be a random variable and A be its expected value ($A = E[X]$) (*527*). By generating n

independent random variables with the same statistical distribution, i.e. probability density function, $X_1, \ldots, X_n$, A can be approximated as described by Eqn. 18.

$$A \approx \hat{A}_n = \frac{1}{n}\sum_{k=1}^{n} X_k \tag{18}$$

The *strong law of large numbers* states that as $n \rightarrow \infty$, $\hat{A}_n \rightarrow A$ *(528)*. The X_k and $\hat{A}_n$ are random and might be different each time the program is run. However, the target number, *A*, is not random.

Besides leading to vast improvements in efficiency and accuracy by simulation of experimental processes, Monte Carlo can also be used to assess the order of magnitude of statistical errors, which is the main source of error in most Monte Carlo computations. Estimates correspond to "error bars", which are essentially statistical confidence intervals (*527*).

Assuming *X* is a scalar random variable and A = E[X] is approximated by Eqn. 19.

$$\hat{A}_n = \frac{1}{n}\sum_{k=1}^{n} X_k \tag{19}$$

The central limit theorem (*528*) states that (see Eqn. 20)

$$R_n = \hat{A}_n - A \approx \sigma_n Z \tag{20}$$

where σ_n is the standard deviation of $\hat{A}_n$ and $Z \sim N(0, 1)$. A simple calculation shows that $\sigma_n = \frac{1}{\sqrt{n}}\sqrt{\sigma^2}$ where $\sigma^2 = var(X) = E[(X - A)^2]$. σ^2 can be estimated using (see Eqn. 21)

$$\widehat{\sigma_n^2} = \frac{1}{n}\sum_{k=1}^{n}\left(X_k - \hat{A}_n\right)^2 \tag{21}$$

then take (see Eqn. 22)

$$\hat{\sigma}_n = \frac{1}{\sqrt{n}}\sqrt{\widehat{\sigma_n^2}} \tag{22}$$

Monte carlo data is usually reported in the form $A = \hat{A}_n \pm \hat{\sigma}_n$. The error bar is the interval $[\hat{A}_n - \hat{\sigma}_n, \hat{A}_n + \hat{\sigma}_n]$ while multiplication of standard deviations with a factor *k* $[\hat{A}_n - k\hat{\sigma}_n, \hat{A}_n + k\hat{\sigma}_n]$ allow to obtain confidence intervals of given statistical probability. The central limit theorem shows that use of an expansion factor of k=1 has a confidence of 66% and use of k=2 gives a confidence of 95% (*528*).

Error propagation analysis to obtain combined measurement uncertainties by using the propagation of errors formula requires the computation of derivatives, which can be quite complicated for larger models. However, the Monte Carlo approach only requires a program for computing f(x,p), where x is an independent variable and p is a set of parameters that fit into the model. Previously, this could only be done with advanced computer programming skills. At present, the Monte Carlo approach can even be done with a simple program, such as Microsoft Excel (*529*), that is able to generate random numbers.

7.2.4. Outlier Testing by Regression Analysis

Outliers are data points that do not follow the pattern of the other data points. They can be detected by dedicated algorithms including Dixon's Q-test and Robust Regression Analysis (*530*), which will be explained in more detail as it was the method of choice for the presented work. Dixon's Q-test can only examine if one (and only one) data point from a small set of data can be rejected as an outlier. In this test, a normal distribution of data is assumed. If the outlier is just an extreme point drawn from the tail of a normal distribution, least squares estimate for regression models, which are highly sensitive to and not robust against outliers, may be used. However, if the outlier was caused by non-normal measurement error or other violation of standard ordinary least squares assumptions, using non-robust regression technique would compromise the validity of the regression results.

When plotting data that are correlated with each other, the slope b_{ij} of a line connecting data point (x_i, y_i) and data point (x_j, y_j) is given by Eqn. 23.

$$b_{ij} = \frac{y_j - y_i}{x_j - x_i} \tag{23}$$

Firstly, the median *med* of the slopes of the *n-1* lines that go through data point (x_i,y_i) is estimated. And then the median of the slope medians is calculated for all *n* data points. Consequently, the regression slope b_{reg} is obtained as described in Eqn. 24.

$$b_{reg} = med_i\left[med_{j \neq i}\left(b_{ij}\right)\right] \tag{24}$$

In order to find the intercept of the regression line for all data pairs a_{reg}, for each data point y_i the intercept a_i is calculated (see Eqn. 25), followed by determination of a_{reg} (see Eqn. 26).

$$a_i = y_i - b_{reg} \tag{25}$$

$$a_{reg} = med_i\,(a_i) \tag{26}$$

The regression line for all data can then be calculated by using Eqn. 27.

$$y_{reg}\,(x) = b_{reg}\,(x) + a_{reg} \tag{27}$$

which allows the determination the residual *Res* for each data point (x_i, y_i) as the difference between its measured value and its value based on the regression line (see Eqn. 28).

$$Res(x_i, y_i) = y_i - y_{reg}(x_i) \tag{28}$$

Part B

Experimental Work

CHAPTER 1: ISOTOPIC LABELING OF BONE FOR ASSESSMENT OF CHANGES IN BONE CALCIUM BALANCE: A COMPARATIVE STUDY IN SHEEP USING ^{41}Ca AND ^{86}Sr

1.1. Introduction

Osteoporosis is a skeletal disease characterized by low bone mass and micro-architectural deterioration of bone, causing an increase in bone fragility and vulnerability to fracture (*2*). Apart from affecting quality of life of the individual, economic burdens arising from osteoporosis treatment and disability management are becoming significant. This makes osteoporosis prevention the preferred strategy for moderating societal impact of osteoporosis in the foreseeable future (*3*).

A major hurdle in identifying effective strategies to improve or maintain bone health is the lack of techniques to assess bone response in human intervention trials at high sensitivity (*531*). Effects of diet and life-style are usually small but become relevant as they may accumulate over a life-time. Radiometric techniques for measuring bone mineral density (BMD) and content (BMC) are insensitive as they can only measure relative but not incremental changes over time, which are rather small as bone turnover is slow. Biomarkers, i.e. compounds in blood or urine that can be associated with bone accretion or resorption, are much more sensitive but subject to significant intra-individual variation (*12, 13*). Conventional approaches therefore require large subject numbers and/or long intervention intervals for reliable detection of changes in bone.

Isotopic labeling of bone is a conceptually different approach that is potentially more sensitive than existing techniques. After giving a tracer orally or intravenously, the tracer is gradually incorporated into bone while tracer not incorporated in bone is washed out from the body over time. Once complete, all tracer in urine has its origin in bone and changes in tracer excretion in urine should reflect bone balance (see Figure B-1). While this concept is simple in theory, it is difficult to put into practice. Conventional calcium radiotracers cannot be used

for bone labeling because of health concerns. Stable calcium isotopes are a safe alternative but doses and therefore costs would be prohibitively high (ca. 70,000 EUR per person) due to the large amounts of natural calcium in the skeleton. These shortcomings have led to the suggestion of ^{41}Ca as the isotope of choice (*277*). It is a radioisotope but at a half-life of 105,000 years it decays very slowly and emission of ionizing radiation per unit time is therefore also low. As such, it can be regarded as stable with respect to time scales of biomedical tracer studies and human life span. In addition, it is virtually non-existent in nature and can be detected with extreme sensitivity by accelerator mass spectrometry (AMS) (*532, 533*). This permits usage of ^{41}Ca doses for skeleton labeling that are so small that resulting radiation burdens are lower by several orders of magnitudes than burdens arising from natural radiation exposure.

Over the past two decades, technicalities of ^{41}Ca labeling of the human skeleton have been explored systematically (*269, 271, 275, 277, 280, 284, 534, 535*). More recently, the ^{41}Ca technique was validated against established techniques (BMD, biomarkers) (*285*) and used successfully in a series of clinical trials which demonstrated the sensitivity of the technique (*285, 536, 537*). Nevertheless, the technique has so far not been used widely because of practical restrictions. Only a handful of laboratories worldwide have access to the necessary instrumental infrastructure for ^{41}Ca analysis. Therefore we have explored strontium stable isotopes as alternatives. Strontium is known for a long time to mimic calcium closely at the level of absorption and discharge from the body, homeostatic control as well as distribution between hard and soft tissues (*14*). Strontium, however, is less abundant than calcium by a factor of approximately 1,000 in the biosphere. This permits the use of strontium stable isotopes at a much lower dose as compared to calcium isotopes. Furthermore, isotopic analysis of strontium is technically much less demanding and can be conducted using mass spectrometric techniques (ICP-MS, TIMS) that are now available at nearly every larger hospital or research university in the industrialized world.

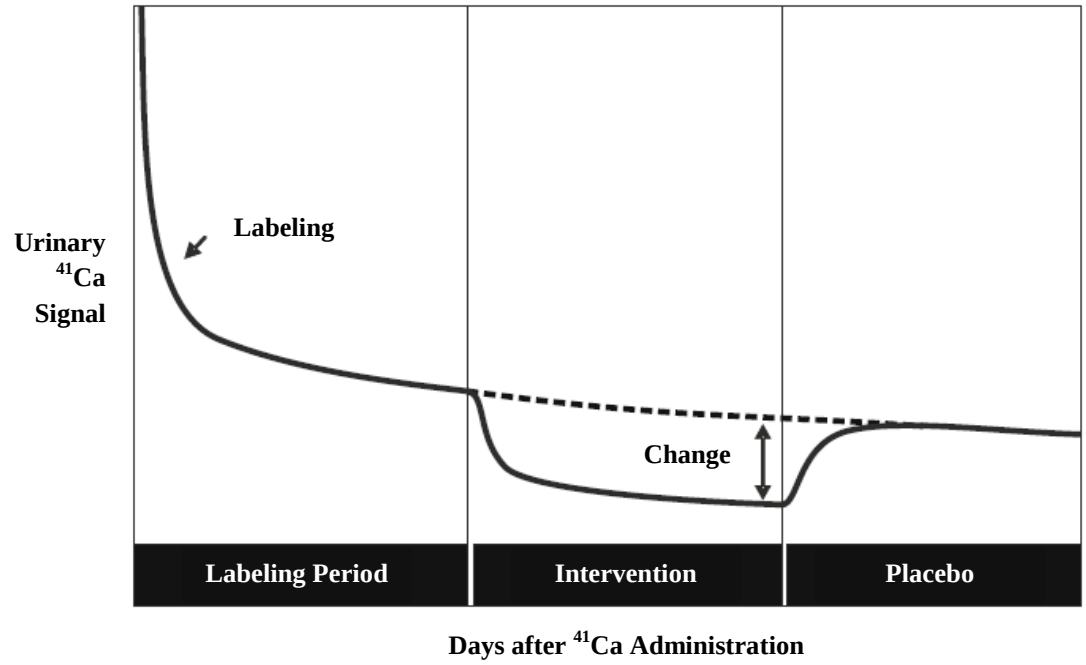

Figure B-1: Basic principle behind the use of ^{41}Ca for monitoring changes in bone metabolism. Following single administration of the isotope, the urinary excretion pattern of the tracer is followed during the labeling period. Once tracer not incorporated into bone has been washed out from the body, all urinary ^{41}Ca can be assumed to originate from bone and the labeling curve can be used as a fixed index against which changes due to an intervention can be measured. Figure is reproduced from Denk *et al.* 2006 with permission (*271*).

Concerns regarding uptake of ^{90}Sr from nuclear accidents, which is highly radioactive as opposed to the stable strontium isotopes ^{84}Sr and ^{86}Sr, led to many *in vivo* studies in the 1950s and 1960s. Technically, these experiments were limited to short-term studies over a few days only (*320*, *538*). Here, we compare for the first time ever urinary tracer kinetics and bone deposition of strontium and calcium in the long-term with the aim to assess (a) how far strontium can be used as a surrogate marker of calcium metabolism (b) if strontium stable isotopes can be used similarly to ^{41}Ca for labeling the bone matrix for monitoring bone calcium balance and (c) if incorporation of strontium and calcium into bone is comparable across skeletal sites. Experiments were conducted in a young adult sheep as a model of human calcium metabolism as sheep bones are big enough to study spatial distribution of the tracers in the skeleton.

1.2. Materials and methods

1.2.1. Test Animal

The study was conducted in a two-year-old female domestic sheep (East Frisean Milk Sheep), which was kept in the experimental animal unit at the animal hospital (Tierspital) of the University of Zurich, Switzerland. The sheep originated from a flock raised in normal environment in a stable with sheep-run and pasture. The animal had access *ad libitum* to standard feed (hay and concentrate, licking stone) and drinking water. On day 1 of the study, the sheep received an intravenous dose of ^{41}Ca and ^{86}Sr. Urine was collected the day preceding tracer administration and shortly before dose injection for measurement of calcium and strontium baseline isotope ratios. A total of 26 urine samples were obtained after tracer administration until the sheep was sacrificed on day 181 to obtain bone samples for analysis of tracer content. Intervals between urine samplings were shorter at the beginning and increased gradually towards the end to account for a faster urinary tracer clearance in the days/weeks following tracer administration. The sheep was kept indoors at a 12h day/night cycle for a period of approximately 70 days during winter and early spring before it was allowed to roam and graze freely outdoors. On day 28 the sheep received antibiotics for three days (Danofloxacin, Carprofen) as well as an intravenous Vitamin B bolus dose (B-Neuron®; 33.3 mg Vit B_1, 33.3 mg Vit B_6, 333.3 µg Vit B_{12}) following a minor leg injury. Body weight was monitored regularly over the course of the study. The study protocol was reviewed and approved by the responsible authority for review and approval of animal studies in Zurich (Kantonales Veterinäramt Zürich).

1.2.2. Tracer Preparation and Administration

The ^{41}Ca tracer used in this study came from the same batch of tracer used in earlier human studies (e.g. (*271, 536, 537*) and has been characterized earlier for isotopic and elemental composition at the Institute for Reference Materials and Measurements (IRMM),

Geel, Belgium (*539*). The ^{41}Ca tracer was administered as $CaCl_2$ with a ^{41}Ca isotopic abundance of 1.25±0.01 %. For administration, 2.85 kBq (22.6 nmol) ^{41}Ca in 1 mL physiological saline solution containing 1.8 µmol carrier calcium were mixed with 465 µmol ^{86}Sr tracer dissolved in 4 mL saline. Isotopically enriched strontium was obtained from Chemgas, Boulogne, France, as $SrCO_3$ with a ^{86}Sr isotopic abundance of 96.40±0.04 %. The strontium tracer was prepared by dissolution of the carbonate salt in 0.5 M HCl and subsequent dilution of the solution with saline. The prepared injection solution was sterile filtered before administration and tracer administration was well tolerated by the sheep.

1.2.3. Urine Sampling and Sample Preparation

Spontaneous void urine samples were collected at dawn before feeding in a cup (around 50 mL). Samples were acidified with 1 mL of 0.5 M HCl, shipped to Singapore and stored at 4°C in polyethylene bottles until analysis. All analytical work was carried out at the National University of Singapore apart from ^{41}Ca analysis which was carried out at the Institute of Particle Physics at ETH Zurich, Switzerland.

For calcium and strontium elemental analysis by Atomic Absorption Spectrophotometry (AAS), concentrated HNO_3 was added to the collected urine until all precipitates formed during storage were dissolved (pH < 1). Organic sample matrix was removed by microwave-assisted wet digestion of the samples using an Ethos 1 digestion unit (Milestone Inc., Sorisole, Italy). Urine aliquots (4-70 mL) were weighed into pre-cleaned Teflon vessels, dried and mineralized using a mixture of 8 mL conc. HNO_3 and 2 mL 30% H_2O_2 and diluted with water to the optimal concentration range for analysis. All chemicals used for sample preparation were of analytical grade unless otherwise noted and only purified water was used (Milli-Q-System, Millipore, MA, USA). Acids were cleaned further by sub-boiling distillation in quartz stills (SAP-902 IR, AHF Analysentechnik, Tuebingen, Germany). Only acid washed plastic or teflon containers/labware was used for sample

preparation. Sample handling was restricted as far as possible to class 10 metal free fume-hoods in a class 10,000 clean-room and monitored routinely for analytical blanks.

For calcium isotopic analysis by accelerator mass spectrometry (AMS), sample calcium was separated from 30 g aliquots of urine by oxalate precipitation following established procedures (*271*). In contrast to element analysis, isotopic analysis does not require quantitative element separation. In brief, urine samples were filtered using 2.0 µm pore size membrane filters (Millex-AP20, Merck Millipore, Darmstadt, Germany). Cooled saturated ammonium oxalate solution (40 mL) was added to the cooled urine samples and the pH of the filtered urine was adjusted to 10 by adding 25% NH_4OH solution. Samples were kept for 1.5 h at 70 ˚C and then immersed into an ice bath for at least 2.5 h to precipitate calcium oxalate. The precipitate was then separated by centrifugation, washed twice with saturated ammonium oxalate solution and once with water. To remove sodium and potassium from the sample, the precipitate was dissolved in 200 µL 5 M HNO_3 and passed through an ion exchange column (AG 50WX-8, Bio-Rad, Switzerland). After washing the column with 0.08 M HNO_3, calcium could be eluted with 5 M HNO_3. After neutralization with NH_4OH solution, the isolated calcium was then precipitated as CaF_2 by addition of 40% HF. The precipitate was washed twice with water and dried at 100°C overnight and shipped to Zurich for AMS analysis.

For strontium isotopic analysis by thermal ionization mass spectrometry (TIMS), strontium was isolated from the sample matrix using a strontium-specific ion exchange resin (strontium-specific, Eichrom, Lisle, USA) following standard procedures (*540, 541*). Before use, the resin was soaked in 0.1 M HNO_3 (1 hour), rinsed with and dispersed in water and loaded into polyethylene columns (Bio-spin, 3 mL; Bio-Rad, California, USA). Columns were washed with water and conditioned with 2 mL 8 M HNO_3. The sample was loaded onto the column in 8 M HNO_3. The column was then washed with 10 mL of 8 M HNO_3 to remove interfering elements including calcium. Strontium was then eluted with 7 mL water. The collected eluate was evaporated to dryness and stored in Eppendorf vials for later analysis.

1.2.4. Bone Sampling and Sample Preparation

After 6 months (day 181 post dose), the sheep was sacrificed using a captive bolt pistol for numbing and killed by bleeding thereafter. Relevant bones were obtained for analysis (tibia, radius, metatarsus, metacarpus and vertebrae). Bones were manually cleaned of cartilage and soft tissues, washed with saline and freeze dried. Bones were sliced and multiple samples were obtained from each type of bone both from cortical and trabecular areas to assess spatial differences in tracers deposition between different type and parts of bone. Slicing was done with a diamond band saw (Exakt 310 Macro Band System, Oklahoma, USA). Slices were cut into smaller pieces with a manual saw which were then sampled using a pair of stainless steel pliers (sample size: ca. 2x2x2 mm, weight: ca. 0.5 mg).

Bone samples were mineralized by microwave-assisted acid digestion (Ethos 1, Milestone) and prepared for elemental analysis by AAS as described before. Calcium and strontium were separated and purified using strontium-specific resin for isotopic analysis,. Digested samples were loaded in 8 M HNO_3 on the columns, calcium was eluted with 8 M HNO_3 and the eluate was dried down. Isolated calcium was converted to CaF_2 for AMS analysis as described earlier. Strontium was eluted from the column with water for TIMS analysis.

1.2.5. Calcium and Strontium Elemental Analysis by AAS

Samples were analyzed for calcium and strontium concentration by AAS (AA240 FS, Varian Pty. Ltd., Victoria, Australia), using standard addition techniques to control for possible matrix effects. Commercial calcium and strontium standards (Titrisol®, Merck, Darmstadt, Germany) were used for element spiking. Calcium was analyzed using a nitrous oxide-acetylene flame with KCl (Scharlau, Barcelona, Spain) as an ionization suppressant at a spiked potassium concentration of 2,000 µg/g in the sample solutions. All analyses were performed in triplicate with absorbances being measured in integration mode and corrected

for analytical blanks. Analytical accuracy was assessed by processing of a certified reference material (SRM1567a, wheat flour; National Institute of Standards and Technology, NIST, Gaithersburg, USA).

Strontium concentrations were measured by graphite furnace AAS (AA240 Zeeman, Varian Pty. Ltd., Victoria, Australia). All analyses were performed in triplicate with absorbances being measured in height mode and corrected for analytical blanks. Analytical accuracy was assessed by analysis of a gravimetrically prepared laboratory standard because of a lack of commercially available reference materials certified for strontium concentration.

1.2.6. ^{41}Ca Isotopic Analysis by AMS

Samples were analyzed for the $^{41}Ca/^{40}Ca$ ratio by AMS at the Laboratory of Ion Beam Physics, ETH Zurich as described earlier (*533*). The sample material (CaF_2) was mixed with silver powder in a ratio of 1:9 (w/w) for improving sample conductivity and ionization efficiency. Calcium was extracted from the sample as CaF_3^- ions (extraction current $\leq$ 100 nA) using a caesium (Cs) sputter ion source (*542*). Measured $^{41}Ca/^{40}Ca$ were normalized using a set of standards certified for their $^{41}Ca/^{40}Ca$ ratio (IRMM-3701) covering a range in $^{41}Ca/^{40}Ca$ ratios between $4 \cdot 10^{-8}$ and $4 \cdot 10^{-11}$ (*539*). Limit of ^{41}Ca detection was 7×10^{-12} for $^{41}Ca/^{40}Ca$ as determined by analysis of blank samples. Analytical blanks from sample preparation and/or carry over effects were monitored by identical processing and analysis of unlabeled urinary samples collected before tracer administration. Average repeatability (2 SD) for independent $^{41}Ca/^{40}Ca$ isotope ratio measurements of the same sample were < 5% for isotope ratios between $1 \cdot 10^{-12}$ and $1 \cdot 10^{-9}$ and < 10% for isotope ratios higher than $1 \cdot 10^{-9}$. A commercially available set of isotopic reference materials certified for $^{41}Ca/^{40}Ca$ isotope ratios (*539*) was used together with a set of in-house standards for quality control.

1.2.7. ^{86}Sr Isotopic Analysis by TIMS

Isolated strontium was reconstituted in 2 µL 8 M HNO_3 for TIMS analysis. The instrument (Triton, Thermo Finnigan, Bremen, Germany) was equipped with an array of 9 moveable Faraday Cups for simultaneous ion detection and operated in positive ionization mode at 10 kV acceleration voltage and 10^{11} Ω resistance. Sample strontium was ionized as Sr^+ ions using a rhenium single filament ion source (filament dimensions: 20 x 0.03 x 0.76 mm, zone refined rhenium, 99.999% purity; H. Cross, Moonachie, NJ, USA) and tantalum pentoxide (Ta_2O_5) as an ionization enhancer (*543*). The Ta_2O_5 activator was prepared by adding 2 g of $TaCl_5$ (99.999% purity, Sigma-Aldrich, MO, USA) to a mixture of 1.2 mL of conc. HF (48% Ultrapur, Merck, Darmstadt, Germany), 1.2 mL of conc. H_3PO_4 (85% EMSURE® ACS, Merck, Darmstadt, Germany), 20 mL of conc. HNO_3 (70%) and 80 mL of water (*544*). About 2 µL of the Ta_2O_5-activator suspension was first loaded onto the filament and dried down electro-thermally at a current of 0.5 A. Sample strontium was added to the loaded activator with a micro-pipette and heated to dryness at a current of 0.5 A. The current was then increased to 1.6 A and held there for one minute or until the sample turned brown. The heating current was increased finally to dull red glow for about 20 seconds before loading into the mass spectrometer.

For sample ionization, the filament current was increased to 2.0 A at a rate of 0.2 A/min and kept at this current until the $^{85}Rb^+$ had fallen below an intensity of 0.001 V for minimizing interference of $^{87}Rb^+$ ions with the $^{87}Sr^+$ signal. After automated focusing of the ion beam and peak centering, the heating current was increased automatically to 2.5 A at a rate of 0.1 A/min and further at a rate of 0.05 A/min to ca. 2.8 A (~1,400 - 1,490°C). Data acquisition was started when the $^{87}Sr^+$ signal intensity reached 8 V. Data were collected during ten blocks comprising of 10 scans each at 16.777 s integration time and 4 s idle time per scan. Baseline signal intensities were recorded before each block for 60 s by automated defocusing of the ion beam and amplifiers were rotated to control for differences in gain between individual amplifiers. Measured strontium isotope ratios of natural standards were

corrected for mass bias by normalization to the natural $^{86}Sr/^{88}Sr$ isotope ratio of 0.1194 as its current consensus value (*545, 546*). An iterative algorithm was used for normalization of isotope ratios of spiked samples. The measured $^{84}Sr/^{86}Sr$ isotope of SRM 987 ($SrCO_3$; NIST, Gaithersburg, MD) was 0.056460±0.000002 (1SD) for 25 independent sample loadings which falls well within its certified range (0.05655±0.00014).

1.2.8. Data Analysis

Similarities and differences between calcium and strontium metabolism were identified via studies of their tracer to tracee ratio, i.e. the number of moles of tracer relative to the number of moles of tracee in the sample. As strontium was tested as a surrogate marker for calcium, the tracee as the non-labeled element to be traced was calcium for both elements.

Measured $^{41}Ca/^{40}Ca$ isotope ratios were converted into $^{41}Ca/Ca$ amount ratios by multiplication with 0.96941 as the natural abundance of ^{40}Ca (*547*). Because of the very low abundance of ^{41}Ca (10^{-14}), all ^{41}Ca in the sample could be assumed to be tracer calcium. This is different for ^{86}Sr which is present in well detectable amounts in urine and in body tissues. Measured $^{86}Sr/^{88}Sr$ isotope ratios were therefore converted first into amount ratios of ^{86}Sr tracer to natural strontium in the sample (mole per mole) following isotope dilution principles (*249*). Amount ratios of ^{86}Sr tracer to natural calcium ($^{86}Sr/Ca$) were then calculated using the calcium to strontium mole ratio in the sample as determined by AAS. The amount ratio of both tracers in a given sample ($^{86}Sr/^{41}Ca$) was obtained by dividing the $^{86}Sr/Ca$ amount ratio in the sample by its corresponding $^{41}Ca/Ca$ amount ratio. Uncertainties in the $^{86}Sr/Ca$ ratio and the $^{86}Sr/^{41}Ca$ amount ratios were estimated using Monte Carlo techniques (*529*). Testing of data for statistical outliers was done by using Robust Outlier Testing.

1.3. Results

The sheep tolerated the treatment well and the sheep remained apparently healthy over the course of the study apart from the leg injury and did not require any intervention or

special treatment. The sheep started to gain weight after the first study month for about six weeks and lost some weight again the following six weeks (see Figure B-2). Changes were within the normal response range to changes in feed, climate and physical activity.

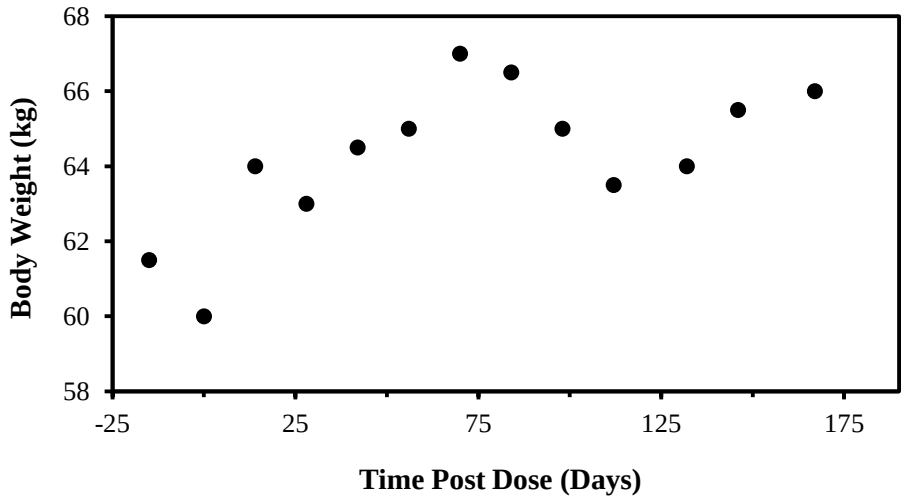

Figure B-2: Weight chart of the sheep during the study period. Each individual point refers to one measurement.

1.3.1. Urinary Tracer Excretion

The tracee to be traced was calcium for both calcium and strontium tracers. Normalization of tracer excretion to natural calcium has the advantage of obtaining a more stable tracer signal in urine as it controls for variations in daily calcium excretion and allows working with spot urine samples as opposed to 24 hour collections. Denk *et al.* (2006) showed in humans that although urinary calcium excretion of calcium and ^{41}Ca excretion varied strongly between days this did not affect the urinary $^{41}Ca/^{40}Ca$ isotope ratio significantly (*271*).

As expected, the ^{41}Ca signal in urine dropped by three orders of magnitude within the first month after tracer administration and fourfold within the following five months (see Figure B-3). The pattern of tracer excretion, however, deviated from similar labeling experiments in animals and man with a slight increase followed by a decrease of the urinary tracer signal from the beginning of the second study month to the end of the fourth study

month. It is well established that excretion of bone seeking tracers can be approximated in mammals using a combined power/exponential law of the form $S(t) = A \cdot t^B + C \cdot e^{D \cdot t}$, where $S(t)$ is the tracer signal at time point t post dose and A, B, C and D are parameters describing individual differences in tracer excretion (*282*, *548*). Accordingly, deviations from these regularities become clearer when urinary ^{41}Ca tracer data are plotted against time on a double logarithmic scale, (see Figure B-4a). For comparison, a typical pattern for urinary ^{41}Ca

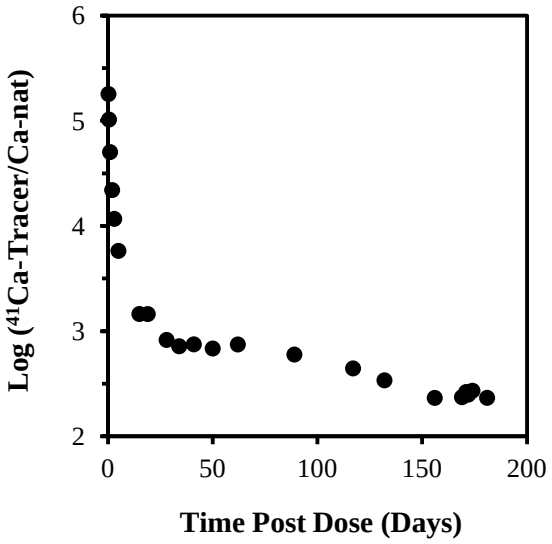

Figure B-3: Changes in urinary ^{41}Ca tracer excretion relative to natural calcium against time. Each individual point represents a spot urine sample from one particular day. Data are plotted on a semi-logarithmic scale. Over the observational period, the $^{41}Ca/Ca$ signal dropped by nearly 3 orders of magnitude. Measurement uncertainties are smaller than the symbol size. Uncertainties for these and all data presented in the following figures are estimated combined measurement uncertainties (95% coverage interval).

excretion in humans from an earlier study (*271*) is shown in Figure B-4b. A clear deviation from a regular excretion pattern could be observed after the first month post dose in the studied sheep with an apparent normalization in ^{41}Ca tracer excretion about three months later, although at a different trajectory. The phase during which the ^{41}Ca tracer signal deviated from its expected excretion pattern coincides remarkably well with the time point at which the sheep has received the antibiotics and B vitamins following a leg injury (day 28) and the phase during which the sheep lost and subsequently gained weight which compares well to the time period where the sheep was kept outdoors (on from day 70, approximately).

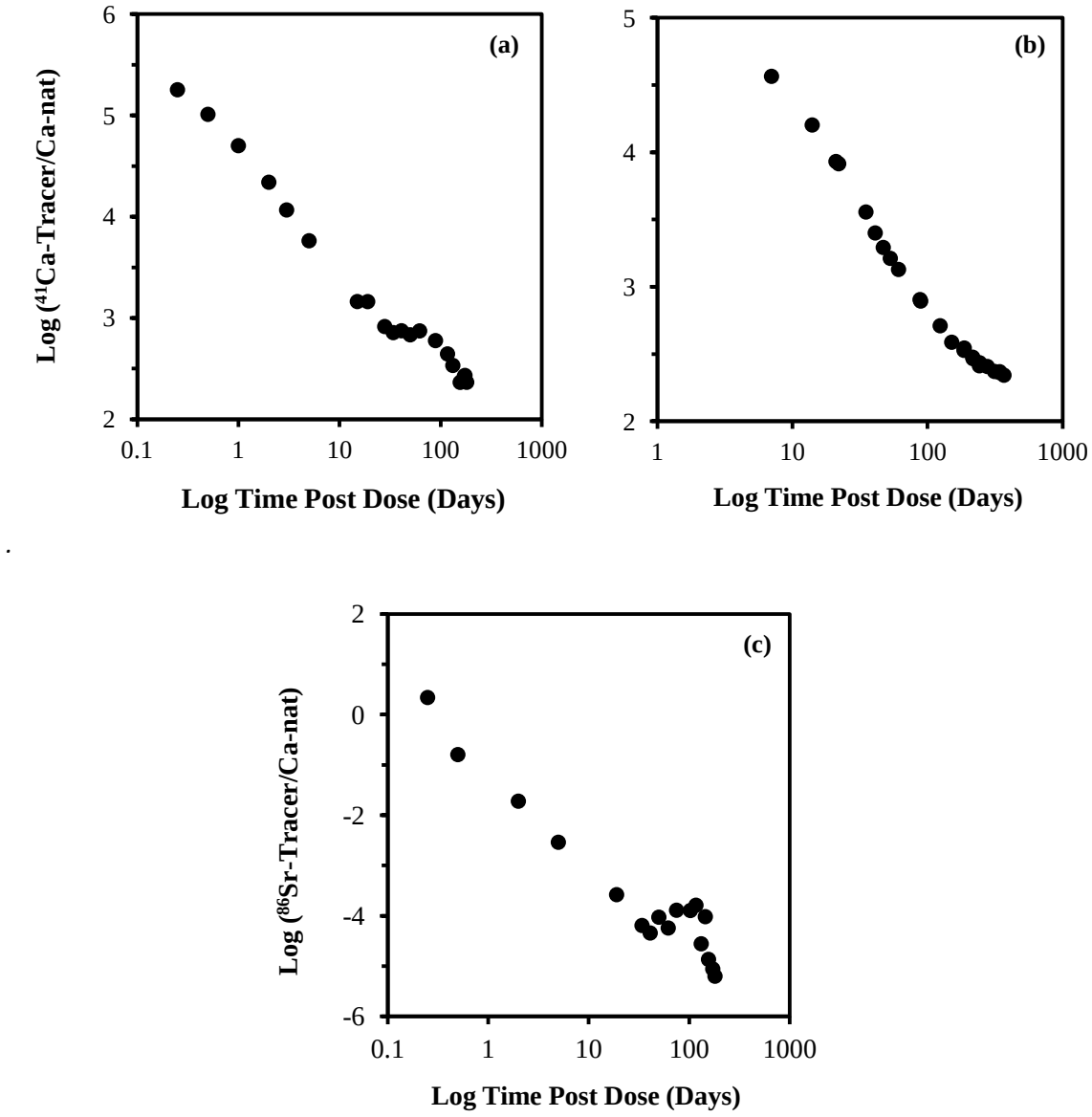

Figure B-4: Changes in urinary excretion of the ^{41}Ca tracer relative to natural calcium against time on a double logarithmic scale in the studied sheep (a) and postmenopausal women (b) from an earlier human study (data from Denk et al. 2006). Urinary excretion of the ^{86}Sr tracer relative to natural calcium against time in the studied sheep is shown in Figure B-4c. A clear deviation from regular tracer excretion patterns as shown for the human subject was found for the studied sheep. Irregularities were stronger for the ^{86}Sr tracer than the ^{41}Ca tracer. Measurement uncertainties are smaller than symbol size.

In agreement with our hypothesis, the ^{86}Sr signal followed the ^{41}Ca signal in urine (see Figure B-5a). Tracer signals were highly correlated (R^2=0.96) when tracer/tracee ratios were plotted against each other on a double logarithmic scale (Figure B-5b). The slope of the trendline was 0.70 which can be expected for a preferential urinary discharge of the strontium

tracer over the calcium tracer as observed in earlier studies. The mole ratio of ^{86}Sr to ^{41}Ca tracer dropped nearly exponentially from 12,200,000 shortly after tracer administration to 27,100 at the end of the study (see Figure B-6). The $^{84}Sr/^{41}Ca$ tracer ratio showed a deviation towards higher values during the same interval when tracer/tracee ratios in urine showed a deviating pattern. Remarkably, deviations from the expected regular excretion pattern were even stronger for the urinary $^{86}Sr/Ca$ ratio as compared to the $^{41}Ca/Ca$ ratio when data were analyzed using a double logarithmic plot (see Figure B-4c).

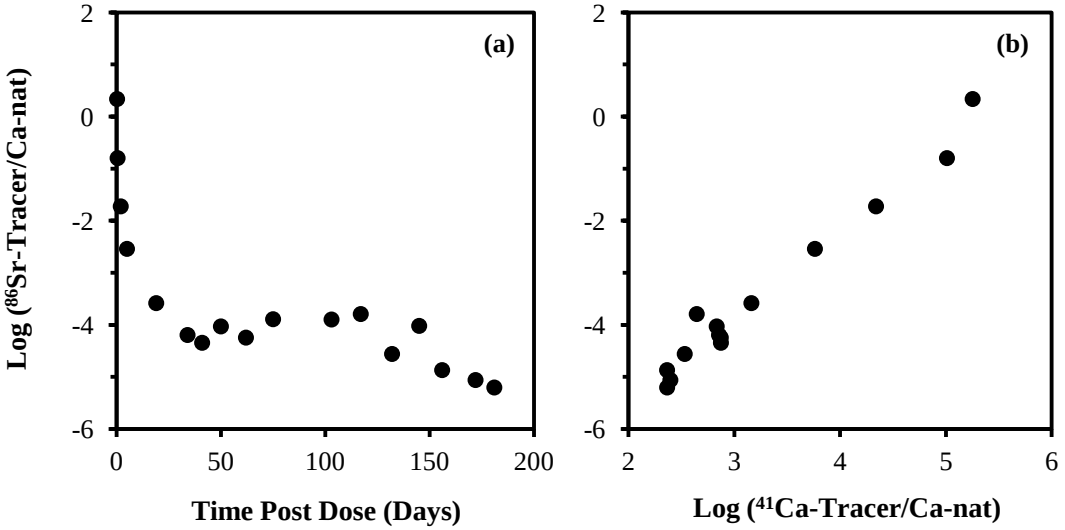

Figure B-5: Changes in urinary excretion of the ^{86}Sr tracer relative to natural calcium against time (a) on a semi-logarithmic scale and (b) against the corresponding $^{41}Ca/Ca$ ratio on a double logarithmic scale. Each individual point refers to spot urine sample from one particular day. Excretion of both tracers was highly correlated during the entire study (R^2=0.96). Measurement uncertainties are smaller than symbol size.

1.3.2. Skeletal Tracer Depositions

Six month after injection of the ^{41}Ca tracer, the tracer was not found to be evenly distributed among bone sites. The $^{41}Ca/Ca$ amount ratio between bone samples varied approximately by an order of magnitude with no indications for a higher tracer deposition in trabecular bone as compared to cortical bone (see Figure B-7). Differences in $^{41}Ca/Ca$ amount ratios in bone across the skeleton, however, can be higher at the microscopic level. Bone samples subjected to isotopic analysis were relatively large (about 10 mm^3) and as such

measured ratios only represent the mean ratio in the analyzed bone volume. As for urine, the ^{86}Sr tracer followed the ^{41}Ca tracer closely as indicated by the $^{86}Sr/^{41}Ca$ amount ratio which was found to be identical for most analyzed samples within the measurement uncertainty (Figure B-8). For $^{41}Ca/Ca$ amount ratios, analytical uncertainties in tracer/tracee ratios were assumed to equal measurement repeatability (ca. 5% RSD). Uncertainties in the $^{86}Sr/Ca$ ratio and the $^{86}Sr/^{41}Ca$ amount ratios, however, were significantly larger (ca. 10% RSD). Uncertainties for these ratios are relatively large as measurement uncertainties in strontium and calcium isotope ratios as well as the measured calcium and strontium concentrations in the sample must be taken into account. Testing of data for statistical outliers revealed that four of the analyzed bone samples carried $^{84}Sr/^{41}Ca$ amount ratios that were significantly lower as compared to other sampling sites ($6.84 \pm 0.98 \times 10^{3}$).

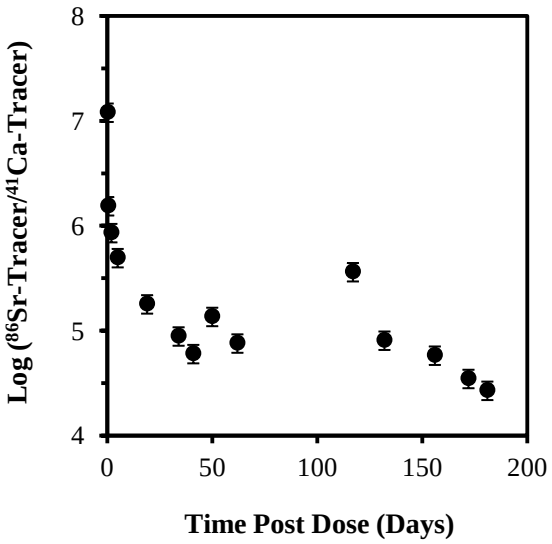

Figure B-6: Progressive changes in the amount ratio of both tracers in urine over time on a semi-logarithmic scale. Each individual point refers to spot urine sample from one particular day.

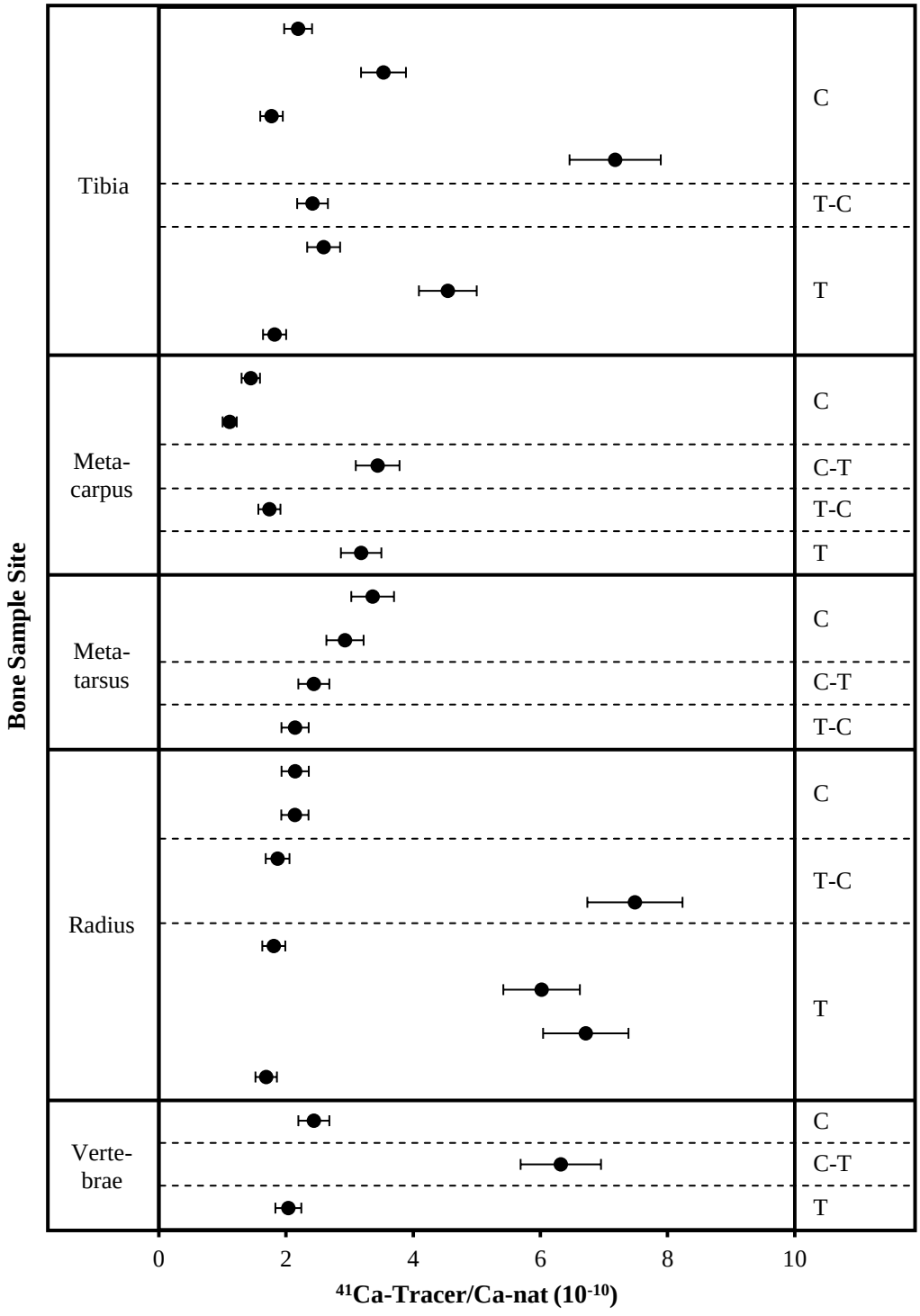

Figure B-7: Amount ratio of ^{41}Ca to natural calcium ratio for different bone sites. Each point represents the analysis of a bone volume of ca. 10 mm^3. Bone sites are listed on the left side while the type of bone is given on the right side (C: cortical bone, T: trabecular bone; C-T: mixed, mainly cortical bone; T-C: mixed, mainly trabecular bone).

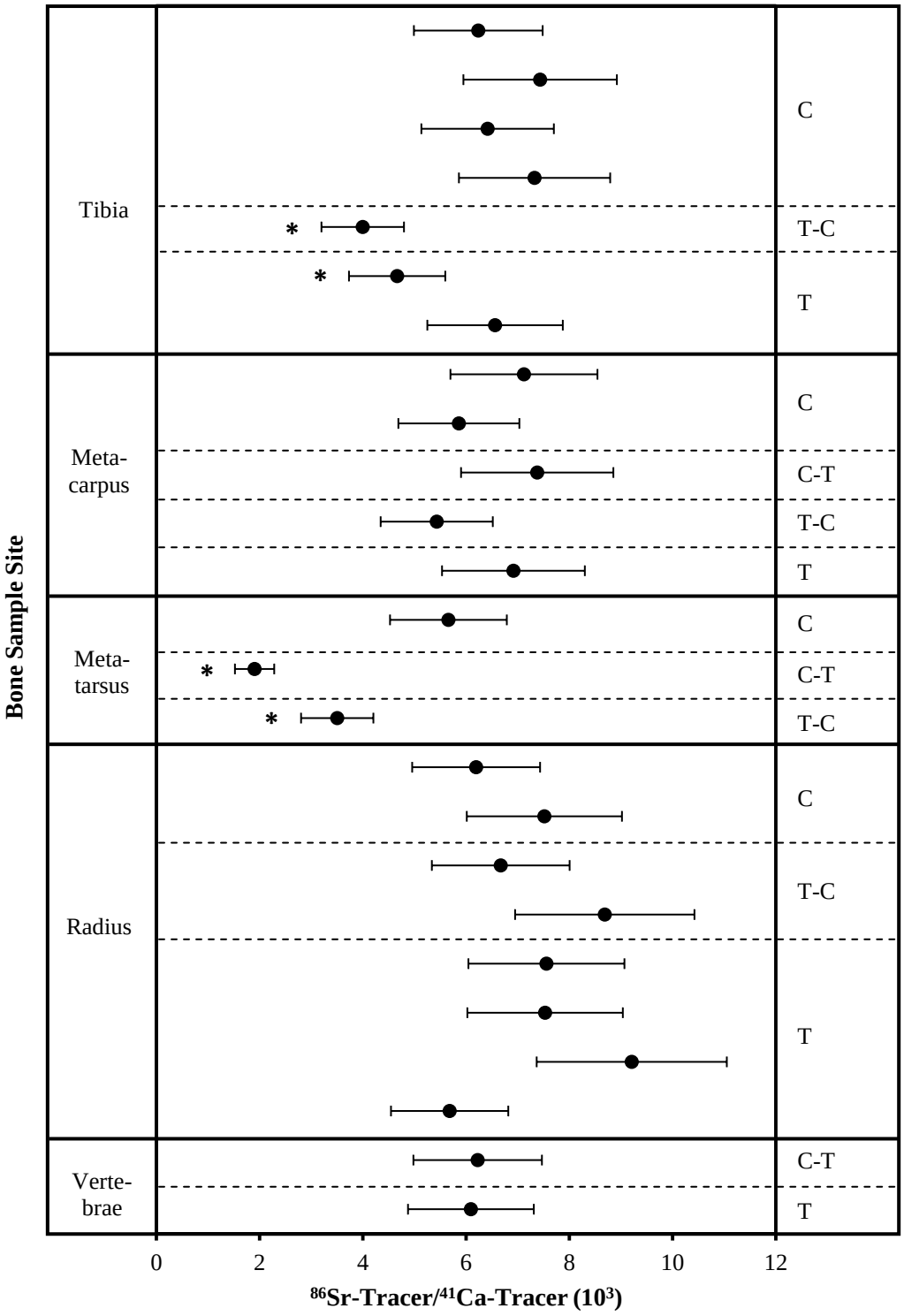

Figure B-8: ^{86}Sr tracer to ^{41}Ca tracer amount ratio for different bone sites and bone types (see Figure B-7 for legend). Samples that were found to be statistically different were by Robust Outlier Testing (95% confidence interval) are marked with an asterisk.

1.4. Discussion

The ^{41}Ca technique is based on isotopic labeling of the skeleton using a single dose of the isotope. After wash out of tracer not incorporated into bone, changes in urinary tracer excretion in response to interventions are supposed to reflect changes in bone mineral balance. To evaluate the usefulness of strontium stable isotopes as an alternative marker, we need to better understand how far the body discriminates strontium against calcium and to what extent this discrimination affects the tracer signal in urine. Discrimination of both elements against each other is studied best by comparing the molar ratio of both elements in body fluids or tissues. For tracer experiments, such as the presented study, comparisons can be made using the molar ratio of both tracers ($^{86}Sr/^{41}Ca$) or by comparing tracer to tracee ratios, i.e. the molar ratio of the tracers (^{41}Ca or ^{86}Sr) relative to the tracee. As strontium follows calcium closely and as we are interested in using a strontium isotope as a surrogate for the ^{41}Ca label, natural calcium and not strontium becomes the tracee. While tracer concentration in urine varies significantly within and between days due to fluctuations in urinary volume, the tracer to tracee ratio in urine is highly constant and identical to that in serum provided no discrimination between tracer and tracee occurs at the level of the kidney. Discrimination is negligible between calcium isotopes and does not affect the $^{41}Ca/Ca$ ratio. This has been demonstrated by Lin *et al.* (*283*) who showed that the $^{41}Ca/^{40}Ca$ ratio in urine equals that of serum within measurement uncertainty. This is not the case for calcium and strontium. The kidney alters the $^{86}Sr/Ca$ ratio due to the lower efficiency at which strontium is known to be reabsorbed in the renal tubules as well as a stronger binding of calcium than strontium to serum proteins (*430, 549*).

Walser and Robinson have tried to describe renal discrimination mathematically based on studies in dogs and humans (*375*). The ratio of strontium excreted in urine Sr_u to the amount of strontium filtered by the kidney Sr_f can be correlated to calcium filtration using the equation $Sr_u/Sr_f = (Ca_u/Ca_f)^{0.7}$. The magnitude of the exponent has been confirmed later independently by Marcus and Wassermann (*550*). However, the sharp drop in the $^{86}Sr/^{41}Ca$

tracer amount ratio at the beginning of our study (see Figure B-5a) is more likely to be due to initial strontium loading rather than discrimination. To achieve a sufficiently high isotopic enrichment in urine over the course of the study, we have administered a dose that exceeded the estimated daily dietary strontium intake of the sheep by a factor of 10 to 20. Injected strontium amounts to ca. 5% of circulating calcium in serum and was given over a few minutes only. This sharp rise in serum levels over a short period of time may have triggered a homeostatic response that resulted in preferential discharge of the strontium tracer relative to the calcium tracer. Their ratio dropped by a factor of 10 within the first day only. This finding indicates that ^{84}Sr might be the tracer of choice for such experiments as it is about 20 times less abundant than ^{86}Sr in nature which we have used in our study. Dose requirements for stable isotope tracers drop proportionally to their natural abundance (*249*).

While homeostatic mechanisms may explain changes in the tracer ratio in urine very early in our study, they fail to explain changes in the following weeks and months. Renal excretion and bone deposition as homeostatic mechanisms can be safely expected to be effective within hours. After this initial discharge, the urinary tracer signal is dominated in the second phase by the continuous release of tracer initially adsorbed at the bone surface. Both calcium and strontium are bone seeking elements that can bind reversibly to the outer layer of bone by ion exchange mechanisms (*551*). In the third phase, remaining tracer at the bone surface has been incorporated into the bone matrix by osteoblast action. Only then, the urinary tracer signal is determined by the amount of tracer resorbed from mineralized bone, the fraction that is recaptured by bone or other tissues before renal elimination and, finally, the efficiency of tubular reabsorption. Because of the dynamic nature of these changes, it is only during the final phase that the urinary tracer signal truly reflects bone mineral balance. In humans, it was found that this point is reached when the urinary tracer signal tends to stabilize approximately after 150 days after dose administration (*271*). This was found to occur earlier in the studied sheep, i.e after 30-50 days post dosing. Due to the strong changes in the tracer ratio during the initial phase (see Figure B-6), strontium is less suitable as a surrogate marker

of bone calcium metabolism as found in earlier studies (*416, 552, 553*). Conclusions might be biased when strontium is not given sufficient time to be fully incorporated into the skeleton.

Discrimination of strontium and calcium during tubular reabsorption clearly affect the tracer ratio in urine. However, this phenomenon does not disqualify strontium stable isotopes per se from being used as a bone label. Because strontium is a bone-seeking element like calcium, it can be safely assumed that virtually all strontium tracer in urine originates from mineralized bone after wash-out of loosely bound strontium from the body. As strontium tracer in the mineralized matrix is gradually replaced during bone remodeling, strontium tracer enters serum and urine steadily. Thus, a strontium tracer signal in urine is obtained that correlates qualitatively with bone calcium release as long as renal discrimination or even discrimination during strontium release from bone is regular. This results in a steadily falling tracer signal that can be used to study the effect of an intervention on bone balance (see Figure B-1).

Following these logics, the observed irregularities in urinary ^{41}Ca excretion would point to a deviation from steady state kinetics of tracer excretion and an effect on bone mineral balance, probably caused by giving the sheep access to the outdoors or in response to the treatment of the sheep with antibiotics or B vitamins. Because antibiotics were given intravenously and over three days only, an effect on bone is unlikely. This is different for the B vitamins which were given once only but at doses exceeding daily recommended intakes by a factor of 30 for Vit B_1 and Vit B_6 and a factor of 100 for Vit B_{12}, respectively. However, such interactions have not yet been described in the literature or studied systematically to the best of our knowledge and are therefore speculative. Nevertheless, observations are most remarkable as they demonstrate the potential of the ^{41}Ca labeling technique to detect even subtle changes in life-style, drugs or diet on bone. Irregularities in ^{41}Ca excretion were paralleled by changes in ^{86}Sr excretion but disturbances were even stronger. Renal discrimination is a possible explanation for the observed differences in response between both tracer signals. When strontium is retained less effectively than calcium by the kidneys,

proportionally more strontium tracer than calcium tracer is expected to be discharged in urine, which we have observed. The question arises now if this attenuating effect is limited to the kidneys or if discrimination between tracers also occurs at the level of the bone.

Tracer to tracee ratios in our study were found to vary by one order of magnitude between skeletal sites (see Figure B-7). This is not unexpected as we have administered tracers only once at the beginning of the study. Bone seeking elements in serum are more likely to be trapped in areas of bone that are actively remodeled. While remodeling occurs at isolated sites across the entire skeleton, osteoblastic activity is higher in areas that are physically loaded than in areas that are less challenged (*554, 555*) which would speak for a higher tracer uptake by trabecular bone. Furthermore, trabecular bone is supposedly remodeled more actively in response to calcium-regulating hormones for maintenance of body calcium homeostasis (*556*). However, we have not observed such a clear trend in our study. This can basically be explained by the relatively large size of the analyzed bone samples. Unless bone is homogenously labeled, which is unlikely as we have administered tracer only once, tracer enrichment of the samples is determined by the number and size of actively remodeled sites in the analyzed bone volume at the time of tracer incorporation which follows its own statistics.

Despite significant differences in tracer deposition density we observed a clear and strong correlation of ^{41}Ca and ^{86}Sr tracer deposition across the skeleton of the sheep (see Figure B-8). Within measurement uncertainty, $^{86}Sr/^{41}Ca$ ratios were mostly undistinguishable between skeletal parts. However, the average $^{86}Sr/^{41}Ca$ ratio of bone (ca. 6,700) was significantly lower than that of the dose material (20,200). This can be explained either by a preferential discharge of the strontium tracer from the sheep's body during the early phase post dosing as discussed earlier or a preferential deposition of calcium over strontium in the skeleton as suggested earlier (*306, 416, 424, 551, 557*). Both mechanisms would have reduced the amount of ^{86}Sr tracer relative to ^{41}Ca tracer in serum available for bone deposition and thus the tracer ratio in bone. Skeletal $^{86}Sr/^{41}Ca$ ratios at sacrifice were generally lower

than ratios in urine which can be explained by a preferential discharge of strontium tracer by the kidneys.

For studying discrimination of strontium and calcium tracer at bone level, our methodology is limited by the rather high measurement uncertainty of ca. 10% for the $^{86}Sr/^{41}Ca$ ratio which is determined both by isotope ratio analysis of strontium and calcium as well as their concentration measurements. Despite this obvious limitation, testing for statistical outliers revealed that four out of 25 analyzed bone samples had a $^{86}Sr/^{41}Ca$ ratio that was lower and statistically different from the other 21 bone samples which were statistically indistinguishable in their $^{86}Sr/^{41}Ca$ ratio. Sites were located in the tibia and metacarpus with a stronger association with samples containing trabecular bone as opposed to samples that were sampled solely from compact bone. Because of the low amount of tracers given relative to bone calcium, it can be safely assumed that the amount ratio of both tracers adsorbed initially on bone surface and later when entering mineralized bone was very similar, if not identical, across all bone sites. If no resorption occurs or if resorption does not discriminate between calcium and strontium, the tracer ratio should be constant within measurement uncertainty in bone. This has not been observed. The fact that the $^{86}Sr/^{41}Ca$ ratio was not homogenous and some samples were clearly depleted in ^{86}Sr tracer can be explained by a preferential release of strontium over calcium from mineralized bone. Because freshly formed bone matrix is less likely to be remodelled within the duration of our study and because of low bone turnover, such adulterations became only apparent in selected bone sites. Mechanistically, strontium can exchange for calcium in the hydroxyapatite crystal in the crystallized areas of bone but its larger ionic radius distorts the crystal lattice. This entails that during recrystallization strontium is slowly eliminated from the interior as crystal perfection is achieved. This is shown by greater discrimination in favor of calcium in older, larger and less rapidly formed crystals (*557*). As an alternative mechanism, a more effective reabsorption of ^{41}Ca tracer than ^{86}Sr in the kidneys would result in a lowered $^{86}Sr/^{41}Ca$ ratio in serum after tracer release from

bone. Non-excreted tracers are expected to bind to the bone surface again and will be incorporated subsequently with their altered $^{86}Sr/^{41}Ca$ ratio.

From the above it appears that strontium mimics calcium closely enough to identify changes in bone calcium balance by monitoring of the strontium tracer signal in urine. Because of a discrimination of strontium over calcium during tubular reabsorption and possibly bone resorption, the urinary strontium isotopic signal appears to accentuate negative changes in bone calcium balance better making it a potentially more sensitive marker than ^{41}Ca. Because changes in ^{86}Sr tracer excretion are consistently proportional to ^{41}Ca tracer excretion (see Figure B-5b) we would expect that a change to a more positive bone balance would also become more visible for the ^{86}Sr tracer than for the ^{41}Ca tracer. Unfortunately, we were not able to show this in our study as the disturbance in tracer clearance made data modeling of steady state kinetics impossible. Modeling would have also revealed more clearly to what extent calcium and strontium are discriminated against each other during bone accretion and resorption, which is still unclear. Such quantitative information would have also been crucial to evaluate to what extent strontium isotopic labeling of the skeleton permits quantitative estimation of changes in bone calcium. The latter is of relevance when comparing the effect of different interventions on bone.

1.5. Conclusions

For technical reasons it has not been possible so far to directly compare strontium and calcium tracer excretion and bone deposition over the long-term. Here we could show for the first time that the mammalian body handles calcium and strontium in a very similar manner once it has been incorporated into the bone matrix. Strontium is preferentially discharged in urine and possibly released more effectively from bone than calcium but discrimination is highly systematic. The high degree of correlation in urinary tracer excretion allows for the use of stable strontium isotopes for bone labeling in order to monitor changes in bone calcium balance. Observed correlations imply that mathematical corrections can possibly be used to

translate changes in strontium tracer excretion into changes in bone calcium balance once comparative data are available from kinetic tracer studies in humans. Our findings also indicate that strontium labeling is potentially more sensitive to detect changes in bone than calcium isotopic labeling of the skeleton. Taken together, strontium stable isotopes can be considered a potential alternative to the ^{41}Ca labeling technique, opening up the possibility of using this ultrasensitive tool for detection of changes in bone calcium more widely in research and perhaps as a diagnostic tool in patient care. Once the skeleton is isotopically labeled, tracer excretion is measurable over decades and may permit early identification of progressive net bone loss or monitoring of therapeutic success in patients.

Acknowledgements

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CHAPTER 2: ISOTOPIC LABELING OF BONE FOR THE ASSESSMENT OF CHANGES IN BONE MINERAL BALANCE: EVALUATION OF SPATIAL TRACER DISTRIBUTION (^{86}Sr) IN MINERALIZED TISSUES

2.1. Introduction

A persistent loss of bone mass during adulthood results ultimately in a weakening of bone structure and an increased fragility of bone later in life. Isotopic labeling of the skeleton is an ultra-sensitive technique to assess the effect of drugs, diet or life-style on bone mineral balance either for the prevention or the treatment of osteoporosis (*285, 536, 537*). After giving a tracer orally or intravenously, the tracer is gradually incorporated into bone while tracer not incorporated in bone is washed out from the body over time. Once washout is complete, all tracer excreted in urine originates from bone and changes in excretion patterns are supposed to reflect changes in bone mineral balance (see Figure B-9). The most ideal isotope for bone

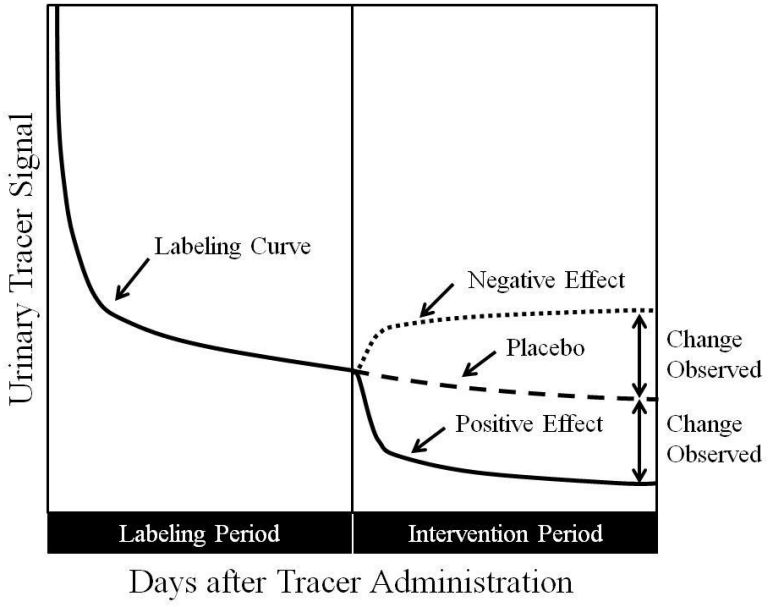

Figure B-9: Basic principle for monitoring bone metabolism using isotopic tracer. Following a single tracer administration, the urinary tracer signal is monitored throughout the labeling period. The tracer that is not incorporated into bone will eventually be washed out before the end of the labeling period. After which, the urinary tracer signal can be assumed to originate mainly from bone. Changes in bone metabolism resulting from an intervention can then be measured against the labeling curve, which is assumed to be a fixed index. Figure is taken from Denk *et al.* (*271*) with modifications.

labeling is ^{41}Ca, a semi-stable radiotracer (*277*). Due to its very long half-life of 105,000 years and the extreme sensitivity by which it can be detected using Accelerator Mass Spectrometry (AMS), ^{41}Ca dose requirements are minimal and effective radiation doses can be kept well below levels arising from natural radiation exposure (*271*).

While several studies have already demonstrated the high sensitivity of the ^{41}Ca technique (*285, 536, 537*), it is unlikely that this technique will become widely available in the future. Analysis of ^{41}Ca requires highly sophisticated instrumentation that is only accessible to a handful of research groups worldwide. We therefore explored strontium stable isotopes as a potential alternative. Strontium and calcium are both bone seeking elements that follow each other closely in nature due to their highly similar chemical and physiological properties. As such, it has already been suggested as a surrogate marker for studying calcium metabolism (*552, 553*). In contrast to ^{41}Ca, strontium stable isotopes can be detected using much less sophisticated instrumentation (TIMS, ICP-MS) that is more widely available than AMS.

To better understand how far the mammalian body discriminates between strontium and calcium over the long-term during bone accretion and resorption and, finally, at the level of the kidneys we have injected ^{41}Ca and ^{86}Sr simultaneously to an adult sheep (*558*). In contrast to many studies in the past comparing metabolism of both elements over days or weeks only, we followed excretion of both tracers in urine for 6 months before sacrifice to study tracer deposition in bone. In this study we could already confirm that short-term handling and long-term handling is different for both elements, that bone deposition and urinary excretion of both tracers were highly correlated over six months and that strontium tracer is discharged more efficiently than calcium tracer in urine and, possibly, bone. The latter makes ^{86}Sr labeling more sensitive to detect changes in net bone loss as compared to ^{41}Ca. For both tracers, however, the question of spatial distribution in the skeleton at the end of the labeling period remains to be answered. Mode and site of tracer deposition affect ultimately the urinary tracer signal due to possible differences in bone turnover and release of the tracer from the skeleton. In our earlier study we were limited to the analysis of bone

biopsies of approximately 10 mm^3 in size. This has made it impossible to identify if areas of bone have been labeled homogenously or in small isolated spots or if tracer has been incorporated preferentially in trabecular bone as found in earlier short-term radiotracer experiments (*306, 422*).

To explore skeletal tracer deposition in our earlier sheep experiment we made use of the potential of laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), an innovative technique that permits elemental as well as isotopic analysis at spatial resolution at the µm scale (*559, 560*). Using this approach we tried (a) to locate the stable strontium tracer in bone that has been given 6 months earlier to the sheep at the microscopic level; (b) to associate tracer deposition with bone site and type; and (c) to relate the tracer deposition with skeletal structures, modeling and remodeling processes. Findings for strontium tracer deposition can be directly extrapolated to calcium isotopic labeling. In our earlier investigations we could show that deposition of both tracers was highly correlated at macroscopic level across all studied skeletal sites.

2.2. Materials and methods

2.2.1. Animal Study

The study was conducted in a two-year-old female domestic sheep (East Frisean Milk Sheep), which was kept in the experimental animal unit at the animal hospital (Tierspital) of the University of Zurich, Switzerland. The sheep was the same as the one described in Part B, Chapter 1. Experimental details are described in an earlier publication (*558*). In brief, the sheep was kept for 6 months with free access to standard feed (hay and concentrate, licking stone) and drinking water. On day 1 of the study, the sheep received an intravenous dose of 2.85 kBq (22.6 nmol) ^{41}Ca and 465 µmol of ^{86}Sr in 4 mL saline. The study protocol was reviewed and approved by the responsible authority for review and approval of animal studies in Zurich (Kantonales Veterinäramt Zürich).

2.2.2. Bone Sampling and Sample Preparation

After 6 months (day 181 post dose), the sheep was sacrificed using a captive bolt pistol for numbing and killed by bleeding thereafter. The tibia as well as one vertebrae (L4) were secured for strontium tracer analysis. Bones were manually cleaned of cartilage and soft tissues, washed with saline and freeze dried. Bones were sliced using a diamond band saw (Exakt 310 Macro Band System, Oklahoma, USA) and mirroring slices were obtained from the proximal and midshaft region of the tibia (see Figures B-10 and B-11) as well as the dorsal plane of the obtained vertebrae (see Figure B-12) together with a slice of one of the vertebrae's transverse processes. For each sampled region, one of the slices was left intact for strontium tracer mapping by LA-ICP-MS while smaller pieces (sample size: ca. 2x2x2 mm, weight: ca. 0.5 mg) were obtained mechanically from the mirroring slices for measuring ^{86}Sr enrichment by TIMS. LA-ICP-MS analysis was conducted at the Division of Analytical Chemistry, University of Natural Resources and Life Sciences (BOKU), Vienna, while samples were analyzed in parallel by TIMS at NutriTrace@NUS.

2.2.3. ^{86}Sr Isotopic Analysis by LA-ICP-MS

LA-ICP-MS analyses were carried out using a laser ablation system (NWR-193, Electro Scientific Industries Inc., Oregon, USA) interfaced with an ICP-MS equipped with a quadrupole mass filter (NexION^{TM} 300D, PerkinElmer Inc., Massachusetts, USA). Bone slices were transferred without further sample preparation into the laser ablation chamber. Instrumental parameters are summarized in Table B-1. Two dimensional distribution of the strontium tracer was assessed by ablating and analyzing bone at a spot size of 10-50 µm and scanning of the bone area in lines. Ablated material was carried to the ICP-MS for isotopic analysis in a helium/argon carrier gas stream. Blank signals in the argon/helium carrier gas were assessed by measuring background intensities of the ion signals of interest for at least 20 s prior to ablation. No elevated analyte signals were found during blank readings. All measured values were blank corrected.

Table B-1: Instrumental parameters of the laser ablation system interfaced with the ICP-QMS for direct analyses of the bone samples.

Parameters	Values
***Laser* (*New wave 'UP 193'*)**	
Ablation mode	Line scan
Wavelength (nm)	193
Pulse length (ns)	3 - 5
Repetition rate (Hz)	20
Spot size (µm)	10 - 50
Scan speed (µm/s)	25 - 50
Ar mix gas / make-up gas (L min^{-1})	1
He mix gas / carrier gas (L min^{-1})	0.9
ICP-QMS (NexION^{TM} 300D)	
RF power (W)	1,300
Auxiliary gas flow (L min^{-1})	1.25
Plasma gas flow (L min^{-1})	18
Analog stage voltage (V)	-1,712
Pulse stage voltage (V)	1,050
Detector	Dual
Autolens	On
Isotopes monitored	^{13}C, ^{24}Mg, ^{26}Mg, ^{31}P, ^{43}Ca, ^{44}Ca, ^{46}Ca, ^{48}Ca, ^{84}Sr, ^{85}Rb, ^{86}Sr, ^{87}Sr, ^{88}Sr
Scanning mode	Peak hopping
Sweeps/reading	1
Readings/replicate	2,700
Replicates	1
Dwell time (ms)	50
Integration time (ms)	135,000

Isotope ratio measurements by ICP-MS are always biased due to mass dependent fractionation of analyte ions arising from mass sensitive transfer of ions from the ion source into the mass analyzer. For matrix matched mass bias correction, a non-spiked sheep bone sample was used. Ten independent line scans were conducted before analysis of the actual bone samples on a given day and a mass bias correction factor was calculated for each of the line scans. The average correction factor was calculated from these scans and used to correct the following measurements of spiked bones. The ratio of ^{86}Sr tracer over natural calcium in

the ablated material was calculated for each data point by using established mathematical algorithms for isotope dilution mass spectrometry (IDMS) analysis (*249*). Data presentation and image development were done by using SigmaPlot 11.0 (Systat Software Inc., CA, USA) and Adobe Photoshop CS5 (Adobe Systems Inc., CA, USA) for enhancement of color contrasts.

2.2.4. ^{86}Sr Isotopic Analysis by TIMS

Samples have been analyzed for bulk strontium isotopic composition using Multicollector-TIMS (Triton, Thermo Finnigan, Bremen, Germany). Methodological details are described elsewhere (*558*). In brief, a strontium-specific ion exchange resin (Sr-Spec, Eichrom, Lisle, USA) was used to isolate strontium from the sample digests. Isolated strontium was ionized as Sr^+ ions using a rhenium single filament ion source with tantalum pentoxide (Ta_2O_5) as an ionization enhancer. Mass bias correction was done on the measured strontium isotope ratios by normalization to the natural $^{86}Sr/^{88}Sr$ isotope ratio of 0.1194 as its current consensus value (*545, 546*). Normalization of isotope ratios of spiked samples was done using an iterative algorithm. Measurements of 25 independent sample loadings of SRM 987 ($SrCO_3$; NIST, Gaithersburg, MD) gave an average $^{84}Sr/^{86}Sr$ isotope ratio of 0.056460±0.000002 (1SD) which falls well within its certified range (0.05655±0.00014). The ratios of ^{86}Sr tracer over natural calcium were calculated based on the measured strontium isotope ratios and calcium concentrations of the samples using established mathematical algorithms for IDMS analysis (*249*).

2.3. Results

Site-specific ^{86}Sr tracer to natural calcium ratio of the proximal tibia and shaft are shown in Figures B-10 and B-11 and of the analyzed L4 vertebrae in Figure B-12. In agreement with our earlier findings (*558*), analysis by LA-ICP-MS and TIMS show both that after 6 month of tracer administration the label is not evenly incorporated into bone. Macroscopic TIMS data (see Figure B-10d) point to a gradual increase in tracer deposition

from the epiphytical area towards the diaphytical area. Microscopic LA-ICP-MS analysis reveals clearly that tracer distribution is patchy and highly scattered with preferential deposition along the subcortical region in the metaphysis and beneath the growth plate where longitudinal growth has most likely occurred. High resolution tracer analysis (see Figures B-10f and B-10g) shows that the tracer has not been deposited only at the bone surface of trabecular cavities but also inside mineralized bone. Outside the areas that showed high tracer deposition, tracer could also be detected but at a much lower level.

The part of the shaft that was analyzed using TIMS covered the area between the endosteum and periosteum (see Figure B-11f). Based on TIMS analysis, the ^{86}Sr tracer to natural calcium ratio of the shaft is very similar to that of epiphytical area. However, LA-ICP-MS analysis shows that there is a strong preferential deposition of the tracer close to the periosteum and there is almost no tracer found in other areas of bone (endosteum and mesosteum). This finding could be confirmed by analysis of three other sites of the shaft pointing to a radial deposition of the tracer as a sharp line (see Figures B-11c-e).

The body of the fourth lumbar vertebrae (L4) was also analyzed for its site-specific ^{86}Sr tracer to natural calcium ratio by using both LA-ICP-MS and TIMS (see Figure B-12). LA-ICP-MS analysis revealed a highly localized deposition of the tracer both in cortical and trabecular bones (see Figure B-12d). While some tracer has been incorporated inside the corticula and trabecular bone, most tracer was found at the bone surface of trabecular cavities similar to the proximal tibia. The *transverse process* was also analyzed for tracer enrichment using TIMS. It shows a much higher enrichment of ^{86}Sr as compared to the vertebral body (see Figures B-12e and B-12f).

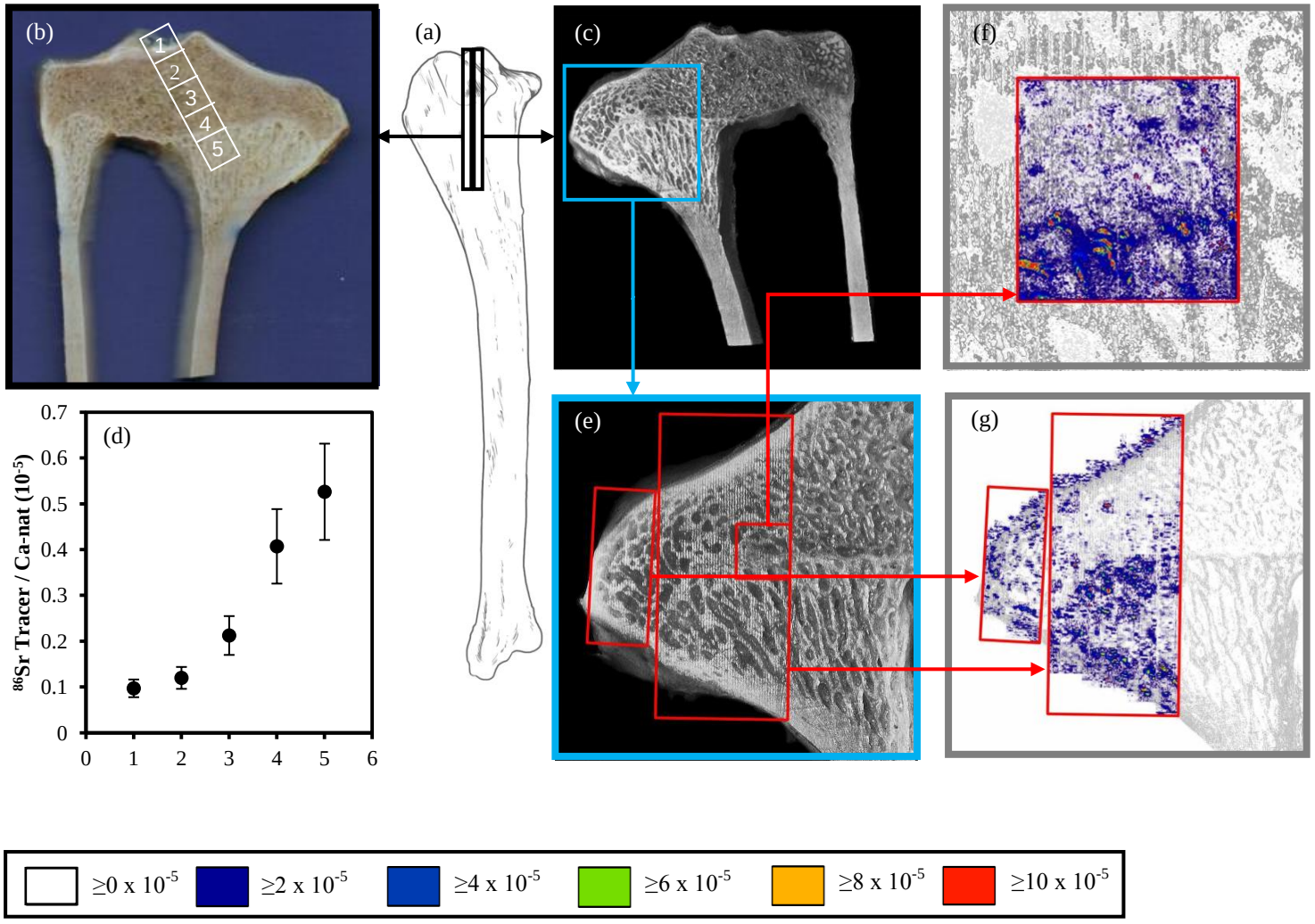

Figure B-10: Analysis of the tibia of the sheep (a) for strontium tracer deposition. The sites that were analyzed are marked by black boxes. Ratio of ^{86}Sr tracer over calcium were analyzed on the two adjacent parts from the epiphysis and metaphysis area (b) and (c). Samples were analyzed for ^{86}Sr tracer at the marked positions (b) relative to calcium by TIMS (d). The mirroring slice (c) was analyzed by LA-ICP-MS (e) for lateral distribution of the ^{86}Sr tracer (f and g) according to the given color scale which codes for ^{86}Sr tracer relative to calcium at the point of analysis. The area shown in (f) visualizes data from strontium mass spectra obtained from ca. 80,000 spot ablations. For better visualization, spots with measured ratios less than 2 x 10^{-5} have not been colorized.

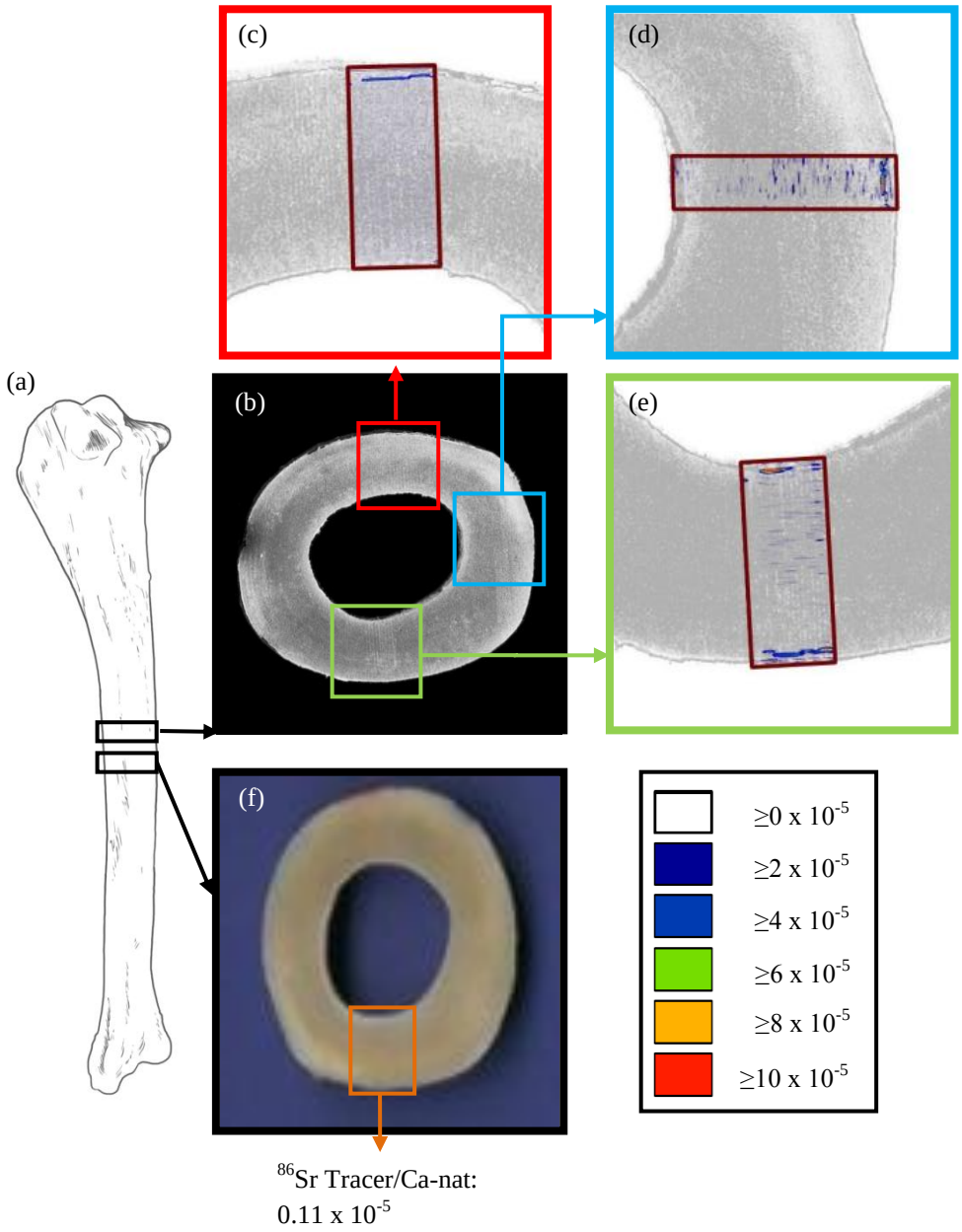

Figure B-11: Analysis of the tibia of the sheep (a) for strontium tracer deposition. Ratio of ^{86}Sr tracer over calcium were analyzed on the two adjacent parts from the shaft (b) and (f). The sites that were analyzed are marked by black boxes. Samples were analyzed for ^{86}Sr isotopic tracer at the marked position (f) relative to calcium by TIMS. The mirroring slice (b) was analyzed by LA-ICP-MS for lateral distribution of the ^{86}Sr tracer according to the given color scale which codes for ^{86}Sr tracer relative to calcium at the points of analysis (c), (d) and (e). For better visualization, spots with measured ratios less than 2 x 10^{-5} have not been colorized.

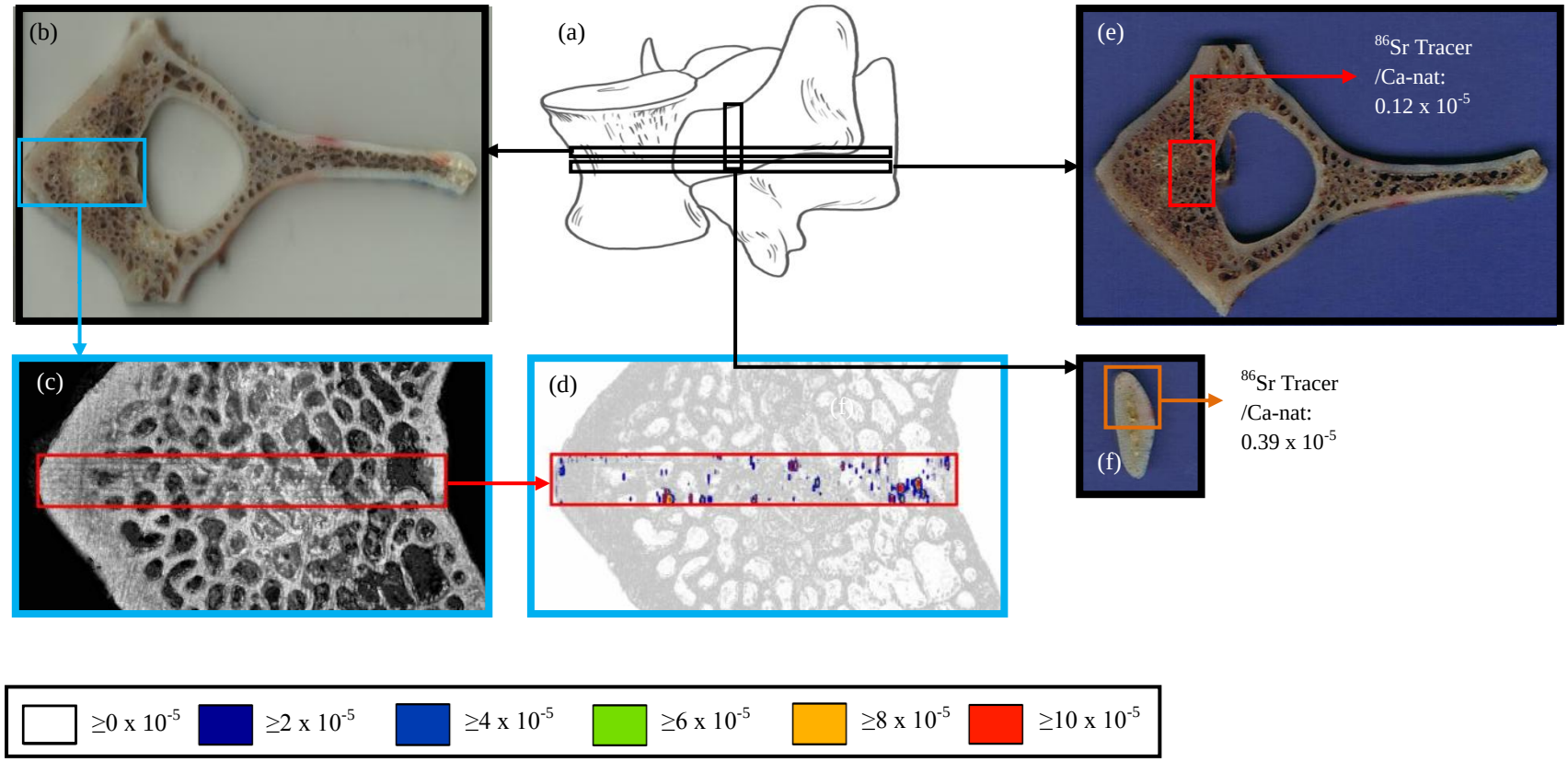

Figure B-12: Analysis of the lumbar vertebrae 4 (L4) of the sheep (a) for strontium tracer deposition. Ratio of ^{86}Sr tracer over calcium were analyzed on the two adjacent parts from the vertebrae body (b) and (e) and on the *transverse process* of the vertebrae (f). The sites that were analyzed are marked by black boxes. Samples were analyzed for ^{86}Sr tracer at the marked positions (e) and (f) relative to calcium by TIMS. The mirroring slice (b) was analyzed by LA-ICP-MS (c) for lateral distribution of the ^{86}Sr tracer (d) according to the given color scale which codes for ^{86}Sr tracer over calcium at the point of analysis. For better visualization, spots with measured ratios less than 2 x 10^{-5} have not been colorized.

2.4. Discussion

For isotopic labeling of the skeleton, an isotopic tracer is given once either orally or intravenously. Short-term studies using radiotracers have shown that osteons undergoing remodeling can take up considerable quantities of tracer rapidly. Osteons which are not completely mineralized take up smaller quantities as mineralization is slowing down. The rest of the bone takes up the tracer uniformly through adsorption processes (*561*).

When administering an isotopic tracer of calcium or strontium, tracer recovery in urine is determined by washout of tracer from plasma within the first hours following tracer administration. In the second phase, tracer initially adsorbed on the bone surface by ion exchange is mobilized and excreted. Only in the third phase, the tracer signal in urine is dominated by release of tracer from the mineralized bone matrix into circulation. From there it may also re-enter bone in a second cycle either following surface deposition or by direct osteoblast uptake at bone areas that are currently remineralized. In agreement with these mechanisms, a fast exchanging pool (bone surface) and a slowly exchanging pool (mineralized bone) could be identified by modeling long-term ^{41}Ca kinetics in earlier studies (*271*). These models showed that it takes approximately 120-150 days in humans until the tracer signal in urine is dominated by tracer resorption from bone (*271*). This compares to approximately 60 days in the studied sheep (*558*). Our observational period of 180 days has therefore been sufficiently long to ensure labeling of the bone matrix itself as the basis for identifying changes in bone mineral balance as the information of interest.

Earlier studies have demonstrated that the $^{41}Ca/Ca$ signal in urine and serum cannot be distinguished experimentally following ^{41}Ca adminsitration (*283*). This allows modeling of serum tracer kinetics based on the kinetics of urinary tracer excretion (*271*). Typical models include serum calcium, a fast exchanging calcium pool probably reflecting calcium bound to bone by adsorption and a slow exchanging pool which probably reflects calcium in mineralized tissue (see Figure B-13). Modeling therefore considers that most but not all tracer recovered in urine comes from mineralized bone. Furthermore, a single slow exchanging pool as in Figure B-13 does not discriminate between more and less active bone sites in terms of

remodeling rate but all bone from which tracer release is slow. Modeling of tracer kinetics delivers therefore quantitative information on calcium uptake (k_2) and release (k_3) from mineralized bone (C_3) per unit time, i.e. net bone calcium balance and turnover. Pharmacokinetic analysis of tracer data, however, requires more frequent measurements of urinary tracer excretion during the labeling phase and during interventions.

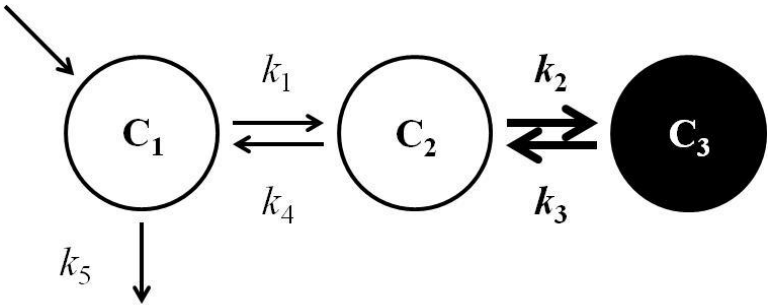

Figure B-13: Three-compartmental model describing ^{41}Ca tracer kinetics. The administrated tracer will first enter the central compartment (C_1), from which it will be transferred to a fast exchanging pool (C_2), which is presumably representing loosely bound calcium, and eventually to a slowly exchanging pool (C_3), which is presumably representing calcium in mineralized tissue.

In accordance with our current understanding of mechanisms of bone strontium uptake we observed a localized deposition of the tracer in the studied bone samples, shown by tracer deposition in clusters (see Figures B-10 to B-12). In the proximal tibia and the analyzed vertebrae, the tracer was deposited in hot spots that compare well in size to the dimensions of an average basic multi-cellular unit (BMU) (see Figures B-10 and B-12). BMUs are groups of tightly packed osteoclasts/osteoblasts at which bone remodeling occurs and are approximately 200 µm in diameter in sheep (*562*). Ratios of ^{86}Sr to natural calcium in these hotspots could be as high as $12x10^{-4}$ exceeding ratios in surrounding tissues by more than an order of magnitude. After 6 months of administration, ^{86}Sr tracer could be found in both trabecular and corticular bone. In trabecular spaces, tracer was mostly not adsorbed at the surface as a thin layer but laid down deeper into bone as it can be expected to result from osteoblastic deposition (see Figures B-10 and B-12). The same holds for corticular bone where tracer was laid down in individual osteons (see Figure B-11). Most of the other bone areas were also

reached by the tracer but at a much lower concentration amounting to a molar ratio of approximately 1-2 x 10^{-5} of tracer to natural calcium. There were only approximately 23% of the bone areas with tracer to natural calcium ratio that was below the detectable limit.

This is in agreement with basic remodeling mechanisms. It can be assumed that strontium tracer has been taken up primarily by BMUs that were in the bone forming phase within hours of tracer administration. At this time, circulating tracer concentration was order(s) of magnitude higher in plasma than in the days and weeks to follow which resulted in the observed patchy tracer distribution. The less tracer is in circulation, the less tracer is available for BMU uptake. Tracer not bound initially by BMUs was loosely adsorbed on bone surface across the skeleton from where it could re-enter circulation with time although at much lower plasma concentration than before. In consequence, much less tracer became available to BMUs than in the early phase of the study which explains the patchy tracer distribution with spots of high tracer enrichment as observed.

In newly formed bone, strontium is incorporated via heteroionic substitutions into the crystals as well as adsorption and exchanges at the surface of crystals (*563*). In older bone, strontium is incorporated mainly via adsorption and exchanges. Hence, clearance of strontium is likely to be higher in old than in new bone. Gradually, more tracer will be incorporated into bone and less will be bound at the bone surface, leaving more tracer in newly formed bone. In the metaphysis, rich with newly formed bone, high tracer deposition was observed along the sides of the growth plate, but not directly on it. The growth plate mainly consists of resting and proliferating cartilage zones, which do not take up tracer. The sections that take up tracer in a diffuse fashion are the hypertrophic cartilage beginning of the first or second row of cells where provisional calcification occurs as well as the metaphyseal bone trabeculae (*564*). Compared to the area above the growth plate, higher tracer deposition was observed beneath the growth plate. This indicates that there was a longitudinal growth of the tibia during the study period. This is in agreement with the finding from a short-term radiotracer study in 10-week-old rabbits that showed high level of calcium tracer beneath the growth plate while the plate itself was almost free of radioactivity (*565*).

Tracer was deposited in the bone matrix as a result of growth and bone remodeling with the latter being regulated mainly by mechanical force (*555*). Therefore, higher tracer enrichment can be expected in areas with elevated mechanical load. In the tibia sample, increased tracer deposition has been observed in the epiphysis and metaphysis, compared to the diaphysis. This can be explained by the fact that the epiphysis and metaphysis regions experience higher load from weight bearing. In addition to that, the epiphysis and metaphysis regions are richer in trabecular bone which undergoes remodeling more frequently and, thus, more new bone tissue. In contrast, the diaphyseal region contains mainly cortical bone which is less frequently remodeled as well as older bone tissue (*302*). Corresponding to this, lower tracer deposition was found in the cortical bone surrounding the epiphysis and metaphysis regions, compared to the trabecular bone inside (see Figure B-10). In the vertebrae, elevated mechanical loading also resulted in higher tracer deposition in the transverse process (see Figure B-12). The mechanical loading results from the muscles being attached to the transverse process (*566*).

The sheep model used in this study was still experiencing bone growth. Increasing mechanical demands can be expected to activate modeling and eventually promote radial bone growth. While undergoing radial bone growth, periosteal bone balance is mildly positive, whereas endosteal bone balance is mildly negative (*18*). Hence, the amount of tracer found in the mesosteal and endosteal surfaces was negligible and the tracer was deposited exclusively in the periosteum. This indicates that there was very little bone remodeling happening in the other areas of the shaft. Several short-term animal studies found that calcium radioisotope was distributed over the entire diaphyseal cortex (*564, 567, 568*). Nevertheless, it is important to note that short-term studies might observe only the initial adsorption of strontium onto the crystal surface, which does not differentiate mesosteum from endosteum and periosteum where bone modeling actually occurs.

Findings from the cortical bone in the diaphysis region of the sheep must be interpreted with caution. Cortical bone of the long bones of large fast-growing animals, such as sheep, typically contains plexiform bone which is uncommon in humans (*569*). Plexiform

bone has a similar structure to laminar bone with a denser vascularization system and a ‘‘brick wall’’ appearance. Cortical bone of a mature sheep’s long bone also consists of Haversian bone and Haversian systems, but they are very scattered on the periosteum and become denser towards the endosteal surface. In contrast to sheep, humans’ cortical bone contains almost exclusively Haversian bone and systems due to the higher load associated with bipedalism and the resulting increase in remodeling rate of the lower limbs.

2.5. Conclusions

Our study shows that isotopic labeling of the skeleton by single administration of an isotopic tracer does not reach all bone. Tracer is preferentially deposited in young bone as well as areas undergoing growth or more frequent remodeling due to a higher mechanical load. This must be considered when extrapolating changes in urinary tracer excretion to changes in bone mineral balance as illustrated in Figure B-9. Firstly, tracer recovered in urine can either originate from bone surface where it was bound loosely by ion exchange mechanisms or from mineralized bone from where it was released by osteoclastic resorption. Secondly, the urinary tracer signal is dominated by tracer release/uptake from bone areas that undergo remodeling more frequently. Accordingly, measurable deviations in tracer excretion from its original excretion pattern cannot be more than a qualitative index for changes in bone mineral balance in response to changes in lifestyle, diet or pharmacological treatment.

Based on our findings and known physiological similarities between calcium and strontium we can conclude that strontium stable isotopes can be used for bone labeling to identify changes in bone mineral balance similar to ^{41}Ca but with some restrictions. As for ^{41}Ca, modeling of urinary tracer excretion is the preferred approach to estimate absolute changes in bone calcium balance in response to interventions. The human body, however, discriminates between calcium and strontium during tubular reabsorption and possibly during uptake and release by the skeleton as shown in the first part of this study (*558*). This lets us conclude that strontium stable isotopes cannot replace ^{41}Ca for measuring bone calcium uptake, release and turnover in absolute terms but that they can be useful for qualitative

assessments. In principle, strontium stable isotopes can be detected similar to ^{41}Ca over decades in urine following a single oral or intravenous administration of the tracer. When being given to individuals in their late 30s or early 40s, regular monitoring of urinary tracer excretion would permit detection of accelerated bone loss or to qualitatively assess response of bone to pharmacological interventions or changes in lifestyle or diet. Because of the high sensitivity and specificity of the bone labeling approach, changes in bone could be detected probably earlier and thus faster as compared to conventional techniques at costs that are comparable or even lower than for radiological measurements of bone mineral density. Instruments that permit strontium isotopic analysis in urine such as inductively coupled plasma mass spectrometry (ICP-MS) are already widely available at university hospitals or commercial clinical laboratories.

Acknowledgements

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CHAPTER 3: UPTAKE AND DEPOSITION OF STRONTIUM IN BONE AND SOFT TISSUES: A DOSE-RESPONSE STUDY IN RATS

3.1. Introduction

Osteoporosis is a disease characterized by low bone mass and micro-architectural deterioration of bone tissue leading to enhanced bone fragility and a consequent increase in fracture risk (*570*). Due to the high morbidity associated with osteoporotic fractures as well as their crippling nature (*571-573*), availability of safe and effective interventions for osteoporosis prevention and treatment is critical.

Shorr and Carter suggested as early as in the 1950s that strontium might be useful in the treatment of osteoporosis after giving moderate doses of strontium lactate to osteoporotic patients (*15*). Strontium ranelate was suggested later as a more suitable strontium salt due to its pharmacological and chemical characteristics and was finally approved as an osteoporotic drug in recent years (*574*). Strontium ranelate is composed of two atoms of stable strontium and ranelic acid as the carrier anion. Strontium was found to act as a dual-action agent by reducing bone resorption and enhancing bone formation simultaneously (*575*). Efficiency of the drug to reduce fracture risks and increasing bone strength has been demonstrated in humans (*455, 574, 576*) as well as laboratory animals (*443, 448, 575, 577, 578*).

Strontium, like calcium, is a bone seeking element. As such ca. 98% of strontium in the body is found in bones (*291*). Strontium and calcium share similar physiological pathways and mechanisms of tubular transport (*300*) with some differences resulting from the larger size of the Sr^{2+} ion (*14*). Excretion of Sr^{2+} via the kidney is approximately thrice that of Ca^{2+} due to a lesser efficiency of Sr^{2+} reabsorption. Sr^{2+} can exchange imperfectly for Ca^{2+} in the bone and other cellular compartments (*300*) and is supposed not to greatly interfere in physiological processes at therapeutic dose level (*14, 300*). However, uptake of one will be affected by the presence of the other due to competitive mechanisms (*14*). While being used

as a potent osteoporotic drug, strontium is not alien to the human body. A normal diet contains 2-4 mg Sr^{2+} per day which comes mostly from vegetables and cereals.

Calcium is the most abundant mineral in bone and an insufficient supply through diet results inevitably in bone loss (*296*). Calcium concentration in extracellular fluids is tightly regulated. Nonetheless, excess calcium in serum results in hypercalcemia. Calcium functions as a sodium-channel blocker and inhibits depolarization of nerve and muscle fibers. As such, hypercalcemia decreases neuromuscular excitability and gastrointestinal contractility (*579*). Furthermore, it can cause arrhythmias and ventricular fibrillation by increasing excitability of the cardiovascular system (*580*). In kidney, excess calcium decreases its concentrating ability and damages renal tubules and can lead to mineral deposits. Calcium can form insoluble salts with phosphates and oxalates in soft tissues (*44, 581*). Calcification risks are higher in patients with compromised kidney function (*404, 582*) and those who obtain calcium from supplements (*38, 39, 583*). The latter has led the US Institute of Medicine to lower the Tolerable Upper Level of Intake (UL) for calcium for adults aged 51 years-old and above from 2,500 mg/day to 2,000 mg/day in 2010 (*479*). Since strontium behaves similarly to calcium and strontium intake from strontium ranelate by osteoporosis patients is comparable to the calcium content of common supplements (ca. 500 mg), strontium treatment may cause the same health problems as high levels of calcium supplementation.

Strontium levels in extracellular fluids are not as tightly regulated as for calcium and high strontium intakes may therefore lead to high serum concentrations (*14*). Because strontium behaves similar to calcium in the body, elevated serum strontium concentrations may cause effects similar to those observed in hypercalcemia. Persistently high strontium intakes can also cause strontium rickets, a condition known to make bone brittle. The mechanism by which excess strontium can cause an adverse effect on bone calcification is not yet confirmed. Due to the larger size of the strontium ion, hydroxyapatite crystals preferentially bind calcium than strontium. However, at high concentrations, strontium may replace calcium by heteroionic exchange (*584*). A mild distortion of the crystal lattice

followed by impaired crystal growth and increased dissolution of mineralized bone may lead to lower bone mineral density (*585*).

A recent evaluation of data from clinical studies in post-menopausal women by the European Medicines Agency's (EMA's) Committee for Medicinal Products for Human Use (CHMP) showed a higher risk of heart attack with strontium ranelate than with placebo, with no observed increase in mortality risk. Taking other known side effects of the drug such as blood clots and serious skin reactions into consideration, it has been recommended to restrict the use of strontium ranelate (Protelos®, Osseor®) to the treatment of severe osteoporosis in postmenopausal women and males at high risk of fracture (*16*) not suffering from heart or circulatory problems. An in-depth evaluation has been initiated.

The aim of this study was to investigate the possible risks associated with high strontium intakes, in particular strontium deposition in soft tissues (heart, kidney and lungs), as well as their effects on turnover and biomechanical properties of bone. Experiments were conducted in adult rats which were fed two different dose levels of strontium through their drinking water. In contrast to earlier studies, animals were sacrificed by hyper-perfusion to study strontium deposition in bones and soft tissues. Hyper-perfusion minimizes risks of artifacts arising from incomplete blood removal from tissues. Strontium and calcium are extra-cellular ions. Their concentrations inside cells are lower by four orders of magnitude as compared to extracellular fluids, including blood. Strontium and calcium concentrations were determined by isotope dilution mass spectrometry as a reference method for delivering element concentrations in biological samples at highest accuracy (*586*). Spatial distribution of calcium and strontium in bone was studied by Particle Induced X-Ray Emission (PIXE) analysis.

3.2. Materials and methods

3.2.1. Test Animals and Sampling Procedures

Adult male Wistar rats (ex-breeders, 6-8 month old at study onset) were chosen as models in this study. Studies were conducted in male and not ovariectomized female rats to assess risks of high strontium intakes uncoupled from pathological conditions. Forty rats with an initial weight ranging from 550 to 700 g were randomly divided into four groups (baseline group, control group, low Sr dose group, high Sr dose group). The low dose group received 0.2% Sr (w/w) and the high dose group 1% Sr (w/w) in the drinking water while no Sr was added to the water given to the control group. The baseline group was sacrificed at study onset to identify changes associated with aging and immobilization during the study period alone. The rats had access to a standard pelleted rodent diet (Harlen Teklad, Madison, Wisconsin) and prepared aqueous strontium solutions *ad libitum*. Strontium lactate (food grade, Dr. Paul Lohmann, Emmerthal, Germany) containing 24% (w/w) Sr was used for the preparation of drinking water which was adjusted to a pH of 5.4±0.2. Weight, water and feed consumption were monitored closely over 12 weeks. Rats were kept under conditions of controlled temperature and a 12 hour light-dark regimen in the vivarium at the Centre for Life Sciences (CeLS), NUS. The experimental protocol was approved by the NUS Institutional Animal Care and Use Committee (IACUC).

After a period of 12 weeks, the rats were sacrificed by hyper-perfusion with heparinized Ringer's solution to effectively remove blood in the sampled organs. The rats were anaesthetized using a mixture of ketamine (0.75 mg/kg rats' body weight) and xylazine (10 mg/kg rats' body weight). A cut was made from the abdomen to the chest cavity. A needle was then inserted 1 cm into the left ventricle of the rat's heart to perfuse tissues and organs with Ringer's solution (ca. 2 L per rat) without the use of a pump or by applying pressure. Fluid was drained through the right atrium where a small cut was made. Ringer's solution was self-prepared from salts of analytical grade quality. Organs were collected after perfusion and cleaned with analytical grade phosphate-buffered saline (PBS; Ultrapure, 1st

Base, Singapore). Bone samples were wrapped with gauze soaked in PBS solution. All samples were stored at -20˚C until further analysis.

3.2.2. Calcium and Strontium Concentration in Tissue Samples

Strontium and calcium concentrations in freeze dried samples were determined by IDMS as described elsewhere (*558*). Isotopically enriched strontium and calcium were both obtained from Chemgas, Boulogne, France, in carbonate form with a ^{86}Sr isotopic abundance of 95.32±0.83 % and a ^{44}Ca isotopic abundance of 98.841±0.079 %, respectively. Both tracers were prepared by dissolution of the carbonate salts in 0.5 M HCl. Samples and suitable amounts of strontium and calcium tracer were weighed into pre-cleaned Teflon vessels, dried and mineralized in a microwave digester (Ethos 1, Milestone Inc., Sorisole, Italy) using a mixture of 8 ml conc. HNO_3 and 2 ml 30% H_2O_2. All chemicals used were of analytical grade unless otherwise noted and only purified water was used (Milli-Q-System, Millipore, MA, USA). Acids were cleaned further by sub-boiling distillation in quartz stills (SAP-902 IR, AHF Analysentechnik, Tuebingen, Germany). Only acid washed plastic or teflon containers/labware were used for sample preparation. Sample handling was restricted as far as possible to class 10 metal free laminar flow hoods in a class 10,000 clean-room and was monitored routinely for analytical blanks.

Strontium was isolated for isotopic analysis by thermal ionization mass spectrometry (TIMS) analysis from the sample digests by column chromatrography using a strontium-specific ion exchange resin (Sr-spec, Eichrom, Lisle, USA). Sample strontium was ionized as Sr^{2+} ions using a rhenium single filament ion source and tantalum pentoxide (Ta_2O_5) as an ionization enhancer (*558*). Measured strontium isotope ratios were corrected for mass bias by normalization to the natural $^{86}Sr/^{88}Sr$ isotope ratio of 0.1194 as its current consensus value (*545, 546*). An iterative algorithm was used for normalization of isotope ratios of spiked samples. The measured $^{84}Sr/^{86}Sr$ isotope of SRM 987 ($SrCO_3$; NIST, Gaithersburg, MD) was 0.056460±0.000002 (1SD) for 25 independent sample loadings which falls well within its

certified range (0.05655±0.00014). Eluted fractions from the strontium columns containing calcium were dried down and calcium was separated from other matrix elements by ion-exchange chromatography. Sample calcium was ionized as Ca^{2+} ions using a rhenium double filament ion source and isotope ratios measured by TIMS (*558*). Measured calcium isotope ratios were corrected for mass bias by normalization to the natural $^{42}Ca/\ ^{44}Ca$ isotope ratio of 0.3104 as its current consensus value (*587*). Isotope ratios were normalized using an exponential law and iterative algorithms for isotope diluted samples (Russell et al. 1978). The measured $^{43}Ca/\ ^{44}Ca$ isotope of SRM 915b ($CaCO_3$; NIST, Gaithersburg, MD) was 0.064613±0.000007 for five independent sample loadings which falls well within the range of the current best measurements accepted by IUPAC of 0.0648±0.0009 (*587*). Measured calcium and strontium isotope ratios of the spiked samples were converted into calcium and strontium concentrations using established mathematical algorithms for IDMS analysis (*249*). Differences in element concentrations between the different groups were identified by ANOVA test at 95% confidence level.

3.2.3. Calcium and Strontium Distribution in Bone Samples

To study spatial distribution of calcium and strontium by PIXE analysis, bone samples were oven dried and embedded in epoxy resin using standard procedures recommended by the manufacturer (EpoFix Kit, Struers Inc., OH, USA). Sections were cut to expose the area near the region of interest using a low speed diamond saw (Isomet, Buehler, IL, USA) under constant water irrigation. Sample heating and tissue damage was minimized by maintaining the cutting rate below 150 rpm. After cutting, samples were re-embedded and the surface to be analyzed was grinded (TegraPol-11, Struers Inc.). Final surface preparation was done by diamond polishing (3 µm and 1 µm grain size, Struers Inc.). Thin slices (< 0.5 mm thickness) for Particle Induced X-ray Emission (PIXE) and Rutherford Backscattering Spectrometry (RBS) analyses were finally cut from the polished resin block by using a low speed diamond saw under similar conditions.

PIXE and RBS analyses were conducted at the nuclear microscopy facility at the Physics Department, National University of Singapore (*588*). The instruments were operated with a 2.1 MeV proton beam focused to a spot size of approximately 1 μm^2 and with an average beam current of 250 pA. The two measurements can be carried out simultaneously for mapping and measurement of the major, minor and trace elements concentrations in biological samples. Since the bone samples are rich in calcium, the area of interest was selected using the calcium images provided by PIXE. Quantitative results were determined by using RBS and PIXE; RBS provided the information on matrix composition and incident charge, while PIXE measured the concentrations of the elements from sodium upwards in the periodic table. X-ray spectra were collected using a lithium-drifted silicon X-ray detector placed at 90˚ to the beam axis and fitted with a filter consisting of 50 micron aluminium and 250 micron of Kapton. This filter is specially designed to reduce the calcium and potassium X-ray intensity to 0.01%, in order to improve the detection of trace amount of strontium in the samples. Initial scans (area: 4mm × 4mm) were carried out on each sample to map the calcium and strontium distributions. Strontium rich areas were identified, and subsequent scans with higher spatial resolution were carried out. The data was collected and recorded in listmode files, so that offline analysis was possible using the GUPIX code (*589*) to extract quantitative strontium and calcium concentrations.

3.2.4. Mechanical Testing

Three-point bending tests were based on the method suggested by Stürmer (*590*) and conducted as described in an earlier publication (*591*) using the instrumentation at the Department of Bioengineering, NUS (Instron 5848, Norwood, MA, USA). Tibia samples were thawed overnight at 4˚C. Bones were kept moist with PBS until mounting on a jig with an aluminum base. The proximal tibia was fixed by a notch on the base of the jig while the distal part remained unfixed to allow the bone to extend along the diaphyseal axis. The stamp was connected to the micro tester by an aluminum stem. The distance between the proximal

end of the tibia (with epiphysis removed) and the center of the roller stamp was set to 3 mm to achieve a consistent loading scenario between different samples. Before the test, the stamp was driven down slowly to the metaphysis of the tibia until the initial loading of 0.25 N was reached. After a final visual check of the correct tibia position, the breaking test was initiated. Maximum load, maximum stress and flexural modulus were recorded directly using the instrument's software (Merlin, Series IX). Yield load which divides the elastic from the plastic region was determined by the offset method. Stiffness and failure load were determined from the load-deformation curve, where stiffness is obtained from the slope of the elastic region and fracture load as the point at which the bone breaks. ANOVA test was used to compare averages of biomechanical parameters between groups at 95% confidence level.

3.2.5. Biomarkers of Bone Turnover

Blood (500 μL) was collected from the lateral tail vein after overnight fasting of the rats at the beginning of the study (week 0) and shortly before sacrifice (week 12). Serum was separated by centrifugation and stored at −20 °C until analysis. Serum levels of bone formation markers, procollagen type 1 N-terminal propeptide (P1NP), and bone resorption markers, C-terminal cross-linked telopeptides of type I collagen (CTX), were measured in duplicate in the control, standard and serum samples by enzyme-linked immunosorbent assay (Immunodiagnostic Systems Ltd, UK). Average values of P1NP and CTX concentration of the serum samples from the different groups were compared using ANOVA test at a confidence level of 95%.

3.3. Results

3.3.1. Treatment Tolerability

The rats tolerated the treatment well and remained apparently healthy over the course of the study. They did not require any intervention or special treatment. However, one rat from the control group died due to natural causes one day before study completion which did

not allow perfusion/sampling of organs. The rats from all groups gained weight steadily from the start until the end of the study (initial weight = 603±22 g; final weight = 684±21 g; refer to Appendix II, Table II-1), with no significant difference in the final weight between groups ($p=0.29$). Their feed consumption remained relatively constant throughout the study period (average consumption: 26±1 g/day; refer to Appendix II, Table II-4) with no significant difference between groups ($p=0.24$). Water consumption increased slightly with time (average consumption: 33±7 g/day; refer to Appendix II, Table II-2) with significant differences between the groups ($p<0.01$). The control group generally consumed more water throughout the study and the high dose group consumed less than the low dose group, respectively. Total strontium intake in the low dose group was 98±15 mg/kg/day and 383±36 mg/kg/day in the high dose group, respectively (refer to Appendix II, Table II-3).

3.3.2. Strontium Absorption and Uptake

Strontium lactate given with the drinking water increased the concentration of strontium in the serum in a dose dependent manner (see Figure B-14a). Serum strontium concentrations were about four times higher in the high dose group (35.6±3.8 µg/g) than in the low dose group (9.3±2.8 µg/g). This compares well to the nearly fourfold higher strontium intake of the high dose group as compared to the low dose group. Relative to the baseline/control group, oral administration of strontium increased its serum concentration about 100 times in the low dose group and about 400 times in the high dose group.

A dose-dependent increase of strontium was also observed in the skeleton (see Figure B-14b). Strontium concentrations in the right tibia of the baseline and control groups were very similar at 0.0300±0.0015 mg/g and 0.0270±0.0017 mg/g, respectively. Strontium administration increased strontium concentration in bone 90-fold to 2.56±0.92 mg/g in the low dose group and 280-fold to 8.0±2.3 mg/g in the high dose group, respectively. While the increase in strontium content relative to the baseline/control group was comparable for bone and serum (90-fold increase versus 100-fold increase), the increase in strontium content

between the strontium supplemented groups was significantly low for bone than serum (three-fold increase versus four-fold increase).

Strontium was distributed across the studied skeletal sites with some areas having higher strontium deposition (see Figures B-15 and B-16). In the femoral head and neck region of the treatment groups, more strontium was incorporated into trabecular bone compared to the cortical shell. Trabecular bone itself was scattered with strontium-rich areas (see Figures B-15b-3 and B-15c-3). The Sr/Ca ratio at the strontium-richest area was as high as 0.074 in the low dose group and 0.257 in the high dose group, respectively. In the mid-shaft region, strontium was incorporated mainly as a band close to the periosteum in the treatment groups (see Figures B-16a-3 and B-16b-3). In the high dose group, the strontium level was also elevated in the endosteum (see Figures B-16b-3 and B-16b-5), but it remained at the baseline level in the mesosteum (see Figures B-16a-3 and B-16b-3). In addition to that, cavities with lower calcium density were observed which appear to be surrounded by a strontium rich mineral phase.

Unlike strontium, calcium distribution in both groups was almost homogenous across the studied bone samples, both in the epiphysis and diaphysis (see Figures B-15a-b and B-16a-b), with a slight decrease in calcium concentration in areas with particularly high strontium enrichment (see Figure B-17). Some exceptions were observed in the mid-shaft region of the high dose group (see Figure B-16b). In sections without high strontium enrichment (blue areas in Figures B-15b-c and B-16a-b), the Sr/Ca ratio was approximately 0.0036-0.0070 in the low group and 0.0089-0.017 in the high dose group. This value was about 10-20 times lower than that for the Sr/Ca ratio in the strontium-richest area in the low dose group and about 15-30 times lower as found for the high dose group. In the control group, the Sr/Ca ratio was significantly lower (ca. 0.00012-0.00014). This shows that strontium was incorporated nearly homogenously into most parts of the skeleton (see Figures B-15a-b and B-16a-b) with the exception of few ‘hot spots’ showing a significantly higher strontium enrichment (see Figure B-17).

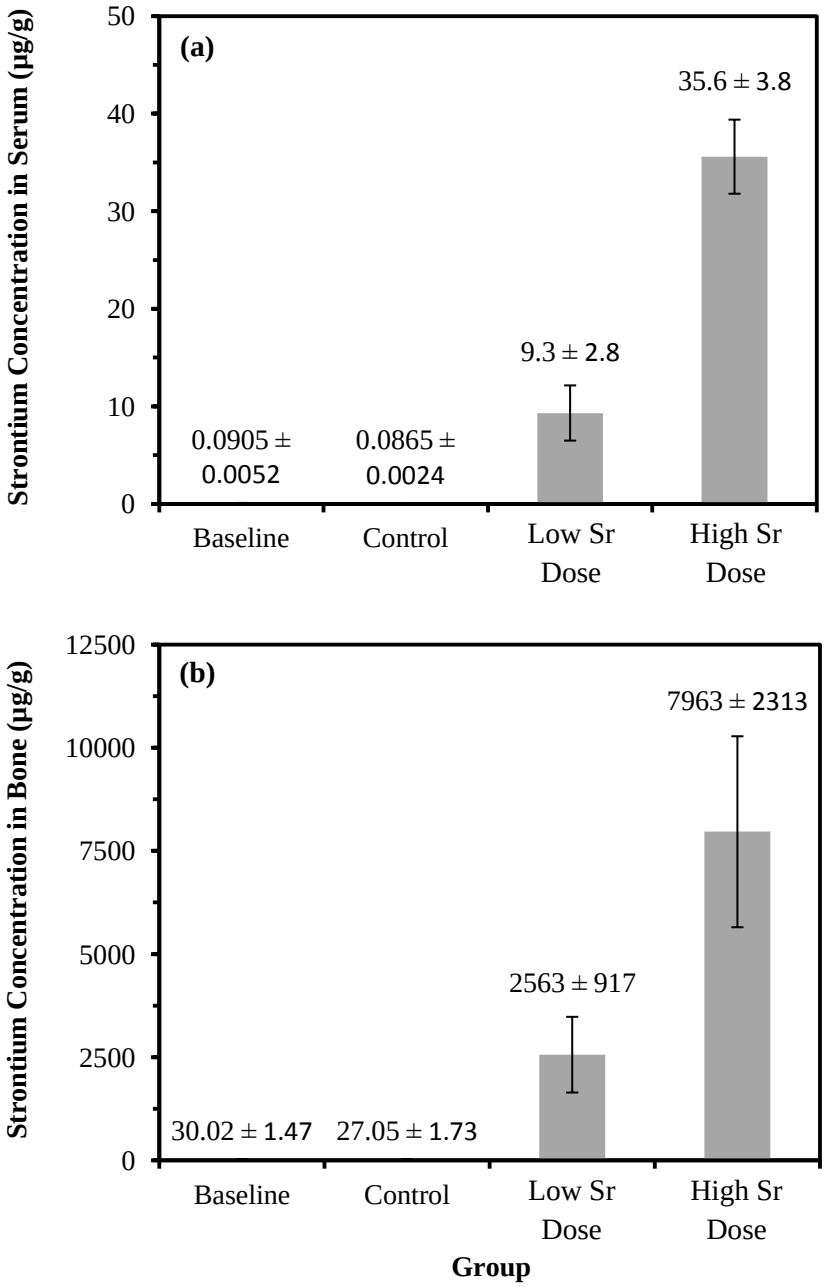

Figure B-14: Strontium concentration in serum samples (a) and bones (b) of the different study groups. The baseline group was sacrificed at the beginning of the study. The control group was sacrificed after 3 month, but did not receive any additional strontium in the drinking water. Average strontium intake from the drinking water was 98±15 mg/kg/day for the low dose group and 383±36 mg/kg/day for the high dose group. Each bar represents the mean strontium concentration (±1 SD) as measured in a given group (n=9 for control group, n=10 for baseline, low dose and high dose group).

In general, a dose dependent increase of strontium concentration was observed in all soft tissues (see Figure B-18b) while calcium concentrations were relatively similar across groups with some minor exceptions (see Figure B-18a). Strontium concentrations in soft

tissues were found to be statistically significant, yet moderately, increased in the low dose group as compared to the control group for the studied tissues. In contrast, increases in strontium concentration were substantial for the high dose group and clearly disproportionate to the increase in strontium intake and serum strontium concentration between groups. Differences in strontium concentration between the low dose and the high dose group were the highest for the kidneys (15x) followed by lungs (6x) and heart (5x). However, due to the much higher calcium content of bone, differences in combined calcium and strontium concentration of organs were statistically insignificant between study groups except for the kidneys (see Figure B-19).

3.3.3. Strontium Effects on Bone Mechanical Properties

The flexural modulus and stiffness of the control group were significantly lower than that of the low dose group ($p<0.05$) (see Figures B-20c and B-20d). Differences in parameters between the high dose group and the other groups were insignificant.

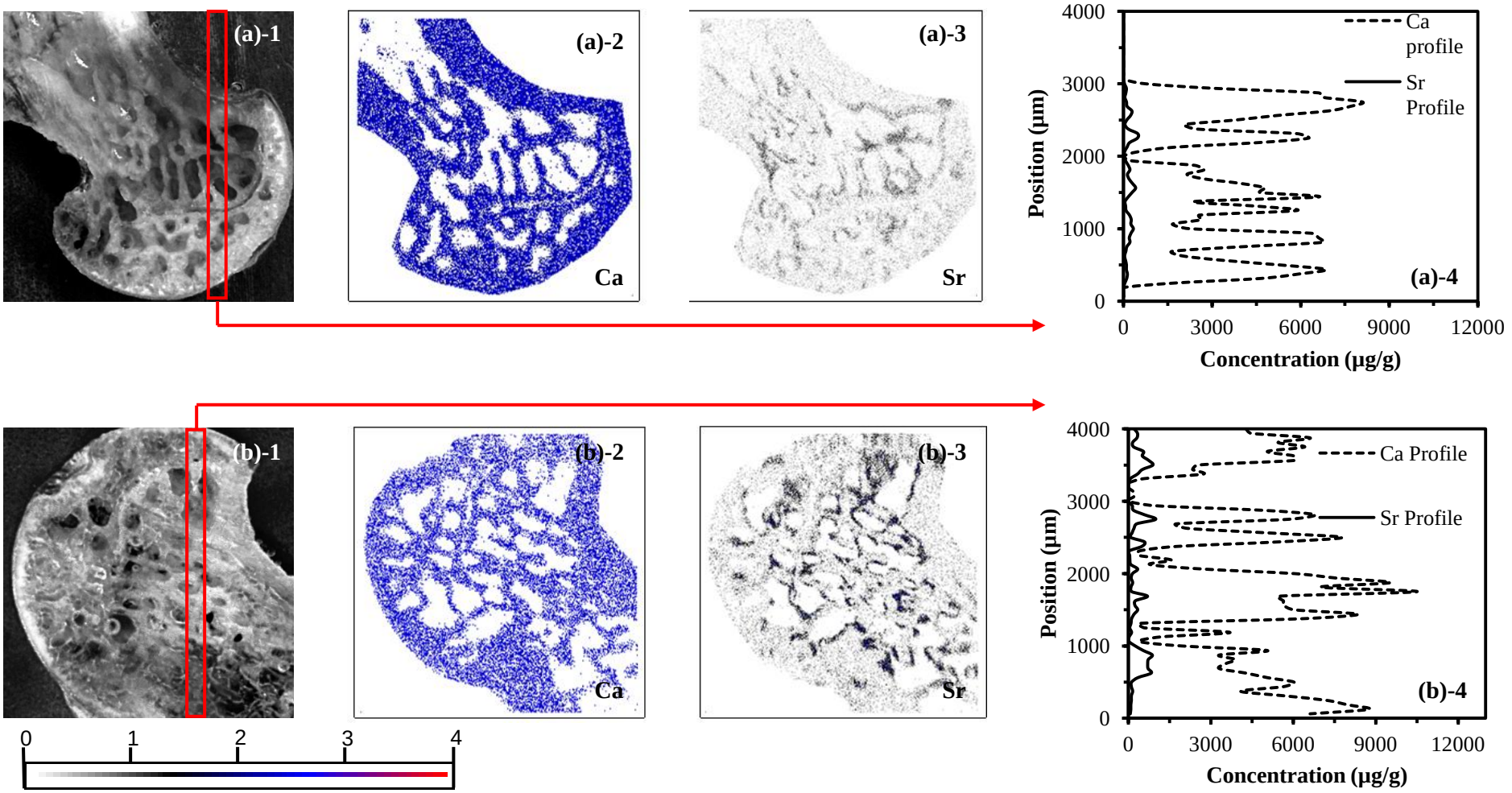

Figure B-15: PIXE scans of femoral heads of (a) low Sr dose group and (b) high Sr dose group. The measured values were corrected for filter factors and expressed in logarithmic scale.

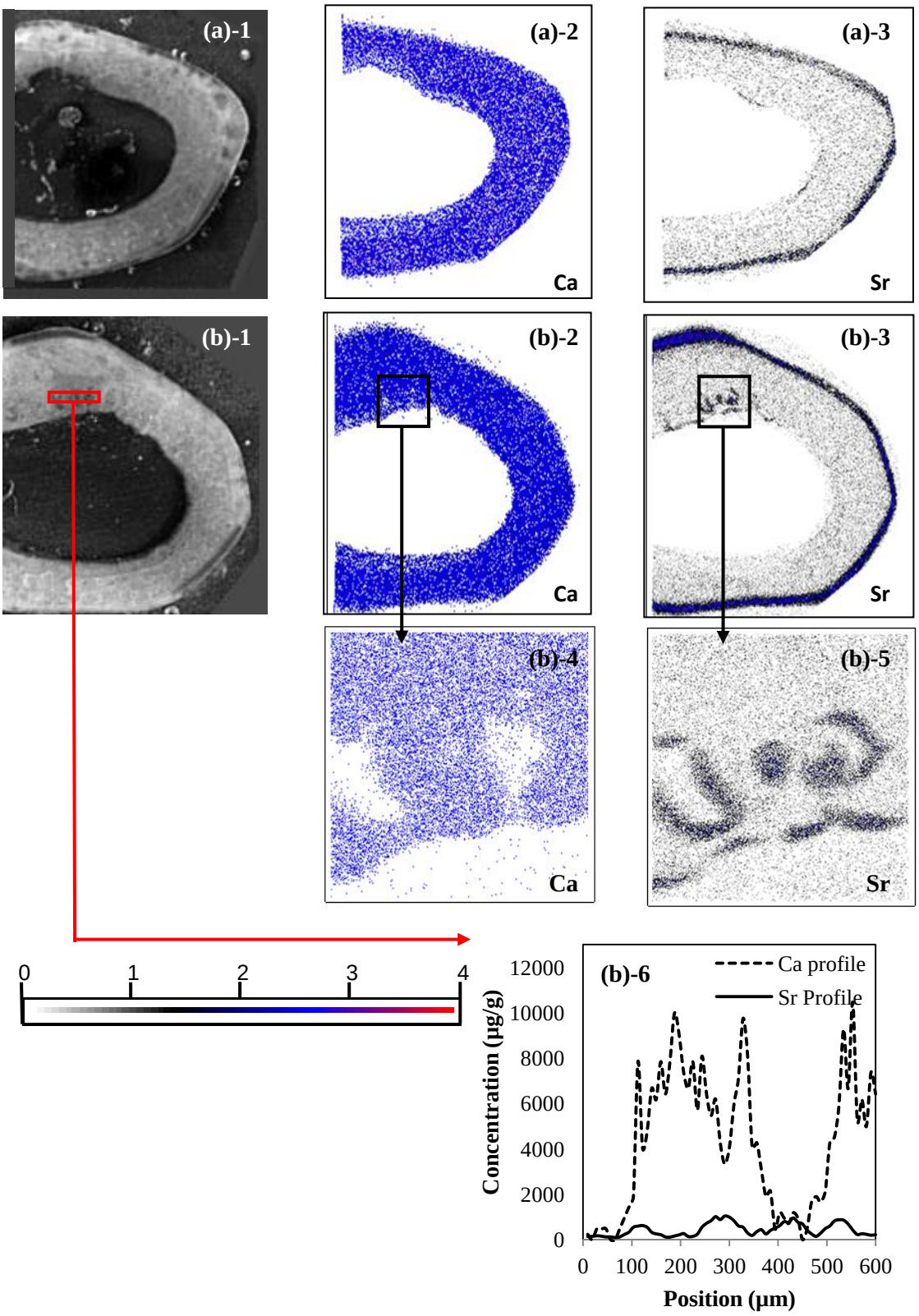

Figure B-16: PIXE scans of mid-shaft femurs: (a) low Sr dose group and (b) high Sr dose group. The measured values were corrected for filter factors and expressed in logarithmic scale.

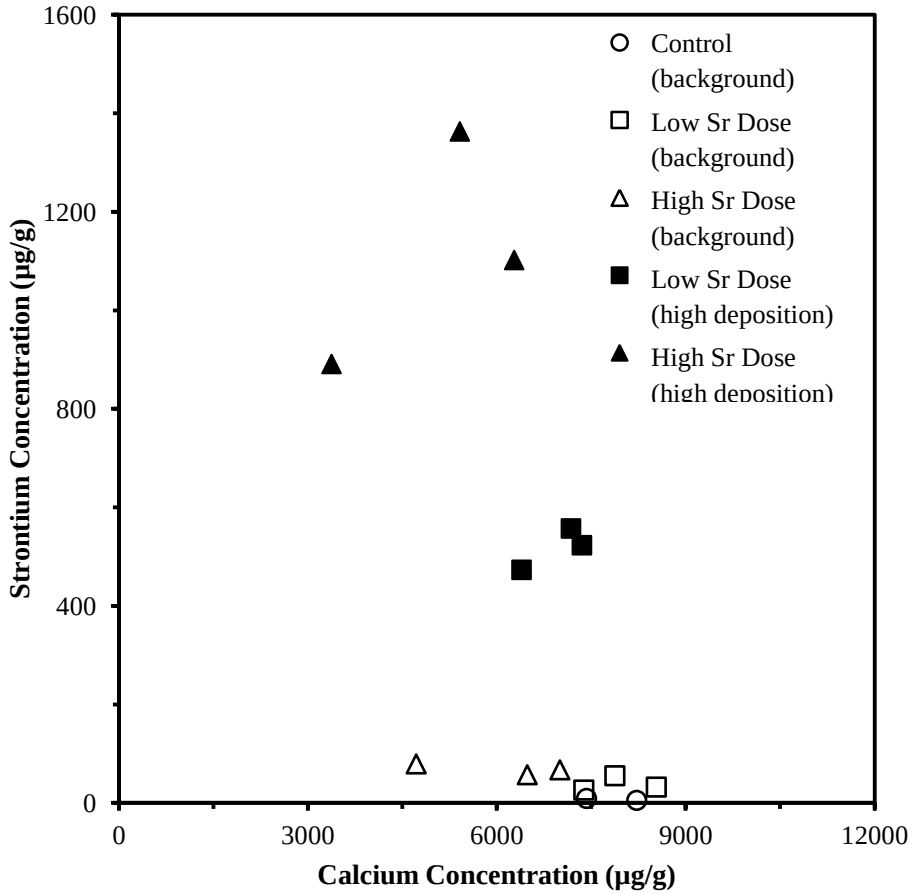

Figure B-17: Correlation between calcium and strontium concentration in the bone samples from the control, low Sr dose and high Sr dose groups. The background readings from the area with no elevated strontium deposition (represented with hollow markers) are scattered at the lower side of the chart. The readings from the area with high strontium deposition (represented with solid markers) are scattered at the middle and upper side of the chart.

3.3.4. Strontium Effects on Bone Biomarkers

Serum concentration of CTX as a biomarker of bone resorption was not significantly different between the different groups (see Figure B-21a). For P1NP as a biomarker of bone formation, serum concentrations for the baseline group were significantly higher compared to all other groups ($p<0.05$; see Figure B-21b). However, no significant difference was observed between the control group and the treatment group.

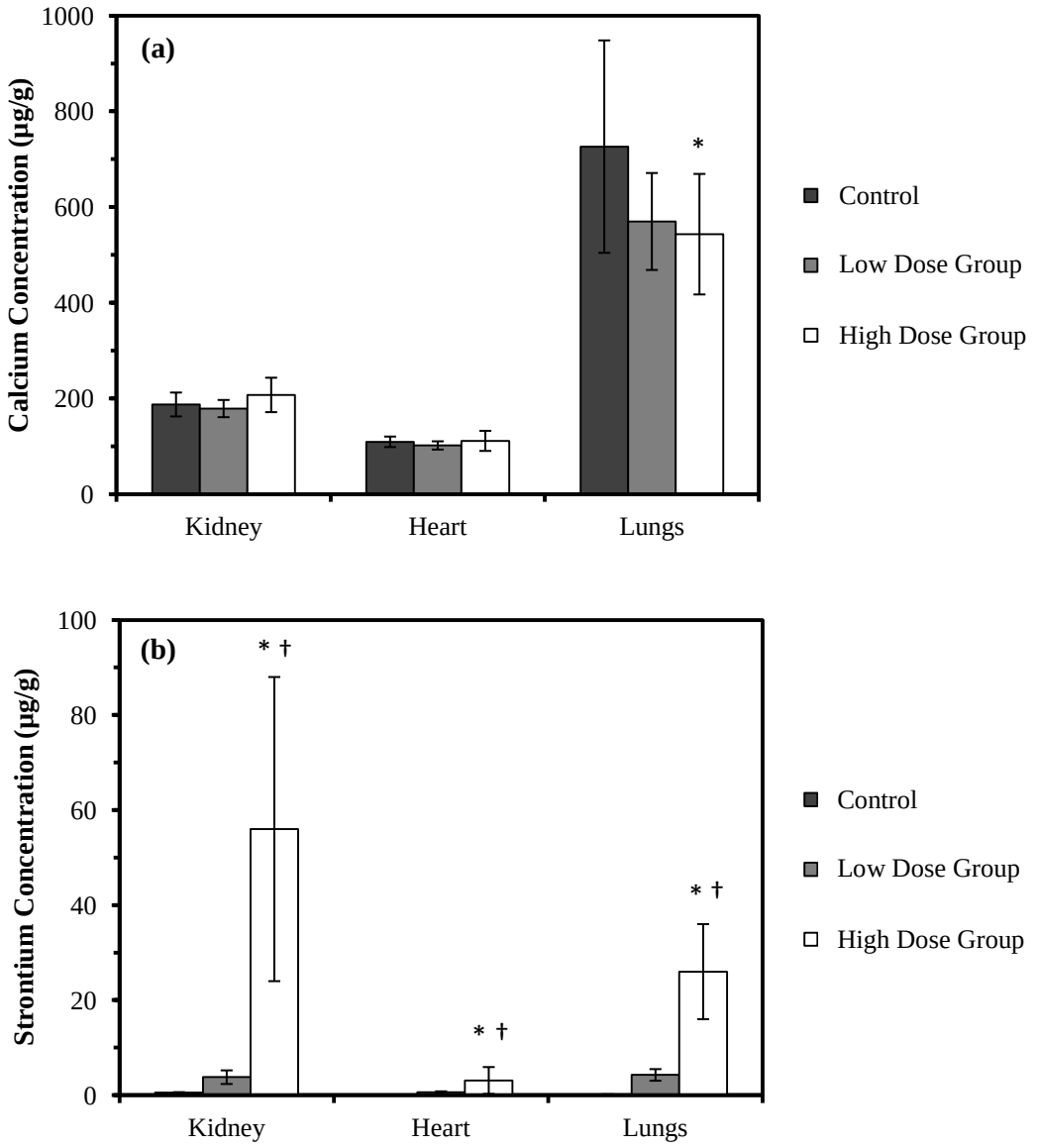

Figure B-18: Concentrations of calcium (a) and strontium (b) in the kidneys, hearts and lungs as measured in the different study groups. Symbols indicate statistically significant differences between groups (p=0.05): *significantly different from control group; †significantly different from low dose group (n=9 for control group, n=10 for low dose and high dose group). Each bar represents the mean strontium concentration (±1 SD) as measured in a given group. A dose dependent increase of strontium concentration was observed in all soft tissues, while calcium concentrations were relatively similar across groups. Strontium concentrations in soft tissues were found to be insignificantly increased in the low dose group as compared to the control group. In contrast, increases in strontium concentration were substantial for the high dose group's soft tissues.

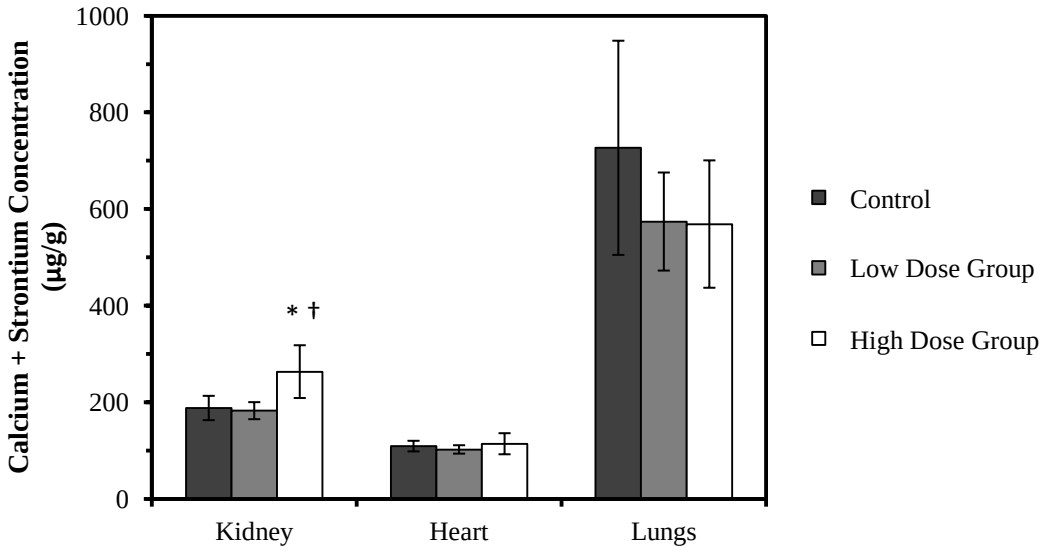

Figure B-19: Total amounts of calcium and srontium in the kidneys, hearts and lungs of rats as measured in the different study groups. Symbols indicate statistically significant differences between groups (p=0.05): *significantly different from control group; †significantly different from low dose group (n=9 for control group, n=10 for low dose and high dose group). Each bar represents the mean strontium concentration (±1 SD) as measured in a given group. Differences in combined calcium and strontium concentration of organs were statistically insignificant between study groups except for the kidneys.

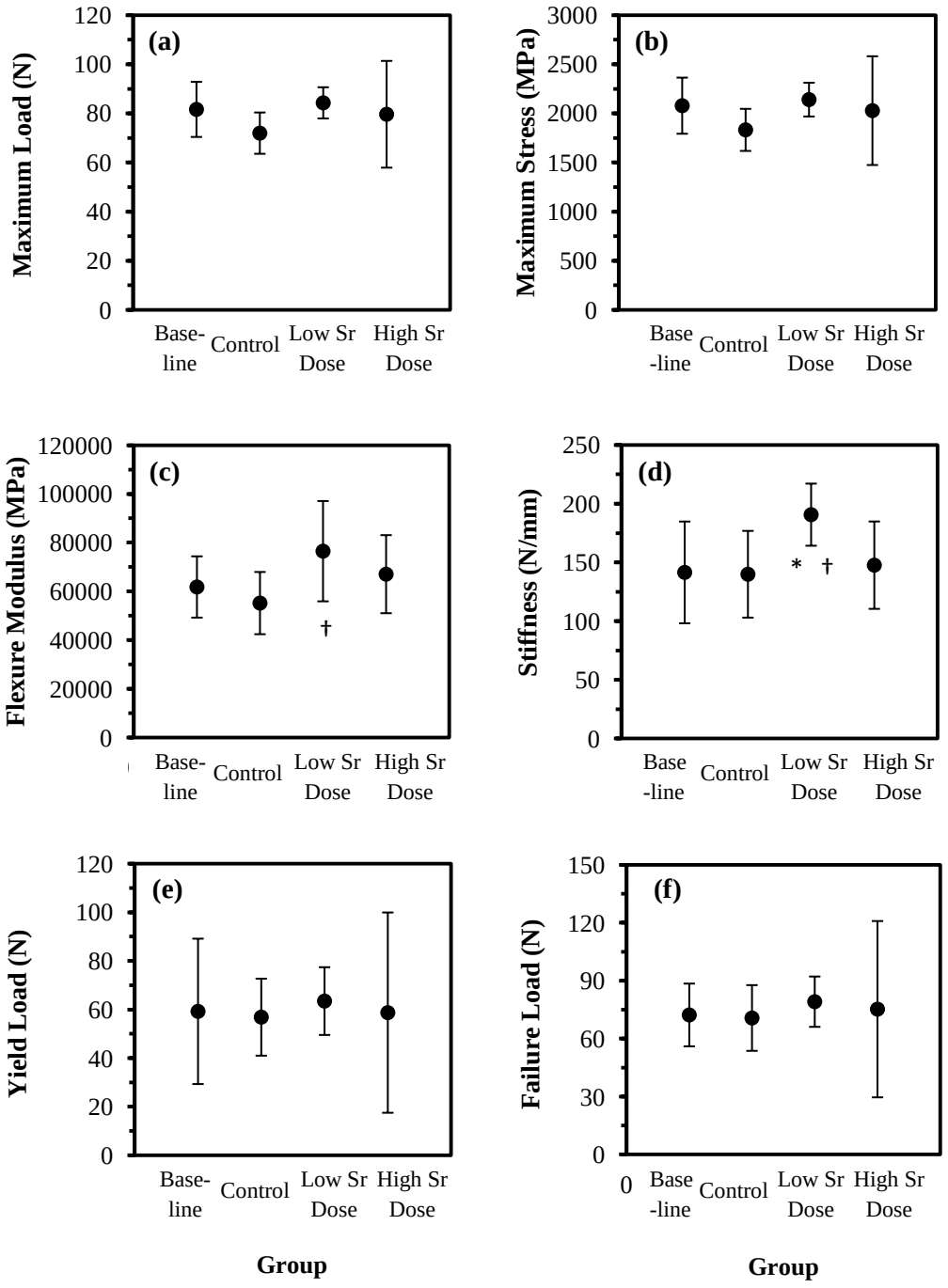

Figure B-20: Mechanical properties of collected bone samples. Symbols indicate statistically significant differences between groups (p=0.05): *significantly different from baseline group; †significantly different from control group. Each bar represents the mean value (±1 SD) as measured in a given group. The flexural modulus and stiffness of the control group were significantly lower than that of the low dose group. Differences in parameters between the high dose group and the other groups were insignificant.

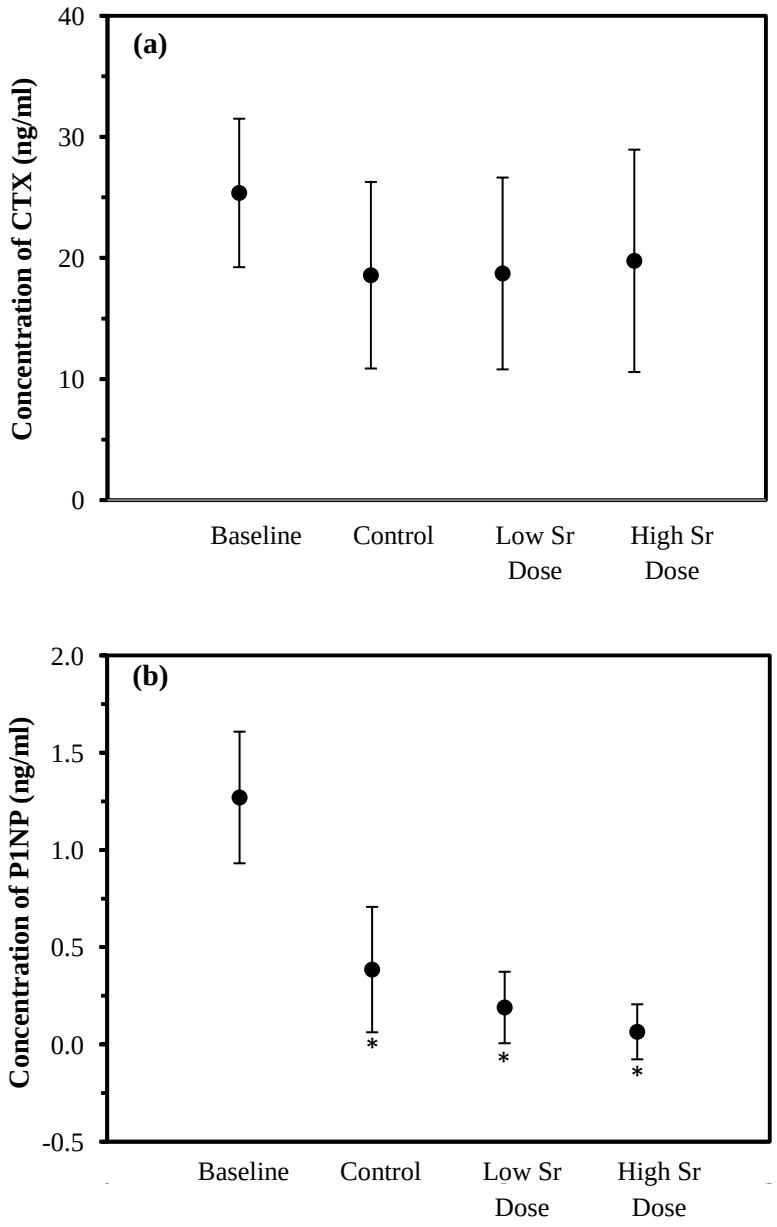

Figure B-21: Serum concentration of bone resorption (a) and formation (b) markers in the studied groups. Symbols indicate statistically significant differences between groups (p=0.05): *significantly different from baseline group. Each bar represents the mean concentration (±1 SD) as measured in a given group. No significant difference in the serum concentration of CTX as a biomarker of bone resorption, was observed. For P1NP as a biomarker of bone accretion, serum concentrations for the baseline group were significantly higher compared to all other groups.

3.4. Discussion

In the present study we observed dose-dependent differences in serum strontium levels at the end of strontium treatment. This is in good agreement with observations by

Marie (*310*) and Ammann *et al.* (*448*) who also found that strontium concentrations in serum stabilized after 3 month of strontium administration. The ratio of serum strontium in the high dose group to the low dose group is approximately 4 in our study (see Figure B-14a) and is equal to the ratio of the intake of strontium from their drinking water. This indicates no suppression of strontium absorption at higher intakes.

Previous studies have shown that strontium exposure in rats receiving 625 mg/kg/day of strontium ranelate for 44 weeks (equivalent to 213 mg/kg/day of elemental strontium) was comparable to that of humans receiving 500 mg of strontium per day in the form of strontium ranelate as preventive treatment for 52 weeks (*592*). Serum strontium concentrations in humans after 1 year of strontium treatment were 12.4±5.9 µg/ml in CL3-032 (*593*), 11.2±5.9 µg/ml in TROPOS (*455*) and 10.2±6.7 µg/ml in SOTI (*184*) as the available phase 3 trials for the drug. This compares well to a serum strontium concentration of 9.3±2.8 µg/ml in the rats in the low dose group in our study that received only 98±15 mg/kg/day of strontium in form of strontium lactate, i.e. more than half times less than in comparable studies using strontium ranelate as the strontium source. After 52 weeks of strontium ranelate treatment at 625 mg/kg/day (equivalent to 213 mg/kg/day of elemental strontium), serum strontium level in ovariectomized rats was only elevated to 9.0±2.3 µg/ml in the study by Bain *et al.* (*594*). However, direct comparisons between studies are difficult due to differences in age, gender and ovariectomization status of the rats.

Lactate and lactic acid were found in earlier studies to enhance the bioavailability of calcium. In absence of comparable studies, the same may hold true for strontium. Both elements share the same pathways of absorption and excretion. Calcium from calcium lactate was found to be better absorbed than calcium gluconate in two human studies (*595, 596*). In another study in rats, calcium lactate was found to have a greater bone-stimulating activity than calcium carbonate and citrate (*597*). It has been suggested that lactate and/or lactic acid binds to calcium in the intestine and increases its solubility at intestinal pH (*598, 599*). Lactic acid also regulates release of the gastric moiety from the stomach to the small intestine (*600*)

which may also affect calcium absorption (*601*). In contrast to these findings, Tsugawa *et al.* (*602*) observed no significant difference between the bioavailability of calcium lactate and carbonate in rats.

Similar to serum, we have also observed a dose-dependent increase in strontium concentration in the skeleton. A three-fold higher strontium content of bone was observed in the high dose group relative to the lower dose group. This compares to a factor of four for the concentration of strontium in serum and feed. Strontium content of rat femur also increased in an earlier study in a dose-dependent manner up to 255 mg/kg/day of strontium in the feed but the relative increase was less from 93.5 to 255 mg/kg/day than from 0 to 93.5 mg/kg/day (*310*).

For the studied soft tissues we found clear indications of strontium accumulation with increasing strontium dosage. Strontium treatment increased strontium concentration in tissues for the low dose group by a factor of 7 to 27 relative to the control group. At a factor of 78 to 164, the increase in strontium concentrations in the high dose group was much more substantial and clearly disproportionate to the increase in strontium intake and serum strontium concentration between groups. Nevertheless, strontium is still overall a trace element and risks of tissue mineralization should be considered together with calcium. In the low dose group, there was no significant increase in its combined strontium and calcium content. However, it could be observed in the kidney in the high dose group. This can be problematic as oxalates and phosphates are present in the kidney and they can form poorly soluble salts both with calcium and strontium. Excess strontium supplementation, however, appears to be of less concern for the heart than for the kidney in terms of tissue mineralization. There was no significant increase in the total amounts of calcium and strontium in heart even at the higher dose. Calcium data for the lungs has been excluded from discussion due to the markedly higher levels of calcium in the control group as compared to the treatment groups and the poor consistency of the results. This was due to the difficulty in

properly perfusing capillary blood vessels in the lungs. Remaining blood may have introduced sufficient amounts of calcium and strontium to artificially inflate results.

Substantial strontium accumulation in the kidney and heart of the treatment group as compared to the control was also observed in the earlier rat study by Skoryna (*460*). The treatment group received strontium in drinking water for 12 weeks at a dose (0.34% $SrCl_2$ w/w) that was similar to the low dose in this study. Their findings are in agreement with ours. The changes in calcium levels were insignificant. As a result, the amount of total calcium and strontium were relatively unaffected. On the other hand, Aranyosiova *et al.* (*603*) who fed 400 mg/kg/day of strontium to a group of rats for five weeks did not observe any strontium deposition in soft tissues at all. This could be explained by their lower measurement sensitivity as they quantified strontium by mass spectrometric imaging and not chemical analysis.

Based on our findings, we suggest that there is a maximum capacity for bone mineral uptake from circulation above which excess calcium or strontium will remain in circulation and increases risks of tissue mineralization. This could explain why disproportionately more strontium was deposited in organs in the high dose group as compared to the low dose group while less strontium was deposited in bone. Serum strontium concentration between treatment groups differed four-fold and was proportional to strontium intake. In contrast, organ strontium concentration was higher by a factor of 5-15 for the high dose group while bone strontium concentration increased by a factor of three only. In general, bone can bind cations including strontium in the calcification process, by later heteroionic exchange against calcium in the hydroxyapatite crystal or by ionic exchange at the surface of mineralized bone (*293*). Kinetic tracer studies in humans point conclusively to calcium pools that differ in the rate by which they exchange calcium with the serum calcium pool (*269, 271, 424, 534*). It has been suggested that the fast exchanging pool comprises mainly of loosely bound calcium while the slow exchanging pool is supposed to represent mineralized bone. Fast exchange of calcium in serum and bone is pivotal for maintaining calcium homeostasis. Bone accretion and

resorption are processes that are much too slow to respond to the bodies' immediate needs. Capacities of bone surface to remove calcium and strontium from circulation by ion exchange, however, are naturally limited.

Besides deposition in bone, risks of tissue mineralization by high strontium intakes might also be mitigated by the competition of strontium and calcium for transfer across biological membranes and, thus, organ uptake. Previous studies showed that although serum strontium concentration increased parallel to oral intake, serum calcium level remained unchanged in the range of 90.3 – 102.1 µg/ml (*460*, *604*). There was a similar finding for organs in our study. Strontium exposure did not decrease tissue calcium content. In agreement with our finding, Skoryna (*460*) showed that although the amount of strontium in the kidney and heart had increased when the rats were fed with strontium, there was no corresponding decrease in the amount of calcium. Hence, strontium does not appear to replace calcium even when it is present at higher levels.

In earlier strontium supplementation studies in animal and human models, calcium concentration in bone at the end of the experiment did not differ between groups (*291*, *302*, *563*, *605*). This shows that strontium uptake by the skeleton does not deplete bone calcium. Li *et al.* showed in bone biopsies of osteoporosis patients treated with strontium ranelate for 3 years that most of the ingested strontium was absorbed on the bone crystal surface while only a limited amount of strontium replaced calcium in hydroxyapatite crystals (*563*).

PIXE analysis in our study revealed that calcium and strontium were deposited nearly homogenously across all bone sites with certain areas having high strontium deposition. Calcium concentration in the mid-cortical region was higher than in the endosteum and periosteum, in general. In the treatment groups, strontium concentration was highest in the periosteum followed by the endosteum while the mid-cortical was lowest. This is in agreement with an earlier study by Li *et al.* and Boivin *et al.* in bone biopsies of patients treated with strontium ranelate (*563*, *606*). Higher strontium concentration in endosteum and periosteum illustrated that therapeutic strontium tends to concentrate in areas where new bone

is actively formed. This is in good agreement with several studies in ovariectomized monkeys and osteoporotic women treated with the drug which also showed that more strontium enters new bone than old bone in therapeutic regimens (*302, 607-609*).

Elemental mapping by PIXE also showed that at certain areas in the skeleton, the strontium to calcium ratio can go up to 0.074 in the low dose group and up to 0.257 in the high dose group. Although Boivin *et al.* found that only 1 in 10 calcium can be replaced by strontium, our finding shows that when the amount of strontium given to the rats is high enough, more strontium can enter and be deposited in the skeleton regardless of the amount of calcium present (*302*). However, a depletion of calcium in the shaft can be observed in the endosteum of the mid diaphysis in the high dose group. In contrast to the mid diaphysis of the low dose group that has a fully calcified surface, some cavities can be observed in that of the high dose group (see Figure B-16). The cavities were surrounded by high strontium deposition. This observation could be related to strontium rachitis. In several studies, chronic exposure to high strontium doses caused several skeletal abnormalities, including calcification defects, a lower mineral content and bone density, etc (*300*).

Growing strontium rachitic or mineralization defects could explain the finding that strontium improved mechanical bone properties only in the low dose group, i.e. the group in which serum strontium concentrations were induced similar to humans undergoing strontium ranelate therapy. In comparison to the control group, the low dose group showed significantly higher flexural modulus and stiffness. For the high dose group, no significant differences relative to the control and the low dose group were observed (see Figure B-20). In agreement with our findings, previous studies in rats revealed that a threshold exists (strontium content in diet < 0.4% w/w) up to which an increased dietary strontium intake increases bone formation rate as well as trabecular bone density (*610, 611*). At higher intakes, strontium can potentially disturb bone mineralization (*466, 471*). Previous studies showed that the action of strontium involves the dissociation of bone formation and resorption, rebalancing bone remodeling towards formation (*612, 613*). This mode of action has been verified in animal

studies as well as clinical trials (*614*). In our study, no significant difference was observed in the serum concentration of CTX as a bone resorption marker between treatment groups and the control group (see Figure B-21a). Similar findings were reported by Ma *et al.*, who also found no indications for a change in bone resorption rate in ovariectomized rats after 3 months of strontium ranelate therapy (*615*). The significant decrease in serum P1NP concentration we have observed in the control, low dose and high dose groups compared to the baseline group could potentially be the result of prolonged immobilization of the rats in their cages. Lack of physical activity is well-known to negatively affect bone health in animals and man (*616*). However, the relatively small sample size in our study must be considered when comparing biomarker data in our study with previous observations. A recent human study by Chavassieux *et al.* (2013) has confirmed that strontium ranelate has a mild negative effect on bone formation and insignificant effect on bone resorption (*617*). However, clinical trials have provided strong evidence for the effectiveness of strontium ranelate in fracture prevention. Hence, it is likely that strontium ranelate works physically, rather than biologically, to offer this fracture prevention capability.

3.5. Conclusion

Several studies in animals and humans have demonstrated the potential of strontium salts to positively affect bone health. This refers in particular to strontium ranelate, the only strontium drug approved so far for osteoporosis treatment. A positive effect on bone could also be confirmed in our study in the low dose group which exhibited strontium concentrations in serum comparable to those observed in patients undergoing strontium therapy.

Concerns have been raised recently by the European Medicines Agency regarding the safety of strontium ranelate treatment. Restrictions to its use have been recommended to mitigate higher risk for heart attacks observed in patients undergoing strontium therapy. As for calcium supplementation, high strontium intakes may exacerbate risks of tissue

mineralization and artherosclerosis by formation of poorly soluble strontium salts. Disproportionate changes in bone and soft tissue content relative to strontium levels in feed and serum let us hypothesize that a threshold level may exist from which strontium is taken up less efficiently by bone and risks for tissue accumulation were increasing. The same may hold true for calcium due to their physiological and chemical similarity.

Strontium ranelate is used at a dose level (500 mg/day of strontium) that is comparable to regular calcium supplementation which has been suggested to increase risks of soft tissues calcification. On top of that, strontium ranelate treatment is recommended to be administered together with up to 1,000 mg of calcium and 400 IU or 800 IU of vitamin D depending on the patients' needs (*478*). The combined strontium and calcium intake may approach or even exceed the tolerable upper intake level of 2,000 mg calcium per day (IOM, 2010). This would point to a reconsideration of dosage levels for strontium therapy and/or practices of concomitant intake of calcium supplements.

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CHAPTER 4: AGE-DEPENDENT DIFFERENCES IN CALCIUM AND MAGNESIUM INTAKE IN SINGAPOREAN CHINESE WOMEN

4.1. Introduction

Osteoporosis is a skeletal disease characterized by low bone mass and microarchitectural deterioration of bone causing an increase in bone fragility and vulnerability to fractures (*2*). Postmenopausal women and the older population are at an elevated risk of developing osteoporosis (*8, 618*). With growing life expectancy (*1*), osteoporosis is on the rise (*9, 619*). Effective strategies are needed for its prevention and treatment not only to alleviate individual burden but also dampen economic impact on public health systems in the coming decades (*5*).

Calcium is the most abundant cation in the human body, of which 99% is bound in the skeleton as hydroxyapatite crystals. The remaining calcium is contained in the extracellular fluid, blood, and soft tissues. Besides its structural role, bone functions as a reservoir of calcium which is readily available to fulfill its roles in multiple physiologic and biochemical processes including neuromuscular functioning, blood coagulation, cell permeability, enzyme activation and hormone secretion (*32*). Negative calcium balance inevitably results in bone loss and a lowered structural stability of bone in the long-term (*620*). Generally accepted strategies to reduce osteoporosis risks at advanced age include maximization of peak bone mass during childhood and puberty and to minimize net calcium losses during adulthood. Hence, a sufficient dietary calcium supply throughout life together with vitamin D for facilitating its absorption is widely considered a major pillar of osteoporosis prevention (*621-623*).

Besides calcium, magnesium is also believed to play a role in bone health, yet its actual role in bone metabolism is poorly understood. Normal magnesium body content is around 20-30 g. About half of it is located in bone where it is mostly present as a surface constituent of the hydroxyapatite crystal (*84*). Because bone undergoes continuous

remodeling, an adequate supply of magnesium as well as other nutrients important to bone is needed to support bone formation. Studies have shown that magnesium deficiency could affect bone growth, osteoblastic and osteoclastic activity, as well as induce osteopenia and bone fragility. It may also alter calcium metabolism by affecting calciotropic hormones (*624*). Furthermore, it is necessary for activation of more than 300 enzyme systems making it an essential nutrient in a plethora of physiological processes. An insufficient magnesium supply has been associated with an increased risk for diabetes mellitus type 2, hypertension and atherosclerosis but findings are still inconclusive (*625-628*).

Singapore is an example of an Asian country that has experienced significant changes in lifestyle including dietary habits in response to rapid socioeconomic developments. This is evident, in particular, in the younger generation which is presumably more westernized and more open to other ethnic cuisines in the local, highly multi-cultural context than the older generation. In this study we tried to assess (1) differences in dietary habits between the younger and older generation in Singapore and how they affect their calcium and magnesium intakes; (2) the adequacy of the mineral intakes with reference to current requirements; and (3) the contribution of food fortification and dietary supplements to calcium and magnesium intake. An integral part of the project was the development of an in-house database for calcium and magnesium content of local foods and the design and validation of a strategy for assessment of calcium and magnesium intake in the Singaporean context. In parallel, we have estimated dietary intake of strontium, a bone seeking element that is known to reduce fracture risk at therapeutic doses (*455, 629*).

4.2. Methods

4.2.1. Subjects

Two groups of Singaporean women of Chinese descent, aged 18-30 years-old (n=94) and ≥ 55 years-old (n=75), were recruited from the local population. The survey was limited to women as the group at higher risk of contracting osteoporosis and to ethnic Chinese as the

dominant ethnic group (74.2%) (*630*). Subjects were balanced for their highest educational degree in each group according to recent demographic data (*630*). Educational level is a major determinant of socioeconomic status and known to influence dietary habits (*631, 632*). General health status and dietary habits were assessed through a guided questionnaire. Subjects who had major dietary changes over the past six months and/or had to restrict their diet were excluded from the study. Vegetarians were not purposely excluded but there were none in both subject groups. Vegetarianism is not widely practiced among Singaporeans of Chinese descent. Written informed consent was obtained from all subjects and the protocol was approved by the Institutional Review Board of the National University of Singapore.

4.2.2. In-house Calcium and Magnesium Database

The in-house database covers calcium and magnesium contents from over 2,700 entries of food and drink items that were considered relevant to the Singaporean context (see Appendix). Data were compiled mainly from six food composition tables: Food Composition Guide Singapore (*633*), McCance and Widdowson's The Composition of Foods (5^{th} Ed) (*634*), Pacific Islands Food Composition Tables (*635*), ASEAN Food Composition Tables (*636*), Thai Food Composition Tables (*637*) and Food Composition and Nutrition Labels (6^{th} Ed) (*638*). Additional information on calcium content of some food and drinks were also obtained from food labels, Singapore Food Facts (*639*) and the United States Department of Agriculture (USDA) National Database for Standard Reference (*640*). For highly relevant food items that are known to have a high mineral content and/or are frequently consumed by the majority of the studied population group, content was determined in-house by Flame Atomic Absorption Spectroscopy (F-AAS). After data compilation, food items were categorized into groups based on their calcium contents and their expected consumption frequency. The nine major food groups were: staple foods and carbohydrate sources; vegetables and vegetable products; meat, poultry, eggs and their products; fish, seafood and their products; soybean products; topping, sauces and condiments; fruits; dessert and snacks;

and dairy products. Under each main group, food items were grouped further and each sub-group was assigned a standard portion size and a calcium and magnesium content, respectively. Assigned values were rounded to only one or two significant figure(s). Mineral contents can vary by more than 50% between batches for the same food items for most foods. Assigned values were based primarily on concentrations determined in-house (if available), followed by those reported in food composition tables or in the scientific literature and, lastly, food labels. If different brands of the same product showed highly variable calcium contents, assignments were brand specific and were based on mineral contents as indicated on the food label. Standard portion sizes for main food items were determined based on information obtained from food composition tables, food packaging labels and weighing of food items purchased from food vendors. In the process of developing the database, the composite dishes and the spices and additives used as ingredients, especially those rich in calcium, were given extra consideration.

4.2.3. Assessment of Mineral Intake

Mineral intake was assessed in this survey by three-day food records. Subjects were briefed both orally (in English or Mandarin) and in writing about the study. They were also provided with two samples of completed food records and a set of picture aids to help them estimate portion sizes. During the study periods (two weekdays and one weekend), subjects were asked to write down all the food and drinks directly after consumption including portion sizes, brands and methods of preparation. For composite meals they were asked to write down major food items in the dish. Portion sizes were standardized for recording in terms of measurable units e.g. number of rice bowls, number of tablespoons, etc. In addition, subjects were asked to write down any nutritional supplements including brand names and number of pills/tablets they have consumed during the recording period. Food Records were analyzed using the developed in-house database for mineral content. Average values for individual

mineral intake were obtained by weighting of data obtained on two weekdays and Saturday/Sunday at a 5/2 ratio.

A duplicate diet study was conducted for method validation. Subjects (n=24) were asked to collect all foods and drinks they consume in duplicate for any two weekdays and one Saturday or Sunday and to complete food records on each of the three days in parallel. Calcium and magnesium content of the duplicate diet samples was analyzed (measured daily intake) using F-AAS as well as estimated using the in-house database. For evaluation, estimated and measured mineral contents were plotted against each other. Slope of the regression line and goodness of fit (R^2) was used to assess accuracy of estimates for mineral intake in population assessment. Strontium intake was estimated by analysing collected duplicate diets for strontium content by Graphite Furnace Atomic Absorption Spectroscopy (GF-AAS). Assessment of strontium intake through the collected food records was not possible as data on strontium content of foods in the literature are scarce and insufficient for the intended purpose.

4.2.4. Sample Analysis

Sampled foods as well as the collected duplicate diets was analyzed for calcium and magnesium content by F-AAS (AA240 FS, Varian Pty. Ltd., Victoria, Australia) and for strontium content by Graphite Furnace AAS (GF-AAS, AA240 Zeeman, Varian Pty. Ltd., Victoria, Australia). For sub-sampling, solid samples were blended with a standard food blender after adding a measured amount of Millipore water (Milli-Q-System) and homogenised using a laboratory blender (SilentCrusher M; Heidolph Instruments GmbH & Co. KG, Kelheim, Germany). Drink samples were stirred until they were mixed well or homogenized when they contained solids. Three aliquots were taken from each sample. Samples were mineralized by microwave-assisted wet digestion using an Ethos 1 digestion unit (Milestone Inc., Sorisole, Italy). Homogenized samples were weighed into pre-cleaned Teflon vessels, mineralized using a mixture of 8 ml concentrated nitric acid, HNO_3 and 2 ml

30% hydrogen peroxide (Merck, Darmstadt, Germany) and diluted with water after digestion to the optimal concentration range for analysis. All chemicals used for sample preparation were of analytical grade. Only ultrapure water was used (Milli-Q-System, Millipore, MA, USA) and acids were cleaned further by surface distillation. Element concentrations were measured by external calibration using commercial standards (Titrisol®, Merck, Darmstadt, Germany). Calcium and strontium were analyzed with KCl (Scharlau, Barcelona, Spain) as an ionization suppressant at a spiked K concentration of 2,000 µg/g in the sample solutions.

The F-AAS was equipped with a 10 mA calcium hollow cathode lamp and was operated at a slit width of 0.5 nm and a wavelength of 422.7 nm. A nitrous oxide-acetylene flame was used for the determination of calcium. The same system equipped with a 4 mA magnesium hollow cathode lamp operated at a slit width of 0.5 nm and a wavelength of 285.2 nm was used for the determination of magnesium with an air-acetylene flame. The GF-AAS was equipped with a 10 mA strontium hollow cathode lamp and was operated at a slit width of 0.5 nm and a wavelength of 460.7nm. High purity argon gas (Soxal, Singapore) was used to flush the graphite tube. All readings were performed in triplicate in integration mode for FAAS and height mode for GF-AAS to determine absorbances and corrected for analytical blanks. For calcium and magnesium, analytical accuracy was assessed by processing a certified reference material (SRM1567a, wheat flour; National Institute of Standards and Technology, NIST, Gaithersburg, USA). For strontium, analytical accuracy was assessed by analysis of a gravimetrically prepared laboratory standard because of a lack of commercially available reference materials certified for strontium concentration.

4.2.5. Statistical Analysis

Testing for distribution was done by Kolmogorov-Smirnov and Shapiro-Wilk test. Since calcium and magnesium intake data were not normally distributed, Mann-Whitney U-test was used to perform non-parametric comparison of mineral intakes between subject groups. Mann-Whitney U-test and Kruskal-Wallis-H test were used to compare mineral

intakes of subjects which were categorized by socio-economic and health factors as well as dietary habits to assess for any possible confounders. Factors assessed using Mann-Whitney U-test included whether the participant had any diagnosed health condition (including hypertension, diabetes, chronic disease(s), etc.). Factors assessed using Kruskal-Wallis H-test included: (1) highest educational degree, (2) body mass index (BMI) and (3) physical activity level. Mann-Whitney U-test was also used to compare the mineral intakes of subjects from each individual food group.

4.3. Results

4.3.1. Subject Characteristics

Average age was 24±3 years for the younger subjects and 63±8 years for the older subjects. Table B-2 shows the subject characteristics assessed including highest educational degree, body mass index (BMI), location where most meals are taken (at home/outside), level of physical activity, diagnosed health conditions and calcium and/or magnesium supplement consumption. Factors which were found to be statistically associated with and therefore might have confounding effects on mineral intakes were location where most meals were taken (food stalls/restaurants versus home) for magnesium and supplement consumption for both calcium and magnesium.

Subject characteristics were within expectations for the Singaporean context. Virtually all young Singaporeans have a Diploma or University Degree while the majority of the older population has joined the workforce after secondary school. Average BMI was higher in the older (23.1 ± 3.6) than in the younger women (20.2 ± 2.6) with more of the younger subjects below cut-offs for underweight (26%) and more of the elderly women above cut-offs for overweight (43%) when applying current WHO recommended for BMI classification for Asian populations (*641*). Physical activity level of the younger and the older women was comparable with about 1/3 in both groups following a sedentary lifestyle. While most of the younger women (91%) had no diagnosed health condition, about 2/3 of the older

Table B-2: Subject groups' characteristics.

Age Group	18 – 30 Years Old		≥ 55 Years Old		Overall	
n	94		75		169	
Average age	24 ± 3 years		63 ± 8 years		41 ± 21 years	
Highest educational degree (n)						
No formal qualification	0	0 %	18	24 %	18	12 %
Primary school	0	0 %	9	12 %	9	6 %
Secondary school	0	0 %	33	44 %	33	22 %
Diploma course	33	35 %	11	15 %	44	25 %
University degree	61	65 %	4	5 %	65	35 %
BMI (n)						
<18.5 kg/m^2	24	26 %	1	1 %	25	13.5 %
18.5-23.0 kg/m^2	55	58 %	44	59 %	99	58.5 %
>23.0 kg/m^2	15	16 %	30	40 %	45	28 %
Physical activity level (n)*						
None	31	33 %	22	29 %	53	31 %
Category 1	28	30 %	29	39 %	57	34.5 %
Category 2	15	16 %	7	9 %	22	12.5 %
Category 3	20	21 %	17	23 %	37	22 %
Diagnosed health condition (n)						
None	86	91 %	28	37 %	114	64 %
Yes	8	9 %	47	63 %	55	36 %
Meals taken mostly at (n)						
Home	29	31 %	45	60 %	74	45.5 %
Outside	65	69 %	30	40 %	95	54.5 %
Ca / Mg supplements						
Ca	4	4 %	14	19 %	18	11.5 %
Mg	0	0 %	0	0 %	0	0 %
Ca + Mg	7	8 %	11	15 %	18	11.5 %
None	83	88 %	50	66 %	133	77 %

*Physical activity level.

None: sedentary lifestyle

Category 1: ca. 20 minutes of moderate-intensity physical activity 5 days per week or 10 minutes of vigorous-intensity physical activity 3 days per week or equivalent.

Category 2: ca. 30 minutes of moderate-intensity physical activity 5 days per week or 20 minutes of vigorous-intensity physical activity 3 days per week or equivalent.

Category 3: ca. 45 minutes or more of moderate-intensity physical activity five days per week or 30 minutes or more of vigorous-intensity physical activity 3 days per week or equivalent.

women were found to be diagnosed with a disease. In agreement with common Singaporean habits, most meals (69%) were taken by the younger women outside at public places while 60% of the older women reported to prepare and eat their meals mostly at home. Consumption of calcium or magnesium supplements was more common in the older subject group (43%) as compared to the younger women (14%). In general, calcium supplements were found to be more commonly consumed than magnesium supplements.

4.3.2. Method Validation

Figure B-22 shows the results of the duplicate diet study. At a slope of correlation lines close to 1.00, measured and estimated average intakes were found to agree remarkably well over the 3-day study period for both elements. Residuals were 18±86 mg/day for calcium and 2±37 mg/day for magnesium. This translates to a 95% confidence interval of ±18% for estimated calcium content and ±5% for estimated magnesium content of the duplicate diet on an individual basis.

4.3.3. Survey

Average calcium intake was found to be lower for the younger women (483±24 mg/day) than for the women aged 55 and above (653±47 mg/day). The same was observed for magnesium intake which was 188±7 mg/day for the younger women and 225±11 mg/day for the older women. Differences (Mann-Whitney U-test) between age groups were highly significant both for calcium ($p=0.024$) and magnesium ($p=0.007$). Distribution of mineral intakes is shown in Figure B-23. The majority of women did not meet the estimated average requirements (EAR) for calcium and magnesium as suggested by the US Institute of Medicine (*479*) and adopted by WHO for this age group. Average calcium intake of the younger women in our survey was 317 mg/day below the current EAR of 800 mg for adults aged 19 - 50 years, while that of the older women was 347 mg/day below the current EAR of 1,000 mg for adults aged ≥ 51 years. The estimated average magnesium intake of 188±7 mg/day for the

younger women and 225±11 mg/day for the older women in our study are noticeably lower than the current EAR of 255 mg/day for women aged 19 - 30 years and 265 mg/day for women aged 51 years and above, respectively (*621*). Strontium intake correlated with calcium intake (R^2=0.25; p<0.001).

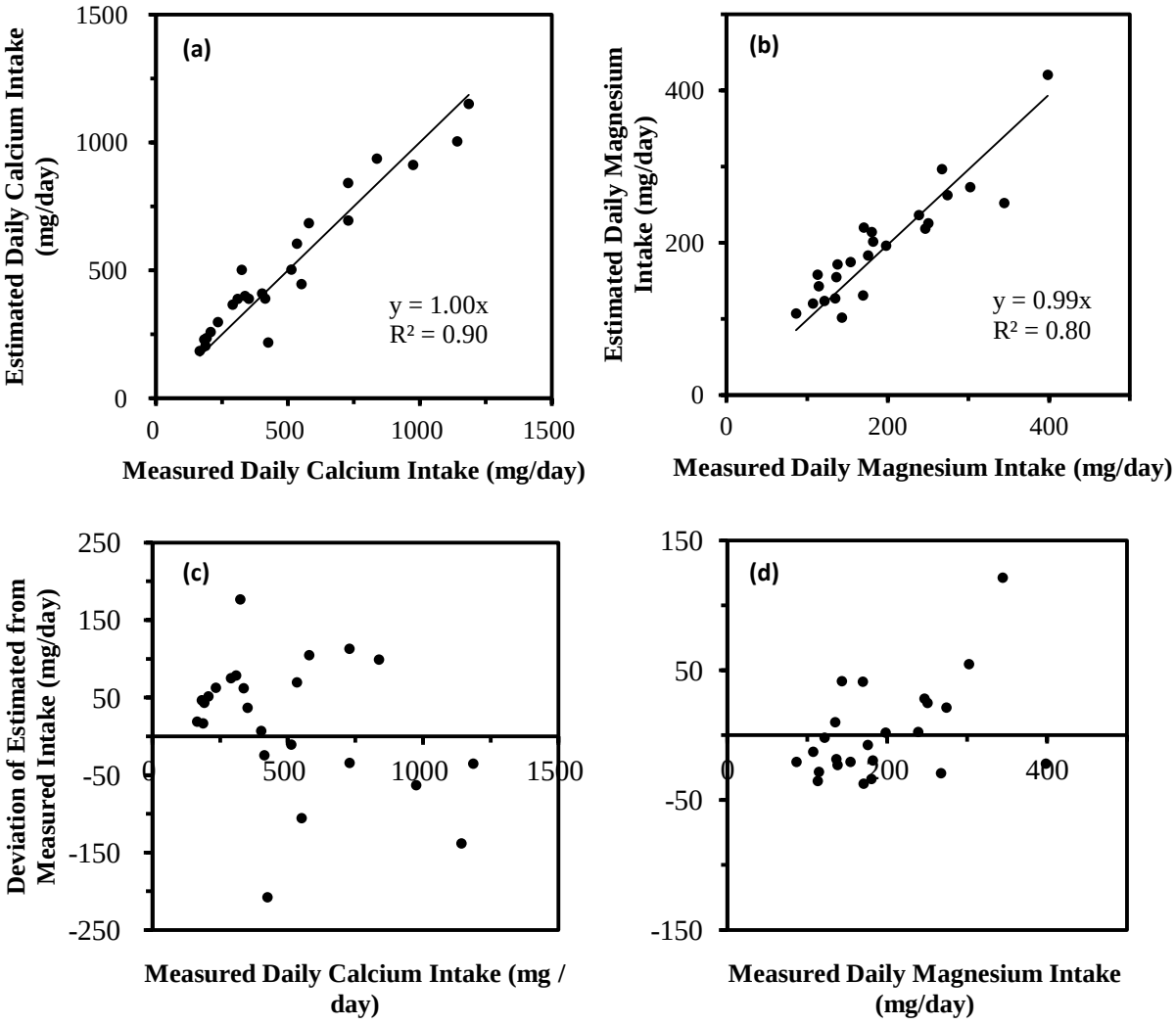

Figure B-22: Correlation between mineral contents as measured in 3-day duplicate diet collections and as estimated using parallel 3-day food records for calcium (Figure B-22a) and magnesium (Figure B-22b) in 24 subjects. Best fit-lines were forced through the origin. Slopes close to 1.00 demonstrate that 3-day food records in combination with an in-house database for calcium and magnesium content of local foods can be used to accurately estimate mineral intake of population groups in the Singaporean context. Absolute residuals for calcium (Figure B-22c) and magnesium (Figure B-22d) are also presented as residual plots. The percentage residuals are 9±20 % and 3±18 % for calcium and magnesium, respectively.

Figure B-24 shows the contribution of non-fortified food and beverages to the average daily calcium and magnesium intake of both age groups. Among food items/groups, major contributors to calcium intake were vegetables, staple foods/carbohydrates and meat products in both groups in that particular order. Together, they accounted for ca. 50% of calcium intake from foods. The contribution from vegetables to calcium and magnesium intakes in both subject groups was comparable (p=0.107 and 0.124, respectively). However, there were statistically significant, yet negligible, differences in the contributions from other food groups, such as staple foods/carbohydrates (p<0.01), meat products (p<0.01) and dairy products (p<0.01). Among the beverages, major sources of calcium were milk and milk products which contributed approximately as much as staple foods/carbohydrates to overall calcium intake. For magnesium, staple foods/carbohydrates contributed ca. one third of magnesium intake from foods with the other two thirds of magnesium coming from the other food groups in much smaller amounts. Intake of magnesium from drinks was ca. 20% of

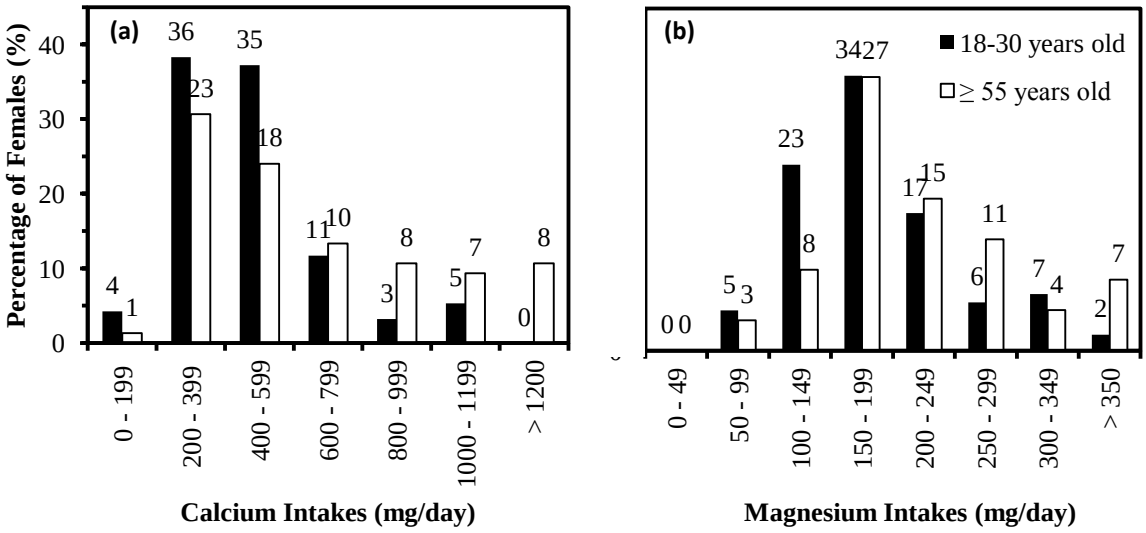

Figure B-23: Distribution of dietary calcium (Figure B-24a) and magnesium (Figure B-24b) intake of female Chinese Singaporeans aged 18 - 30 years old (■) and ≥ 55 years old (□). The average calcium intake for the group aged 18 - 30 years old and that for the group aged ≥ 55 years old were 483±24 and 653±47 mg/day, respectively. Average magnesium intake for the group aged 18 - 30 years old and that for the group ≥ 55 years old were 188±7 and 225±11 mg/day, respectively.

intake from foods. There were significant differences in the contributions from unfortified beverages, such as milk ($p=0.015$), milk products (0.019), as well as coffee and tea ($p<0.01$) to calcium and magnesium intakes in both subject groups.

Figure B-25 shows the contribution of fortified foods and supplements to overall calcium and magnesium intake. While intake of calcium from non-fortified foods was comparable for both age groups ($p=0.94$), intake of calcium from non-fortified beverages ($p=0.009$) and magnesium from non-fortified foods ($p=0.001$) and beverages ($p=0.035$) were significantly different. Fortified foods and beverages and supplements contributed to a much larger extent to calcium intake of the older women as compared to the younger women in our study ($p=0.011$ and 0.001, respectively). For magnesium, intake from fortified foods and beverages did not contribute to the age-dependent differences ($p=0.640$), but intake from supplements contributed significantly ($p=0.040$). Observed age dependent differences in calcium intake of Singapore Chinese women can thus be attributed primarily to a higher proportion of fortified foods and beverages in the diet of the older generation, such as milk powder ($p<0.01$), and their higher supplement intake. For magnesium, the observed age dependent differences in intake can be attributed to a higher supplement intake and differences in consumption of non-fortified foods and beverages that has been elaborated earlier.

4.4. Discussion

We have hypothesized that mineral intake may differ between older and younger Singaporean women of Chinese descent. Because identification of general age related differences in dietary preferences has not been the subject of this survey, we could focus on the accurate assessment of mineral intake for method optimization. This is still challenging in a multi-ethnic society such as Singapore and the openness of Singaporeans to cross culinary borders. Major difficulties arise from the diversity of food ingredients used, the variety of composite meals and processed foods as well as the common habit of Singaporeans to enjoy

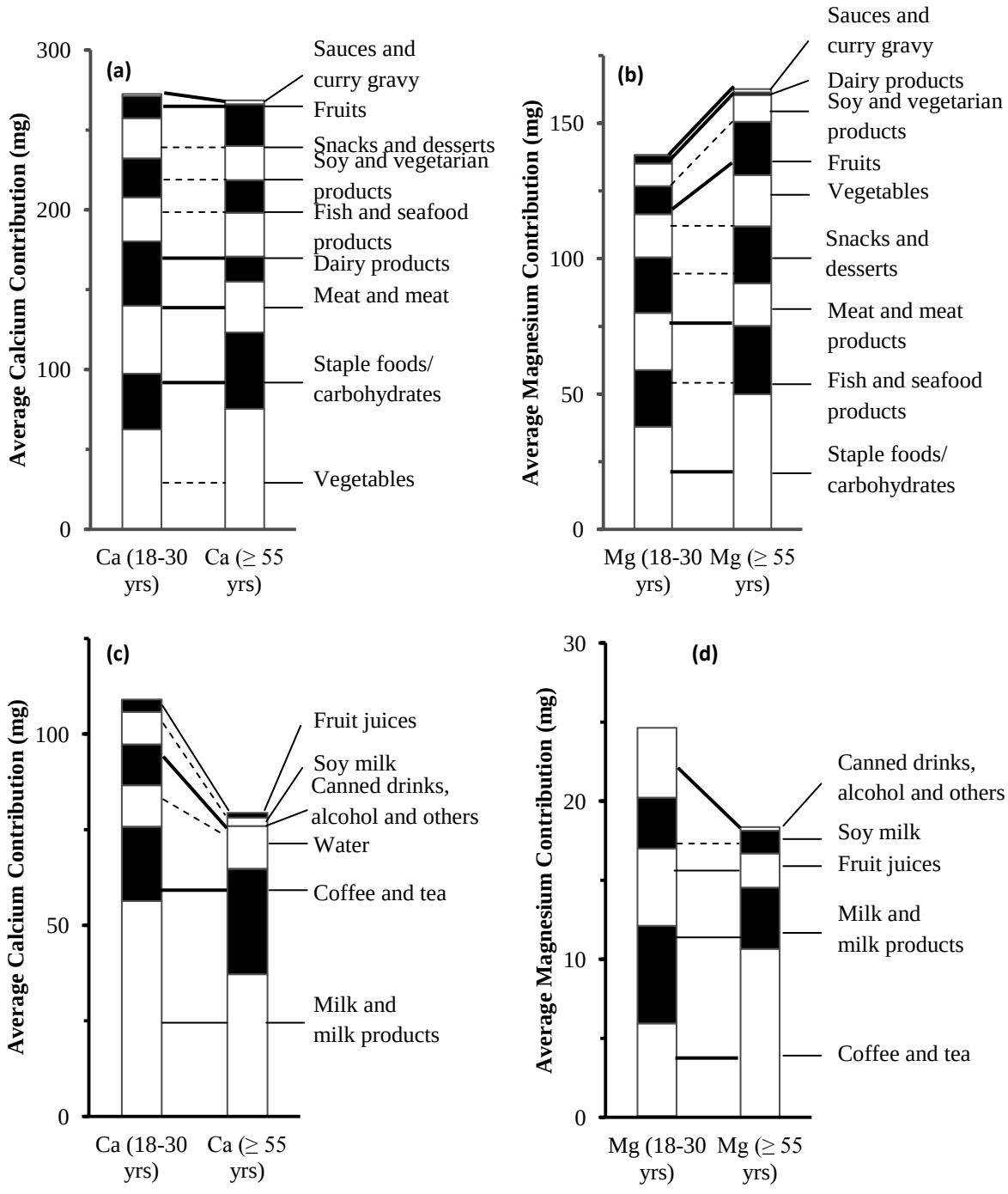

Figure B-24: Contribution of individual non-fortified food items (Figure B-24a and B-24b) and beverages (Figure B-24c and B-24d) to daily average calcium (Figure B-24a and B-24c) and magnesium (Figure B-24b and B-24d) intake of age groups 18 - 30 years old and ≥ 55 years old. Dashed lines (------) show no significant difference in the contribution of the respective food group to daily average mineral intake of the two subject groups. Thin (———) and thick (———) lines show significant difference in the contribution of the respective food group to daily average mineral intake of the two subject groups ($p<0.05$ and $p<0.01$, respectively).

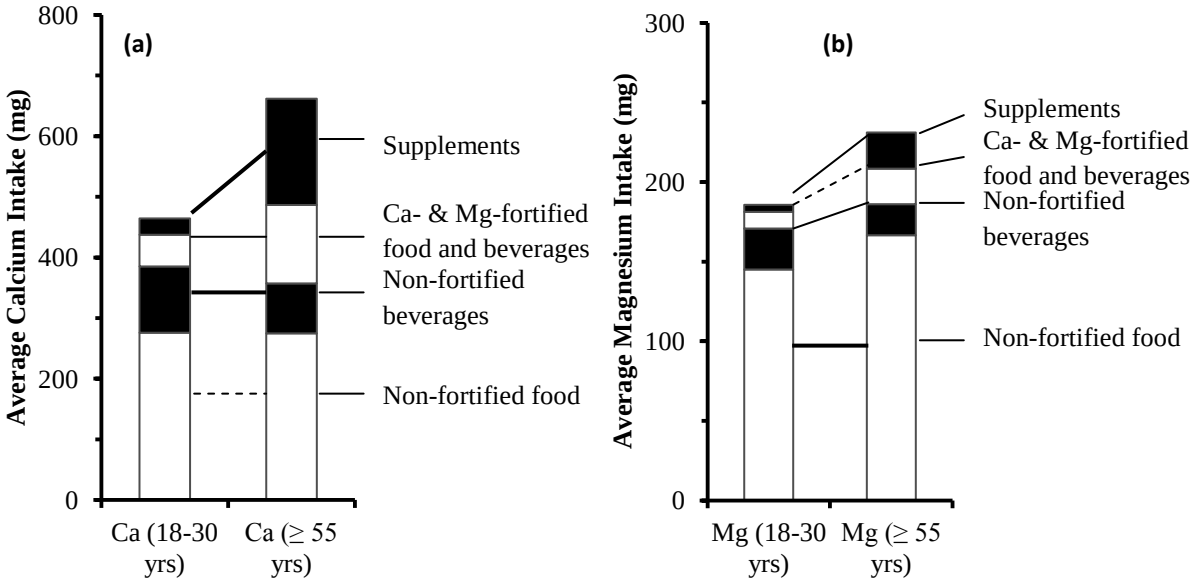

Figure B-25: Breakdown of non-fortified foods, non-fortified beverages, calcium- and magnesium-fortified foods and beverages, and calcium/magnesium supplements and their contributions to daily average calcium (Figure B-25a) and magnesium (Figure B-25b) intake of age groups 18 - 30 years old and ≥ 55 years old. Dotted arrows show no significant difference in the contribution of the respective food group to daily average mineral intake of the two subject groups. Dashed lines (------) show no significant difference in the contribution of the respective food group to daily average mineral intake of the two subject groups. Thin (———) and thick (———) lines show significant difference in the contribution of the respective food group to daily average mineral intake of the two subject groups ($p<0.05$ and $p<0.01$, respectively).

food outside at food stalls and restaurants rather than cooking at home. A recent market survey showed that about 90% of Singaporeans ate at food courts and food stalls at an average of 16 times per month and 82% of consumers ate at an average of seven times a month at fast-food outlets and five times monthly at restaurants (*642*).

Initially we attempted to assess mineral intake through a validated food frequency questionnaire (FFQ) but this approach turned out to result in a significant overestimation of food intake. The developed FFQ was rather complex as it had to consider relevant Southern Chinese, Southeast Asian, Indian and Western dishes and drinks which are all equally available across the country. It is well established that an overly complex FFQ tends to diminish data quality as it may lead to over-reporting of food intake (*643*, *644*). Instead, we have assessed mineral intake using food records. Significant day-to-day variations in food

consumption make food records less suitable to estimate nutrient intake in the individual but they are useful to assess nutrient intakes of population groups as intended in this survey (*643, 644*). Under the typical assumption that records reflect actual food intake on the assessment day, the mean of a single-day intake for a group is equal to the mean of the usual intake distribution (*645*). To increase study power, we have used 3-day food records as this allows a better control of bias due to day-to-day variations in food intake of the individual subject and, thus, the entire population group.

Conversion of food intake data into mineral intake was another difficulty we had to address. With a few exceptions such as eggs and farmed sea-fish, all food sold in Singapore is imported either from neighboring countries or elsewhere in order to satisfy the diverse demands of Singapore's multi-ethnic and multi-national population. We have therefore compiled data both from Asian and non-Asian databases. As our survey was nutrient specific, we could reduce the complexity of the database substantially by grouping relevant foods according to their calcium and magnesium content and assigning estimates for average mineral content of portion sizes typical for the local context. Groupings were done based on the assumption that variability in nutrient content for a given food is at least ±50%, i.e. a food item with a reported calcium content of 100 mg calcium per serving cannot be differentiated from a food item with a reported content of 200 mg calcium per serving. Seasonal variations, genotype, production method, water content, geographic origin and highly variable losses during food preparation, to name a few out of many possible factors that can affect food mineral content substantially (*646*). As can be seen from Figures B-22a and B-22b, this approach yielded a very good agreement between estimates of calcium and magnesium content and actual mineral contents as measured in the collected duplicate diets. Confidence intervals (95%) were relatively small and allowed the statistical resolution of differences in estimated mineral intake between both subject groups larger than 59 mg for calcium and 13 mg for magnesium.

Nutrient requirements differ between individuals. As such, the EAR value represents an estimate of the median of the amount of nutrient that is required by a population to satisfy physiological needs. Nutrient supply of a population is therefore sufficient if not more than 50% of individuals show a nutrient intake that is below their EAR. In the present study we found that the calcium intake of 80% of Singaporean women was below their current EAR of 1,000 mg calcium per day while frequency was even higher at 91% for the younger women. The low calcium intake of the younger women (483±24 mg/day) and the older women (653±47 mg/day) in our study agree well with observations in other Asian populations. Estimated calcium intake of females was found to be in the range of 390 - 570 mg per day in Hongkong (*616, 647, 648*), 640±240 mg/day in Taiwan (*649*) and 230 - 724 mg/day in mainland China with regional differences (*616, 650*). In Japan, a trend towards a lower calcium intake by younger women has also been observed. In the Japanese National Nutritional Survey (1999), it was found that calcium intake by women aged 20-29 years-old (497±242 mg/day) was generally lower than that of women aged 50-59 years-old (606±280 mg/day), 60-69 years-old (610±283 mg/day) and 70-79 years-old (570±275 mg/day) (*651*). Similar to calcium, we observed a suboptimal supply of Singaporean Chinese women with magnesium. At an estimated magnesium intake of 188±7 mg/day, 71% of the older women did not meet their current EAR while magnesium intake of 84% of the younger women (225±11 mg/day) was lower than their EAR. This compares to estimates of magnesium intake of women aged 35 to 64 years of 333±3 mg per day in Tianjin, China (*652*), 306±130 mg per day in women aged 31 to 50 years from Jiangsu province, China (*653*) and 214 - 318 mg per day in various regions in mainland China (*650*).

The rather low calcium intake of most women in the study can be explained, at least partly, by the rather low intake of milk and dairy products. Together they account for less than 10% of total dietary calcium intake in both age groups with milk intake being higher in younger women and tea/coffee consumption in older women. About one third of dietary calcium was found to come from vegetables, staple foods (primarily rice and noodles) and

meat at similar proportions. This is in good agreement with other studies in Chinese populations which showed consistently that milk and dairy products contribute little to total calcium intake and that vegetables are a more relevant source of calcium (*654-656*). In Caucasian populations, milk and dairy products account for up to two-thirds while vegetables contribute to less than 5% of total calcium intake from food sources (*657, 658*). Tofu is a more relevant source of calcium than milk and dairy products in the traditional Asian diet (*659*). Gypsum ($CaSO_4 \cdot H_2O$) or $CaCl_2$ are commonly used in tofu production to precipitate protein from soy milk which is rather low in native calcium content similar to soy beans. While tofu was found to be a major dietary source of calcium in China and Hongkong (*650, 654, 660*), we found that it is of little relevance in the Singaporean context (<5% of total calcium intake), irrespective of age group. We also found that whole dried small fish (ikan bilis), shrimp or shrimp paste (*belachan*) as common condiments of high calcium content in Southeast-Asian dishes were not significant sources of calcium in the studied population group.

Sources of magnesium in the diet and their proportional contribution to total magnesium intake were mostly similar to calcium in our study. Exceptions were staple foods which contributed proportionally more and vegetables which contributed less to total dietary intake of magnesium in comparison to calcium. The suboptimal magnesium intake we have observed in both age groups can be attributed to the rather low intake of whole grain products as a major source of magnesium in the human diet (*650*). Polished rice and noodles prepared from low extraction wheat flour appear to remain the more popular choice among Singaporeans despite attempts of local authorities to promote consumption of whole grain products. Other than that, magnesium is found in green leafy vegetables, legumes, nuts, seeds, fish and hard water (*26*). Although most fluid intake of Singaporeans comes from tap water, it is a minor source of magnesium in the local context. Due to extensive cleaning procedures which include reversed osmosis, local tap water is very low in minerals including calcium and magnesium.

Dietary intake of strontium as estimated by duplicate diet analysis was much lower than for calcium and magnesium. The estimated strontium intake was 1.03±0.08 mg strontium per day in this study, which is within published range (0.20 to 2.36 mg) (*486, 487*). This finding is also in agreement with findings other South-East Asia countries, including Vietnam (1.3 mg/day) and Philippine (1.1 mg/day) (*486*). In UK and Finland, the average daily intakes of strontium were estimated to be 1.3 mg/day and 1.9 mg/day (*486, 488*), which were comparable with the strontium intake in South-East Asia. In Germany and Switzerland, the strontium intake could reach an even higher value and it was estimated to be between 1.1 to 4.5 mg/day (*489, 490*). There are a few major dietary sources of strontium, including cereal (2,440 µg/kg), vegetables (530 – 940 µg/kg), full cream milk (180 to 2,800 µg/L), meat and poultry (450 ug/kg), seafood (274 µg/kg), water (20 – 60 µg/L) (*491, 492*). Higher strontium content in water have been reported in Europe (10.6 – 12,200 µg/L) (*493*), Riyadh (25.4 – 406.4 µg/L) (*494*) and Japan (1.96 - 4539 µg/L) (*495*). As with magnesium, water is not a relevant strontium source in Singapore due to intensive water processing and infrequent consumption of bottled, mineral rich natural waters. Contents of strontium and calcium of the duplicate diets were correlated, which was not unexpected. Both elements follow each other in water, soil and foods due to their similar chemical properties (*14*). Overall, the strontium content of the Singaporean diet was found to be lower by two orders of magnitude than the therapeutic dose of strontium used in osteoporosis treatment (ca. 500 mg strontium per day in the form of strontium ranelate). As such, native strontium in the Singaporean diet has supposedly a negligible effect on bone.

When looking at mineral intake from non-fortified foods and beverages alone, we found no statistically significant difference in intake for calcium between both age groups, but we found significant difference in intake for magnesium. Sources of both minerals in the diet were also relatively similar for both age groups. The differences that need to be highlighted were higher consumption of dairy products, such as cheeses and milk by the younger group of females, resulting in higher contribution to calcium and magnesium intake. This finding is not

surprising considering higher acceptance of Westernized food items by the younger generation. While consumption of coffee and tea, typical traditional Chinese beverages, was higher in the older subject group. This is different when taking calcium and magnesium intake from fortified food products as well as supplements into account. In fact, age dependent differences in total calcium and magnesium intake that we have observed in this study can be mostly attributed to calcium and magnesium intake from these non-traditional sources. Mineral intake from supplements and fortified food products was higher by a factor of three for calcium and by a factor of two for magnesium in the older women as compared to the younger women. Dietary supplements are very popular in Singapore. On average, every family that consumes health supplements spends US$120 per month and it was estimated that in 2010 Singaporeans spent US$260 million on supplements (*661*). Furthermore, Singaporeans are relatively well informed on health consequences of calcium deficiency in older woman through the mass media and advertisement campaigns by manufacturers of foods and dietary supplements (*642*). Age related differences in the intake of supplements and fortified foods can be explained by income gaps between generations and that older women are usually more concerned with their bone health than younger women. Furthermore, the older generation tends to prepare food more often themselves. This makes them more likely to consume fortified food products such as fortified breads, instant beverages and milk powder which are typically prepared and consumed at home. A trend toward higher use of supplements among older age groups was also observed by Mangano *et al.* (*662*) and Bailey *et al.* (*663*) in non-Asian populations. Contribution of supplements and fortified foods to magnesium intake was much lower in comparison. This can be explained by a greater awareness of calcium as a limiting nutrient in the Asian diet and maintenance of bone health. In our study, we found that supplemental magnesium has only been consumed as part of multi-vitamin and mineral supplements. Communication with regards to health impacts of a suboptimal magnesium intake remains difficult as it cannot be associated easily with a particular disease such as calcium or iron (*664*). Being much less studied than other nutrients

despite its multitude of functions it is often considered as the "forgotten mineral" in our diet (*665*).

Our study demonstrates that a suboptimal calcium and magnesium intake from the diet as commonly observed in Asians of Chinese descent can easily be boosted by the use of dietary supplements and fortified food products. Such a strategy, however, is not without risk. Supplements can close the gap between dietary intake and physiological needs. Increasing mineral intake beyond this threshold level has no additional health benefits. This may explain, at least in part, unequivocal findings regarding the effect of calcium supplementation with or without Vitamin D on reduction of fracture risk (*666, 667*). Increasing mineral intake beyond this threshold can be more harmful than useful. Excessive calcium intake may result in vascular and soft tissue calcification, in particular of heart and kidneys, and may increase risks for developing cardiovascular disease (*37-39, 404*). This increasing body of evidence has resulted recently in the lowering of the Tolerable Upper Level of Intake (UL) for calcium by the US Institute of Medicine from 2,500 mg per day to 2,000 mg per day for males and females 51 years and above (*479*). The current UL, i.e. the regular Ca intake from which harmful effects of calcium are increasing, is difficult to reach through diet. It can be reached, however, by excessive intake of calcium supplements. A recent epidemiological study points to calcium supplementation rather than dietary calcium intake as a concern (*39*). Consumption of calcium fortified food products is less problematic as fortification levels of food products are commonly not associated with any harmful effect when looked at in isolation. They can be of concern, however, when calcium fortified foods are given preference over unfortified foods and taken in combination with the aim to maximize bone health.

Because of reasonable grounds for doubting the safety of calcium supplements, in particular in the older generation, the question arises if supplementation should be recommended to correct low calcium and magnesium intakes in Chinese Singaporeans. This refers in particular to the younger generation for which we have observed a very low calcium intake that amounted, on average, to less than two-third of the EAR. While the role of calcium

nutrition and calcium supplementation in fracture prevention in the elderly remains debatable, the importance of calcium nutrition in the first three decades of life is largely unquestioned. A sufficient calcium supply during growth is pivotal to reach the genetically pre-determined peak bone mass to delay the onset of osteoporosis later in life (*26*). Further studies are necessary to elucidate if calcium intakes during childhood and puberty are as low as for the young women in our study and not only confined to Singaporean women of Chinese descent. With a greater openness of the younger generation to consume milk and dairy products despite a deep penetration of lactose intolerance in Chinese Asians, there is some untapped potential to increase calcium intake by the younger generation without relying on supplements.

Our study has shown that calcium and magnesium intake of Singaporean Chinese women is well below the current EAR. However, it remains open if low mineral intakes are actually a true concern and have, finally, an impact on fracture risk. Our knowledge on calcium requirements is based largely on studies in Caucasian populations. Ethnic differences have already been identified for Africans while studies are still scarce for Asian populations. Recent absorption studies using stable calcium isotopes showed that calcium absorption in Chinese is apparently more efficient than in Caucasians (*668, 669*). A more recent study points to lower calcium requirements of Asian than White US children (*670*). No such studies exist for magnesium. Differences in calcium requirements may explain the Asian paradox. Despite a much lower dietary calcium intake as compared to Caucasians, age corrected fracture incidence is significantly lower in Asian populations than in the West (*671*). Until actual mineral requirements of Asian populations are known, it will be difficult to make evidence based recommendations to Singaporeans and other Asian populations regarding optimal mineral intakes that are not of harm in the long-term.

Acknowledgment

The author would like to thank Ms. Steffiana Yuliani Wijaya, Ms. Yeoh Chooi Syn and Ms. Tay Hui Huang for their inputs in setting up the method for the dietary intake assessment and their contributions in parts of the data collection.

Part C

Conclusion

CONCLUSION

Osteoporosis is a skeletal disease characterized by low bone mass and micro-architectural deterioration of bone, resulting in an increase in bone fragility and vulnerability to fracture. Besides affecting the quality of life, osteoporosis treatment and management cause considerable economic burdens. The main challenge in recognizing effective strategies to maintain and improve bone health is the absence of techniques that are sensitive enough to evaluate the skeletal response to interventions. The commonly used techniques, measurement of BMD and biomarkers, are either insensitive or subjected to significant intra-individual variation.

On the other hand, isotopic labeling of bone is able to serve as an ultra-sensitive tool to detect changes in bone metabolism, which allows the assessment of short-term and soft interventions, such as diet and lifestyle changes. Technically, either stable or radioisotopes of calcium can be used for isotopic labeling. However, due to health concerns, conventional radiotracers are no longer used. Although stable isotopes are safe, the doses required, and consequently the costs involved, would be very high. Alternatively, there is a very long-living radiotracer (^{41}Ca), that is safe to use and only required in a minute amount. Nevertheless, its technically challenging analysis has hindered its use in routine applications.

The similarities between strontium and calcium have pointed to the possibility of using strontium stable isotopes as a surrogate marker for calcium in bone metabolism studies. Strontium, like calcium, is a bone seeking element. It has similar chemical properties and passively follows calcium in the body. However, it requires much less sophisticated instrumentation for isotopic analysis.

The first aim of this thesis was to explore the potential of strontium stable isotopes as a diagnostic tool to study bone mineral metabolism. The first study described in this thesis (Part B, Chapter 1) has shown that once calcium and strontium have been incorporated into the bone matrix, the body handles them in a very similar manner. Although strontium is preferentially discharged in urine and possibly released more effectively from bone than

calcium, the discrimination follows certain correlations, allowing the use of strontium stable isotopes for bone labelling in order to monitor changes in bone calcium balance. This implies that once comparative data are available from kinetic tracer studies in humans, mathematical corrections can possibly be used to translate changes in strontium tracer excretion into changes in bone calcium balance. Up to now, several human studies have used only ^{41}Ca for skeletal labelling to monitor changes in bone calcium balance

The second study described in this thesis (Part B, Chapter 2) has revealed some setbacks of using strontium stable isotopes to study bone mineral metabolism. It has been shown in this study that isotopic labelling of the skeleton by single administration of an isotopic tracer does not reach all bone. Tracer is preferentially deposited in young as well as areas undergoing growth or more frequent remodelling due to a higher mechanical load. Tracer recovered in urine can either originate from bone surface where it was bound loosely by ion exchange mechanisms or from mineralized bone from where it was released by osteoclastic resorption. However, the urinary tracer signal is dominated by tracer release/uptake from bone areas that undergo remodeling more frequently. Accordingly, measurable deviations in tracer excretion from its original excretion pattern cannot be more than a qualitative index for changes in bone mineral balance in response to changes in lifestyle, diet or pharmacological treatment. This applies to all tracers administered for the purpose of skeletal labeling. Quantitative evaluations are still possible but they require more complex kinetic modeling of tracer data which can be used to differentiate changes in flux rates for slow exchanging and fast exchanging bone pools as opposed to measuring differences in urinary tracer excretion alone.

Similar to ^{41}Ca, strontium stable isotopes can be detected over decades in urine following a single oral or intravenous administration of the tracer. When being given to individuals in their late 30s or early 40s, regular monitoring of urinary tracer excretion would permit detection of accelerated bone loss or to qualitatively assess response of bone to pharmacological interventions or changes in lifestyle or diet. Because of the high sensitivity and specificity of the bone labeling approach, changes in bone could be detected probably

earlier and thus faster as compared to conventional techniques at costs that are comparable or even lower than for radiological measurements of bone mineral density. This technique can also be used as an ultra-sensitive tool to evaluate the skeletal response to interventions, including those short-term and soft interventions. Instruments that permit strontium isotopic analysis in urine such as ICP-MS are already widely available at university hospitals or commercial clinical laboratories. Hence, this technique can overcome not only the insensitivity of the conventional techniques, like BMD and biomarker measurement, but the technical challenges of using calcium isotopes.

Strontium has also played a role as a therapeutic agent for osteoporosis prevention and treatment. Since the 1950s, strontium has been suggested to be useful for osteoporosis treatment. A few decades later, strontium ranelate has finally been approved as an osteoporotic drug. In principle, strontium ranelate increases bone formation and decreases bone resorption. It also improves bone biomechanical properties including bone strength (*672*). While strontium binding to human plasma proteins is low (approximately 25%), it has a high affinity for bone tissue (*672*). The manufacturer of the drug has claimed that strontium does not accumulate in non-calcified tissues (*672*). However, a recent data evaluation from clinical studies by the European Medicines Agency's Committee for Medicinal Products for Human Use showed a higher risk of heart attack in strontium ranelate group than with placebo, with no observed increase in mortality risk.

The second aim of this thesis was to investigate the role of strontium as a therapeutic substance to improve bone health, together with its side effects on soft tissues. The third study described in this thesis (Part B, Chapter 3) has confirmed a positive effect of strontium ranelate on bone although less clearly as opposed to other studies, probably due to sample size restrictions. It has also showed that the absorption of strontium lactate based on the strontium level in the serum samples was comparable if not higher than for other types of strontium salts, including strontium ranelate shown by earlier studies (*594*). However, a direct comparison and a solid conclusion on this matter could not be drawn due to the differences in the experimental set ups (strain and age of rats, study duration, sample size etc.).

Based on the concerns raised by the European Medicines Agency regarding the safety of strontium ranelate treatment, restrictions to its use have been recommended to mitigate higher risk for cardiovascular events observed in patients undergoing strontium therapy. In principle, this would be consistent with the fact that strontium and calcium show similar physiological behavior. As for calcium supplementation, high strontium intakes may exacerbate risks of tissue mineralization and atherosclerosis by formation of poorly soluble strontium salts. Disproportionate changes in bone and soft tissue content relative to strontium levels in feed and serum let us hypothesize that a threshold level may exist on from which strontium is taken up less efficiently by bone and risks for tissue accumulation are increasing.

In clinical settings, strontium ranelate is used at a dose level (500 mg/day of strontium) that is comparable to regular calcium supplementation which has been suggested to increase risks of soft tissues calcification. On top of that, strontium ranelate treatment is recommended to be administered together with up to 1,000 mg of calcium and 400 IU or 800 IU of vitamin D depending on the patients' needs. The combined strontium and calcium intake may approach or even exceed the tolerable upper intake level of 2,000 mg per day which is the current upper limit for dietary intake of the US DRI for calcium. This would point to a reconsideration of practices of concomitant intake of calcium supplements and/or dosage levels for strontium therapy.

Although strontium is used as a therapeutic agent for osteoporosis, it is not a foreign element to human. In fact, it can be found in many types of regular foods and beverages. The third aim of this thesis was to assess the intake of strontium, as a dietary component with potential positive effects on bone, in a group of Singaporean Chinese. The last study described in this thesis (Part B, Chapter 4) showed that the estimated strontium intake was 1.03±0.08 mg strontium per day. It also showed that contents of strontium and calcium of the duplicate diets were correlated, which was expected. Overall, the strontium content of the Singaporean diet was found to be lower by two orders of magnitude than the therapeutic dose of strontium used in osteoporosis treatment (ca. 500 mg strontium per day in the form of

strontium ranelate). As such, intake of strontium from the Singaporean diet has supposedly a negligible effect on bone.

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Appendix I

Supplementary Data for Isotopic Labeling of Bone for Assessment of Changes in Bone Calcium Balance: A Comparative Study in Sheep Using ^{41}Ca and ^{86}Sr (Part B, Chapter 1)

Table I-1: Changes in urinary ^{41}Ca tracer excretion relative to natural calcium against time. The spot urine samples from the sheep were collected over 181 days. The $^{41}Ca/^{40}Ca$ measurements ($n(^{41}Ca)/n(^{40}Ca)$) were obtained from single measurements by using AMS with relative measurement uncertainty of 5% (coverage factor (k) = 1). The amount ratios of ^{41}Ca tracer over natural calcium ($n(^{41}Ca_{tracer})/n(Ca_{nat})$) were calculated based on the isotopic abundance of ^{40}Ca. Column numbers are indicated in square brackets.

Day	**$n(^{41}Ca)/n(^{40}Ca)$ (10^{-12} mol/mol)**	**$n(^{41}Ca_{tracer})/n(Ca_{nat})$ (10^{-12} mol/mol)[a]**	**Log ($n(^{41}Ca_{tracer})/n(Ca_{nat})$ (10^{-12}))[b]**
[1]	**[2]**	**[3]**	**[4]**
0.25	186509	178862	5.25
0.5	106635	102263	5.01
1	52321	50176	4.70
2	22780	21846	4.34
3	12132	11635	4.07
5	6023	5776	3.76
15	1512	1450	3.16
19	1510	1448	3.16
28	857	822	2.91
34	746	716	2.85
41	778	746	2.87
50	710	681	2.83
62	776	744	2.87
89	622	597	2.78
117	460	441	2.64
132	354	339	2.53
156	241	231	2.36
169	245	235	2.37
171	274	263	2.42
172	259	248	2.40
174	282	270	2.43
181	241	231	2.36

[a] Amount ratio of ^{41}Ca tracer to natural calcium was calculated by multiplying the measured amount ratio of ^{41}Ca tracer to ^{40}Ca in the sample (column [2]) with the natural isotopic abundance of ^{40}Ca of 0.959.

[b] Log of amount ratio of ^{41}Ca tracer to natural calcium was calculated by logarithmizing the amount ratio of ^{41}Ca tracer to natural calcium (column [3]).

Table I-2: Changes in urinary ^{86}Sr tracer excretion relative to natural calcium against time. The spot urine samples from the sheep were collected over 181 days. The $^{86}Sr/^{88}Sr$ measurements ($n(^{86}Sr)/n(^{88}Sr)$) were obtained from triplicate measurements by TIMS. The amount ratios of ^{86}Sr tracer over natural strontium ($n(^{86}Sr_{tracer})/n(Sr_{nat})$) were calculated following IDMS principles. Natural strontium and calcium concentrations ($c(Sr_{nat})$ and $c(Ca_{nat})$) were obtained from triplicate measurements by AAS. ^{86}Sr tracer concentration ($c(^{86}Sr_{tracer})$) and amount ratio of ^{86}Sr tracer to natural calcium ($n(^{86}Sr_{tracer})/n(Ca_{nat})$) were calculated based on the amount ratio of ^{86}Sr tracer to natural strontium, the concentration of natural strontium and the concentration of natural calcium. Column numbers are indicated in square brackets.

Day	$n(^{86}Sr)/n(^{88}Sr)$	$n(^{86}Sr_{tracer})/n(Sr_{nat})$ (mol/mol)[a]	$c(Sr_{nat})$ (mol/g)	$c(^{86}Sr_{tracer})$ (mol/g)[b]	$c(Ca_{nat})$ (mol/g)	$n(^{86}Sr_{tracer})/n(Ca_{nat})$ (mol/mol)[c]	Log $(n(^{86}Sr_{tracer})/n(Ca_{nat}))$[d]
[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]
0.25	11.180585	12.8422	6.17×10^{-8}	7.92×10^{-7}	3.63×10^{-7}	2.18	3.38×10^{-1}
0.5	6.208150	6.1047	1.55×10^{-8}	9.47×10^{-8}	5.93×10^{-7}	1.60×10^{-1}	-7.96×10^{-1}
2	1.125028	0.8849	3.61×10^{-9}	3.19×10^{-9}	1.69×10^{-7}	1.89×10^{-2}	-1.72
5	0.347933	0.1974	2.08×10^{-9}	4.10×10^{-10}	1.42×10^{-7}	2.89×10^{-3}	-2.54
19	0.173447	0.0465	2.81×10^{-9}	1.31×10^{-10}	4.98×10^{-7}	2.62×10^{-4}	-3.58
34	0.147021	0.0238	3.45×10^{-9}	8.21×10^{-11}	1.28×10^{-6}	6.40×10^{-5}	-4.19
41	0.144499	0.0216	4.95×10^{-9}	1.07×10^{-10}	2.36×10^{-6}	4.54×10^{-5}	-4.34
50	0.142047	0.0195	2.92×10^{-9}	5.69×10^{-11}	6.08×10^{-7}	9.36×10^{-5}	-4.03
62	0.130075	0.0092	1.06×10^{-9}	9.75×10^{-12}	1.71×10^{-7}	5.71×10^{-5}	-4.24
75	0.138143	0.0162	3.69×10^{-9}	5.96×10^{-11}	4.63×10^{-7}	1.29×10^{-4}	-3.89
103	0.130043	0.0092	3.73×10^{-10}	3.43×10^{-12}	2.70×10^{-8}	1.27×10^{-4}	-3.90
117	0.140274	0.0180	1.48×10^{-9}	2.65×10^{-11}	1.64×10^{-7}	1.62×10^{-4}	-3.79
132	0.127098	0.0067	1.75×10^{-9}	1.16×10^{-11}	4.20×10^{-7}	2.77×10^{-5}	-4.56

Table I-2 (cont'd): Changes in urinary ^{86}Sr tracer excretion relative to natural calcium against time.

Day	$n(^{86}Sr)/n(^{88}Sr)$	$n(^{86}Sr_{tracer})/n(Sr_{nat})$ (mol/mol)[a]	$c(Sr_{nat})$ (mol/g)	$c(^{86}Sr_{tracer})$ (mol/g)[b]	$c(Ca_{nat})$ (mol/g)	$n(^{86}Sr_{tracer})/n(Ca_{nat})$ (mol/mol)[c]	Log $(n(^{86}Sr_{tracer})/n(Ca_{nat}))$[d]
145	0.126190	0.0059	7.72×10^{-10}	4.54×10^{-12}	4.73×10^{-8}	9.59×10^{-5}	-4.02
156	0.125975	0.0057	1.47×10^{-9}	8.34×10^{-12}	6.15×10^{-7}	1.36×10^{-5}	-4.87
172	0.124040	0.0040	2.69×10^{-9}	1.08×10^{-11}	1.24×10^{-6}	8.74×10^{-6}	-5.06
181	0.122916	0.0031	3.25×10^{-9}	9.94×10^{-12}	1.58×10^{-6}	6.28×10^{-6}	-5.20

[a] Amount ratio of ^{86}Sr tracer to natural strontium was calculated based on IDMS prinicples (Eqn. 2).

[b] ^{86}Sr tracer concentration in the sample was calculated by multiplying the amount ratio of ^{86}Sr tracer to natural strontium (column [3]) with the concentration of natural strontium (column [4]).

[c] Amount ratio of ^{86}Sr tracer to natural calcium was calculated by dividing ^{86}Sr tracer concentration in the sample (column [5]) with the natural calcium concentration (column [6]).

[d] Log of amount ratio of ^{86}Sr tracer to natural calcium was calculated by logarithmizing the amount ratio of ^{86}Sr tracer to natural calcium (column [7]).

Table I-3: Changes in ratio of ^{86}Sr to ^{41}Ca urinary tracer excretion against time. The spot urine samples from the sheep were collected over 181 days. The concentrations of ^{86}Sr tracer in the samples ($c(^{86}Sr_{tracer})$) were calculated in Table I-2. The amount ratios of ^{41}Ca tracer to natural calcium ($n(^{41}Ca_{tracer})/n(Ca_{nat})$) were calculated in Table I-1. Natural calcium concentrations ($c(Ca_{nat})$) were obtained from triplicate measurements using AAS. ^{41}Ca tracer concentration in the sample ($c(^{41}Ca_{tracer})$) and amount ratio of ^{86}Sr tracer to ^{41}Ca tracer ($n(^{86}Sr_{tracer})/n(^{41}Ca_{tracer})$) were calculated based on the amount ratio of ^{41}Ca tracer to natural calcium, the concentration of natural calcium and the ^{86}Sr tracer concentration in the sample. Column numbers are indicated in square brackets.

Day	**$c(^{86}Sr_{tracer})$ (mol/g)**	**$n(^{41}Ca_{tracer})/n(Ca_{nat})$ (10^{-12} mol/mol)**	**$c(Ca_{nat})$ (mol/g)**	**$c(^{41}Ca_{tracer})$ (mol/g)[a]**	**$n(^{86}Sr_{tracer})/n(^{41}Ca_{tracer})$ (mol/mol)[b]**	**Log $(n(^{86}Sr_{tracer})/n(^{41}Ca_{tracer}))$[c]**
[1]	**[2]**	**[3]**	**[4]**	**[5]**	**[6]**	**[7]**
0.25	7.92×10^{-7}	178862	3.63×10^{-7}	6.50×10^{-14}	1.22×10^{7}	7.09
0.5	9.47×10^{-8}	102263	5.93×10^{-7}	6.06×10^{-14}	1.56×10^{6}	6.19
2	3.19×10^{-9}	21846	1.69×10^{-7}	3.68×10^{-15}	8.66×10^{5}	5.94
5	4.10×10^{-10}	5776	1.42×10^{-7}	8.20×10^{-16}	5.00×10^{5}	5.70
19	1.31×10^{-10}	1448	4.98×10^{-7}	7.21×10^{-16}	1.81×10^{5}	5.26
34	8.21×10^{-11}	716	1.28×10^{-7}	9.17×10^{-16}	8.95×10^{4}	4.95
41	1.07×10^{-10}	746	2.36×10^{-6}	1.76×10^{-15}	6.08×10^{4}	4.78
50	5.69×10^{-11}	681	6.08×10^{-7}	4.14×10^{-16}	1.37×10^{5}	5.14
62	9.75×10^{-12}	744	1.71×10^{-7}	1.27×10^{-16}	7.67×10^{4}	4.88
117	2.65×10^{-11}	441	1.64×10^{-7}	7.23×10^{-17}	3.67×10^{5}	5.56
132	1.16×10^{-11}	339	4.20×10^{-7}	1.43×10^{-16}	8.16×10^{4}	4.91
156	8.34×10^{-12}	231	6.15×10^{-7}	1.42×10^{-16}	5.87×10^{4}	4.77
172	1.08×10^{-11}	248	1.24×10^{-6}	3.08×10^{-16}	3.52×10^{4}	4.55
181	9.94×10^{-12}	231	1.58×10^{-6}	3.66×10^{-16}	2.71×10^{4}	4.43

[a] ^{41}Ca tracer concentration in the sample was calculated by multiplying the amount ratio of ^{41}Ca tracer to natural calcium (column [3]) with the concentration of natural calcium (column [4]).

[b] Amount ratio of ^{86}Sr tracer to ^{41}Ca tracer was calculated by dividing ^{86}Sr tracer concentration in the sample (column [2]) with ^{41}Ca tracer concentration in the sample (column [5]).

[c] Log of amount ratio of ^{86}Sr tracer to ^{41}Ca tracer was calculated by logarithmizing the amount ratio of ^{86}Sr tracer to ^{41}Ca tracer (column [6]).

Table I-4: Strontium isotopic ratio and strontium and calcium elemental contents in urine samples. The spot urine samples from the sheep were collected over 181 days. The $^{86}Sr/^{88}Sr$ measurements ($n(^{86}Sr)/n(^{88}Sr)$) were obtained from triplicate measurements by TIMS. Natural strontium and calcium concentrations ($c(Sr_{nat})$ and $c(Ca_{nat})$) were obtained from triplicate measurements using AAS. This table shows the individual measurements, average and %RSD for each spot urine sample.

Day	$n(^{86}Sr)/n(^{88}Sr)$			$c(Sr_{nat})$			$c(Ca_{nat})$		
	Measured	Average	%RSD	Measured (mol/g)	Average (mol/g)	%RSD (mol/g)	Measured (mol/g)	Average (mol/g)	%RSD (mol/g)
	11.18041			6.25×10^{-8}			3.76×10^{-7}		
0.25	11.18187	11.18058	0.011%	5.83×10^{-8}	6.17×10^{-8}	4.91%	3.59×10^{-7}	3.63×10^{-7}	3.00%
	11.17948			6.42×10^{-8}			3.55×10^{-7}		
	6.20708			1.60×10^{-8}			5.88×10^{-7}		
0.5	6.20818	6.20815	0.017%	1.82×10^{-8}	1.55×10^{-8}	4.27%	5.94×10^{-7}	5.93×10^{-7}	0.79%
	6.20919			1.50×10^{-8}			5.97×10^{-7}		
	1.12535			3.60×10^{-9}			1.80×10^{-7}		
2	1.12528	1.12503	0.044%	4.16×10^{-9}	3.61×10^{-9}	0.14%	1.57×10^{-7}	1.69×10^{-7}	9.40%
	1.12445			3.61×10^{-9}			3.08×10^{-7}		
	0.34795			2.04×10^{-9}			1.44×10^{-7}		
5	0.34792	0.34793	0.003%	2.10×10^{-9}	2.08×10^{-9}	1.68%	1.37×10^{-7}	1.42×10^{-7}	2.88%
	0.34793			2.09×10^{-9}			1.44×10^{-7}		
	0.17344			2.67×10^{-9}			4.87×10^{-7}		
19	0.17344	0.17345	0.008%	2.85×10^{-9}	2.81×10^{-9}	4.25%	5.07×10^{-7}	4.98×10^{-7}	2.00%
	0.17346			2.90×10^{-9}			5.00×10^{-7}		

Table I-4 (cont'd): Strontium isotopic ratio and strontium and calcium elemental contents in urine samples.

Day	$n(^{86}Sr)/n(^{88}Sr)$			$c(Sr_{nat})$			$c(Ca_{nat})$		
	Measured	Average	%RSD	Measured (mol/g)	Average (mol/g)	%RSD	Measured (mol/g)	Average (mol/g)	%RSD
	0.14700			2.86×10^{-9}			1.34×10^{-6}		
34	0.14701	0.14702	0.015%	3.59×10^{-9}	3.45×10^{-9}	5.76%	1.28×10^{-6}	1.28×10^{-6}	4.49%
	0.14705			3.31×10^{-9}			1.23×10^{-6}		
	0.14450			5.30×10^{-9}			2.38×10^{-6}		
41	0.14450	0.14450	0.001%	4.98×10^{-9}	4.95×10^{-9}	0.83%	2.29×10^{-6}	2.36×10^{-6}	2.46%
	0.14450			4.93×10^{-9}			2.40×10^{-6}		
	0.14205			2.78×10^{-9}			5.83×10^{-7}		
50	0.14204	0.14205	0.003%	2.93×10^{-9}	2.92×10^{-9}	4.50%	6.42×10^{-7}	6.08×10^{-7}	4.99%
	0.14205			3.04×10^{-9}			5.98×10^{-7}		
	0.13007			1.02×10^{-9}			1.80×10^{-7}		
62	0.13007	0.13007	0.002%	1.10×10^{-9}	1.06×10^{-9}	3.79%	1.68×10^{-7}	1.71×10^{-7}	4.97%
	0.13008			1.05×10^{-9}			1.64×10^{-7}		
	0.13814			3.75×10^{-9}			4.74×10^{-7}		
75	0.13814	0.13814	0.003%	3.85×10^{-9}	3.69×10^{-9}	5.25%	4.46×10^{-7}	4.63×10^{-7}	3.31%
	0.13815			3.48×10^{-9}			4.69×10^{-7}		
103	0.13004	0.13004		3.73×10^{-10}	3.73×10^{-10}		2.70×10^{-8}	2.70×10^{-8}	

Table I-4 (cont'd): Strontium isotopic ratio and strontium and calcium elemental contents in urine samples.

Day	$n(^{86}Sr)/n(^{88}Sr)$			$c(Sr_{nat})$			$c(Ca_{nat})$		
	Measured	**Average**	**%RSD**	**Measured (mol/g)**	**Average (mol/g)**	**%RSD**	**Measured (mol/g)**	**Average (mol/g)**	**%RSD**
	0.14027			1.50×10^{-9}			1.64×10^{-7}		
117	0.14028	0.14027	0.001%	1.42×10^{-9}	1.48×10^{-9}	3.02%	1.66×10^{-7}	1.64×10^{-7}	1.42%
	0.14027			1.50×10^{-9}			1.62×10^{-7}		
	0.12710			1.67×10^{-9}			4.13×10^{-7}		
132	0.12710	0.12710	0.003%	1.72×10^{-9}	1.75×10^{-9}	5.00%	4.26×10^{-7}	4.20×10^{-7}	1.53%
	0.12710			1.84×10^{-9}			4.21×10^{-7}		
145	0.12619	0.12619		7.72×10^{-10}	7.72×10^{-10}		4.73×10^{-8}	4.73×10^{-8}	
	0.12598			1.48×10^{-9}			6.01×10^{-7}		
156	0.12599	0.12598	0.013%	1.51×10^{-9}	1.47×10^{-9}	4.08%	6.03×10^{-7}	6.15×10^{-7}	3.58%
	0.12596			1.40×10^{-9}			6.40×10^{-7}		
	0.12400			2.61×10^{-9}			1.28×10^{-6}		
172	0.12400	0.12404	0.055%	2.70×10^{-9}	2.69×10^{-9}	3.02%	1.23×10^{-6}	1.24×10^{-6}	2.71%
	0.12412			2.77×10^{-9}			1.21×10^{-6}		
	0.11945			2.39×10^{-9}			1.55×10^{-6}		
181	0.12464	0.12292	2.443%	2.98×10^{-9}	3.25×10^{-9}	11.72%	1.57×10^{-6}	1.58×10^{-6}	2.53%
	0.12466			3.52×10^{-9}			1.63×10^{-6}		

Table I-5: Amount ratio of ^{41}Ca tracer relative to natural calcium for different bone sites. The $^{41}Ca/^{40}Ca$ measurements ($n(^{41}Ca)/n(^{40}Ca)$) were obtained from single measurements using AMS with relative measurement uncertainty of 5% (coverage factor (k) = 1). The ratios of ^{41}Ca tracer over natural calcium ($n(^{41}Ca_{tracer})/n(Ca_{nat})$) were calculated based on the isotopic abundance of ^{40}Ca. Column numbers are indicated in square brackets.

Sample	**Bone Type**[a]	**Bone Site**	**$n(^{41}Ca)/n(^{40}Ca)$ (10^{-12} mol/mol)**	**$n(^{41}Ca_{tracer})/n(Ca_{nat})$**	
				(10^{-12} mol/mol)[b]	**(mol/mol)**
[1]	**[2]**	**[3]**	**[4]**	**[5]**	**[6]**
Tibia	C	T A2 a	228	219	2.19×10^{-10}
	T	T A2 b	270	259	2.59×10^{-10}
	C	T A2 c	368	353	3.53×10^{-10}
	C	TH	185	177	1.77×10^{-10}
	C	T P2 e	748	718	7.18×10^{-10}
	T	T P2 c	474	454	4.54×10^{-10}
	T	T P2 b	190	182	1.82×10^{-10}
	T-C	T P2 a	252	242	2.42×10^{-10}
Metacarpus	T-C	MC A2 a	181	174	1.74×10^{-10}
	C	MC A2 b	151	145	1.45×10^{-10}
	C	MC E	116	111	1.11×10^{-10}
	T	MC J3 a	332	318	3.18×10^{-10}
	C-T	MC J3 b	359	344	3.44×10^{-10}

Table I-5 (cont'd): ^{41}Ca tracer relative to natural calcium for different bone sites.

Sample	Bone Type[a]	Bone Site	$n(^{41}Ca)/n(^{40}Ca)$ (10^{-12} mol/mol)	$n(^{41}Ca_{tracer})/n(Ca_{nat})$	
				(10^{-12} mol/mol)[b]	(mol/mol)
Metatarsus	T-C	MT A2 a	223	214	2.14×10^{-10}
	C	MT A2 b	351	336	3.36×10^{-10}
	C	MT E	305	293	2.93×10^{-10}
	C-T	MT J3 b	254	244	2.44×10^{-10}
Radius	T-C	R A3 a	195	187	1.87×10^{-10}
	T	R A3 b	188	181	1.81×10^{-10}
	T	R A3 c	628	602	6.02×10^{-10}
	T	R A3 d	700	671	6.71×10^{-10}
	T-C	R A3 e	781	749	7.49×10^{-10}
	C	R H	224	214	2.14×10^{-10}
	C	R K	223	214	2.14×10^{-10}
	T	R N	176	169	1.69×10^{-10}
Vertebrae	C-T	V B	659	632	6.32×10^{-10}
	C	V F a	254	244	2.44×10^{-10}
	T	V F b	212	204	2.04×10^{-10}

[a] C refers to cortical bone. T refers to trabecular bone. C-T refers to a mix of cortical and trabecular bone, dominated by the cortical portion. T-C refers to a mix of trabecular and cortical bone, dominated by the trabecular portion.

[b] Amount ratio of ^{41}Ca tracer to natural calcium was calculated by multiplying the measured amount ratio of ^{41}Ca tracer to ^{40}Ca in the sample (column [4]) with the natural isotopic abundance of ^{40}Ca of 0.959.

Table I-6: ^{86}Sr tracer relative to ^{41}Ca tracer for different bone sites. The $^{86}Sr/^{88}Sr$ measurements ($^{86}Sr/^{88}Sr$) were obtained from single measurements by using TIMS with relative measurement uncertainty of 10 ppm (k=1). The amount ratios of ^{86}Sr tracer over natural strontium ($n(^{86}Sr_{tracer})/n(Sr_{nat})$) were calculated following IDMS principles. Natural strontium and calcium concentrations ($c(Sr_{nat})$ and $c(Ca_{nat})$) were obtained from triplicate measurements using AAS. The amount ratio of ^{41}Ca tracer to natural calcium ($n(^{41}Ca_{tracer})/n(Ca_{nat})$) was calculated in Table I-5. ^{86}Sr tracer concentration ($c(^{86}Sr_{tracer})$), ^{41}Ca tracer concentration ($c(^{41}Ca_{tracer})$) and amount ratio of ^{86}Sr tracer to ^{41}Ca tracer in the sample ($n(^{86}Sr_{tracer})/n(^{41}Ca_{tracer})$) were calculated based on the amount ratio of ^{86}Sr tracer to natural strontium, the concentration of natural strontium, the amount ratio of ^{41}Ca tracer to natural calcium and the concentration of natural calcium. Column numbers are indicated in square brackets.

Sample	**Bone Type**[a]	**Bone Site**	**$n(^{86}Sr)/n(^{88}Sr)$ (mol/mol)**	**$n(^{86}Sr_{tracer})/n(Sr_{nat})$ (mol/mol)**	**$c(Sr_{nat})$ (mol/g)**	**$c(^{86}Sr_{tracer})$ (mol/g)**[b]	**$n(^{41}Ca_{tracer})/n(Ca_{nat})$ (mol/mol)**	**$c(Ca_{nat})$ (mol/g)**	**$c(^{41}Ca_{tracer})$ (mol/g)**[c]	**$\frac{n(^{86}Sr_{tracer})}{n(^{41}Ca_{tracer})}$ (mol/mol)**[d]
[1]	**[2]**	**[3]**	**[4]**	**[5]**	**[6]**	**[7]**	**[8]**	**[9]**	**[10]**	**[11]**
Tibia	C	T A2 a	0.126871	0.0065	1.28×10^{-6}	8.26×10^{-9}	2.19×10^{-10}	6.05×10^{-3}	1.33×10^{-12}	6.24×10^{3}
	C	T A2 c	0.132086	0.0109	1.37×10^{-6}	1.50×10^{-8}	3.53×10^{-10}	5.72×10^{-3}	2.02×10^{-12}	7.43×10^{3}
	C	TH	0.124877	0.0047	1.58×10^{-6}	7.50×10^{-9}	1.77×10^{-10}	6.60×10^{-3}	1.17×10^{-12}	6.42×10^{3}
	C	T P2 e	0.145326	0.0223	1.45×10^{-6}	3.23×10^{-8}	7.18×10^{-10}	6.15×10^{-3}	4.41×10^{-12}	7.33×10^{3}
	T	T P2 c	0.131061	0.0101	9.81×10^{-7}	9.87×10^{-9}	4.54×10^{-10}	4.66×10^{-3}	2.12×10^{-12}	4.66×10^{3}
	T	T P2 b	0.125590	0.0054	1.10×10^{-6}	5.89×10^{-9}	1.82×10^{-10}	4.93×10^{-3}	8.97×10^{-12}	6.56×10^{3}
	T-C	T P2 a	0.124243	0.0042	1.02×10^{-6}	4.30×10^{-9}	2.42×10^{-10}	4.46×10^{-3}	1.08×10^{-12}	4.00×10^{3}

Table I-6 (cont'd): ^{86}Sr tracer relative to ^{41}Ca tracer for different bone sites.

Sample	Bone Type[a]	Bone Site	$n(^{86}Sr)/n(^{88}Sr)$ (mol/mol)	$n(^{86}Sr_{tracer})/n(Sr_{nat})$ (mol/mol)	$c(Sr_{nat})$ (mol/g)	$c(^{86}Sr_{tracer})$ (mol/g)[b]	$n(^{41}Ca_{tracer})/n(Ca_{nat})$ (mol/mol)	$c(Ca_{nat})$ (mol/g)	$c(^{41}Ca_{tracer})$ (mol/g)[c]	$\frac{n(^{86}Sr_{tracer})}{n(^{41}Ca_{tracer})}$ (mol/mol)[d]
Meta-carpus	T-C	MC A2 a	0.124959	0.0048	1.13×10^{-6}	5.45×10^{-9}	1.74×10^{-10}	5.78×10^{-3}	1.00×10^{-12}	5.43×10^{3}
	C	MC A2 b	0.124407	0.0043	1.47×10^{-6}	6.37×10^{-9}	1.45×10^{-10}	6.19×10^{-3}	8.95×10^{-13}	7.12×10^{3}
	C	MC E	0.123102	0.0032	1.42×10^{-6}	4.57×10^{-9}	1.11×10^{-10}	7.00×10^{-3}	7.79×10^{-13}	5.86×10^{3}
	T	MC J3 a	0.131057	0.0101	1.16×10^{-6}	1.17×10^{-8}	3.18×10^{-10}	5.31×10^{-3}	1.69×10^{-12}	6.92×10^{3}
	C-T	MC J3 b	0.132145	0.0110	1.48×10^{-6}	1.63×10^{-8}	3.44×10^{-10}	6.41×10^{-3}	2.21×10^{-12}	7.38×10^{3}
Meta-tarsus	T-C	MT A2 a	0.123382	0.0035	1.13×10^{-6}	3.92×10^{-9}	2.14×10^{-10}	5.22×10^{-3}	1.12×10^{-12}	3.50×10^{3}
	C	MT E	0.129610	0.0088	1.14×10^{-6}	1.01×10^{-8}	2.93×10^{-10}	6.09×10^{-3}	1.78×10^{-12}	5.66×10^{3}
	C-T	MT J3 b	0.122080	0.0023	1.26×10^{-6}	2.96×10^{-9}	2.44×10^{-10}	6.38×10^{-3}	1.55×10^{-12}	1.90×10^{3}

Table I-6 (cont'd): ^{86}Sr tracer relative to ^{41}Ca tracer for different bone sites.

Sample	Bone Type[a]	Bone Site	$n(^{86}Sr)/n(^{88}Sr)$ (mol/mol)	$n(^{86}Sr_{tracer})/n(Sr_{nat})$ (mol/mol)	$c(Sr_{nat})$ (mol/g)	$c(^{86}Sr_{tracer})$ (mol/g)[b]	$n(^{41}Ca_{tracer})/n(Ca_{nat})$ (mol/mol)	$c(Ca_{nat})$ (mol/g)	$c(^{41}Ca_{tracer})$ (mol/g)[c]	$\frac{n(^{86}Sr_{tracer})}{n(^{41}Ca_{tracer})}$ (mol/mol)[d]
Radius	T-C	R A3 a	0.125862	0.0056	1.15×10^{-6}	6.44×10^{-9}	1.87×10^{-10}	5.17×10^{-3}	9.65×10^{-13}	6.67×10^{3}
	T	R A3 b	0.125178	0.0050	1.39×10^{-6}	6.94×10^{-9}	1.81×10^{-10}	5.08×10^{-3}	9.18×10^{-13}	7.56×10^{3}
	T	R A3 c	0.144344	0.0215	1.08×10^{-6}	2.31×10^{-8}	6.02×10^{-10}	5.10×10^{-3}	3.07×10^{-12}	7.53×10^{3}
	T	R A3 d	0.146565	0.0234	1.38×10^{-6}	3.22×10^{-8}	6.71×10^{-10}	5.21×10^{-3}	3.50×10^{-12}	9.21×10^{3}
	T-C	R A3 e	0.149815	0.0262	1.42×10^{-6}	3.72×10^{-8}	7.49×10^{-10}	5.72×10^{-3}	4.28×10^{-12}	8.69×10^{3}
	C	R H	0.126619	0.0062	1.42×10^{-6}	8.84×10^{-9}	2.14×10^{-10}	6.66×10^{-3}	1.43×10^{-12}	6.19×10^{3}
	C	R K	0.126930	0.0065	1.47×10^{-6}	9.56×10^{-9}	2.14×10^{-10}	5.94×10^{-3}	1.27×10^{-12}	7.52×10^{3}
	T	R N	0.124787	0.0047	9.68×10^{-7}	4.52×10^{-9}	1.69×10^{-10}	4.71×10^{-3}	7.96×10^{-13}	5.68×10^{3}

[a] C refers to cortical bone. T refers to trabecular bone. C-T refers to a mix of cortical and trabecular bone, dominated by the cortical portion. T-C refers to a mix of trabecular and cortical bone, dominated by the trabecular portion.

[b] ^{86}Sr tracer concentration in the sample was calculated by multiplying the amount ratio of ^{86}Sr tracer to natural strontium (column [5]) with the concentration of natural strontium (column [6]).

[c] ^{41}Ca tracer concentration in the sample was calculated by multiplying the amount ratio of ^{41}Ca tracer to natural calcium (column [8]) with the concentration of natural calcium (column [9]).

[d] Amount ratio of ^{86}Sr tracer to ^{41}Ca tracer was calculated by dividing ^{86}Sr tracer concentration in the sample (column [7]) with ^{41}Ca tracer concentration in the sample (column [10]).

Appendix II

Supplementary Data for Uptake and Deposition of Strontium in Bone and Soft Tissues: A Dose-Response Study in Rats

(Part B, Chapter 3)

Table II-1: Rats' body weights. The body weight of each rat was monitored weekly throughout the study period. Numbers in the table header indicate individual rats (n=10) in each of the groups.

Day	Body Weight (gram)											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Mean ± SD**	
Control Group												
1	580	624	608	670	642	640	613	577	654	615	622	30
4	580	626	604	669	638	642	614	585	660	619	624	29
11	590	637	619	694	637	671	633	602	686	634	640	34
18	601	650	628	696	646	670	631	594	694	632	644	35
25	602	669	626	707	653	684	641	607	706	644	654	37
31	603	675	637	714	658	683	651	611	716	638	659	39
38	614	699	645	714	670	694	654	626	729	648	669	38
45	618	707	657	735	678	710	669	643	752	653	682	43
54	628	720	659	740	690	714	679	652	760	663	691	42
60	634	733	663	741	698	721	677	654	769	669	696	44
66	640	743	670	750	701	732	685	666	782	675	704	45
73	642	758	677	756	700	723	681	669	781	672	706	46
83	642	758	673	756	690	728	681	673	-	674	697	41
Mean	**613**	**691**	**644**	**719**	**669**	**693**	**655**	**627**	**728**	**649**	**669**	**39**
Low Dose Group												
4	653	597	582	668	586	591	615	668	618	571	615	36
11	655	604	576	669	582	606	616	681	622	574	619	38
28	676	631	594	706	606	625	620	712	602	580	635	47
32	674	629	593	701	615	626	626	711	630	581	639	43
39	674	644	595	710	615	634	629	721	644	583	645	45
47	683	645	600	711	604	645	638	730	651	593	650	46
56	695	649	601	728	614	655	652	744	664	594	660	51
60	693	658	596	727	618	658	650	742	663	596	660	50
67	705	668	597	734	624	672	656	741	663	588	665	52
76	716	688	603	734	610	683	668	750	668	600	672	54
83	715	687	612	750	623	691	662	761	677	596	677	56
Mean	**678**	**635**	**590**	**702**	**604**	**633**	**634**	**713**	**640**	**584**	**641**	**44**

Table II-1 (cont'd): Rats' body weights.

Day	Body Weight (gram)											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Mean ± SD**	
High Dose Group												
0	623	728	702	697	694	613	690	692	655	659	675	37
6	623	724	692	695	690	608	684	692	648	663	672	36
13	629	736	688	713	684	611	689	678	636	669	673	39
20	630	754	698	719	698	618	695	689	648	695	684	41
28	640	764	703	734	713	617	705	677	651	702	691	45
34	644	770	695	736	706	615	714	688	660	710	694	45
41	650	781	702	740	715	611	735	689	665	716	700	49
56	666	788	700	761	724	601	749	698	667	737	709	55
62	668	787	702	768	730	608	754	673	679	744	711	55
69	649	800	707	758	733	608	750	590	675	755	703	70
77	650	821	710	767	744	608	742	566	660	764	703	80
83	628	792	708	757	733	604	735	578	652	767	695	75
Mean	**643**	**770**	**700**	**736**	**712**	**611**	**720**	**668**	**659**	**711**	**693**	**47**

Table II-2: Rats' drinking water consumption. The amount of water consumed by each rat was monitored regularly (2-3 times weekly) and average daily water consumption was calculated. Empty cells show that no reading was taken on those days and the average was based on the following monitoring periods. Numbers in the table header indicate individual rats (n=10) in each of the groups.

Day	Average Amount of Water Consumed (gram/day)											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Mean ± SD**	
Control Group												
0	20.3	19.6	23.6	26.9	19.0	30.5	22.9	21.5	25.3	24.4	23.4	3.6
1	16.8	23.8	21.2	22.3	17.8	25.0	24.0	25.2	26.8	28.4	23.1	3.7
4	20.9	26.2	24.6	23.6	21.5	26.4	22.5	26.9	29.9	33.1	25.6	3.8
5	24.6	30.3	32.5	35.7	20.7	39.9	33.5	46.2	50.4	46.1	36.0	9.7
8	31.1	32.0	32.4	31.6	30.7	54.2	37.2	39.2	43.9	47.7	43.6	4.3
11	33.2	33.9	35.9	34.7	37.2	43.4	40.7	33.0	49.0	51.3	39.23	6.7
13	-	-	-	-	-	-	-	44.7	39.7	56.1	46.8	8.4
18	35.5	35.0	31.2	33.9	33.2	40.1	45.5	41.7	45.4	54.2	39.6	7.2
25	31.5	33.1	32.2	33.4	36.1	41.8	42.1	42.4	40.5	54.0	38.7	6.9
32	36.2	40.8	33.8	30.3	38.4	45.7	51.3	34.2	49.7	57.1	41.8	8.8
36	35.1	36.4	37.8	32.6	36.3	45.3	56.5	35.4	46.7	58.5	42.1	9.3
39	34.5	36.9	34.2	36.4	37.5	47.9	45.5	38.3	44.9	58.7	41.5	7.7
43	34.4	30.1	31.3	40.7	32.5	42.2	43.1	37.1	42.7	61.9	39.6	9.2
46	42.5	36.1	36.6	42.6	37.3	45.2	45.9	54.2	49.8	61.7	45.2	8.2
49	40.6	33.8	30.9	32.4	31.8	48.0	37.6	43.3	42.1	63.4	40.4	9.9

Table II-2 (cont'd): Rats' drinking water consumption.

Day	Average Amount of Water Consumed (gram/day)											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Mean ± SD**	
53	36.5	32.3	36.3	34.3	31.9	47.3	41.5	37.1	42.2	62.9	40.2	9.3
56	37.3	29.4	34.1	30.5	31.1	41.4	36.5	37.3	41.2	53.4	37.2	7.1
61	34.5	32.0	33.9	30.0	28.3	28.5	36.8	32.3	41.7	40.4	33.8	4.6
63	36.8	32.1	28.7	30.6	31.1	38.8	38.6	33.7	41.4	42.0	35.4	4.7
67	32.0	34.3	29.4	32.8	28.2	39.3	49.9	36.0	37.7	56.4	37.6	9.0
71	38.2	31.5	31.8	31.7	23.9	46.2	43.9	44.0	42.4	57.5	39.1	9.7
74	37.3	30.7	32.2	31.0	23.5	47.7	45.3	40.8	37.6	56.6	38.3	9.7
78	36.8	33.9	31.7	29.1	25.6	49.7	49.6	38.5	-	52.5	38.6	9.8
Low Dose Group												
0	22.3	39.4	30.4	36.8	29.5	28.0	27.7	31.5	26.2	23.3	29.5	5.4
4	23.3	34.9	28.9	35.4	29.9	29.5	27.0	28.2	26.3	23.0	28.6	4.2
8	25.3	33.8	29.6	37.1	31.1	33.2	25.7	33.2	29.1	22.3	30.0	4.6
11	26.8	35.2	30.1	34.2	33.0	26.5	25.6	33.9	29.9	22.3	29.7	4.3
15	26.7	39.5	33.5	45.4	39.1	26.2	23.4	32.8	34.3	24.1	32.5	7.4
20	26.9	35.1	33.3	40.2	40.2	29.4	25.4	35.3	36.3	26.6	32.9	5.5
22	-	-	-	-	-	-	-	32.7	33.9	28.1	31.6	3.1
25	18.7	42.0	39.5	37.8	41.8	42.3	49.3	41.4	28.7	29.9	37.1	8.9

Table II-2 (cont'd): Rats' drinking water consumption.

Day	Average Amount of Water Consumed (gram/day)											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Mean ± SD**	
28	20.0	23.8	22.2	46.6	29.7	17.9	17.9	41.6	14.9	27.3	26.2	10.5
32	26.4	36.4	30.1	37.9	35.8	27.5	26.6	32.9	43.3	27.6	32.5	5.8
35	23.3	38.2	31.8	43.5	41.5	28.4	24.0	38.0	38.7	25.7	33.3	7.6
39	27.4	39.9	32.7	41.9	38.1	24.7	26.0	39.8	43.1	26.4	34.0	7.3
42	27.8	41.8	33.9	41.0	34.2	27.6	25.7	42.6	36.8	22.8	33.4	7.2
47	28.2	43.5	31.7	42.6	32.0	27.7	25.0	34.8	44.4	26.0	33.6	7.5
49	32.2	38.2	35.9	37.8	32.1	26.9	26.6	37.0	36.8	25.2	32.9	5.1
53	29.8	39.7	32.7	44.5	36.0	28.4	27.8	37.0	34.0	25.3	33.5	6.0
56	24.9	34.4	28.0	42.7	35.3	24.0	24.0	30.4	34.0	26.7	30.4	6.1
60	26.9	38.0	31.3	48.2	32.9	29.4	25.8	34.2	34.5	27.4	32.9	6.6
63	27.1	38.2	30.4	36.7	32.8	27.9	25.1	30.3	31.1	24.0	30.4	4.6
67	28.5	37.3	26.8	29.6	36.0	29.6	24.6	38.0	36.4	25.9	31.3	5.1
70	28.0	37.0	29.8	36.6	34.7	27.6	25.2	31.8	31.6	24.7	30.7	4.4
76	22.8	33.5	33.7	36.5	34.9	26.2	23.8	31.8	33.9	23.2	30.0	5.4
81	26.2	38.7	33.1	41.4	33.6	26.9	22.3	26.8	34.0	21.4	30.4	6.7
High Dose Group												
0	19.3	19.0	16.0	24.9	18.5	17.5	20.3	21.3	18.7	19.5	19.5	2.4

Table II-2 (cont'd): Rats' drinking water consumption.

Day	Average Amount of Water Consumed (gram/day)											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Mean ± SD**	
3	19.3	26.9	22.3	25.5	30.2	23.1	25.9	24.5	19.5	26.0	24.3	3.4
6	19.1	28.9	24.4	28.7	24.0	24.3	27.6	22.2	19.4	26.8	24.5	3.5
13	19.5	30.6	28.1	28.7	23.3	26.8	25.5	25.3	22.7	28.8	25.9	3.4
20	20.7	31.6	28.4	31.2	25.5	26.1	28.7	24.3	23.4	27.0	26.7	3.4
28	21.6	29.1	26.6	31.4	21.5	17.0	26.4	23.5	24.3	29.1	25.1	4.3
34	20.9	31.5	27.7	30.0	22.4	25.3	27.1	23.7	26.2	26.7	26.1	3.3
37	21.4	33.2	28.3	28.8	25.4	23.1	31.1	23.9	25.2	26.7	26.7	3.7
40	21.2	34.0	30.5	31.7	25.1	26.0	28.7	26.9	26.9	29.0	28.0	3.6
44	21.1	35.2	30.4	30.3	26.3	25.3	25.8	27.9	26.8	28.5	27.7	3.8
49	21.5	34.6	31.0	34.5	26.6	20.9	29.4	27.9	27.8	36.8	29.1	5.3
54	30.3	24.8	28.3	37.5	21.5	13.6	9.5	16.9	30.6	26.8	24.0	8.6
57	25.3	36.5	39.2	26.5	29.2	36.3	35.9	49.5	27.6	29.0	33.5	7.5
61	23.6	36.7	35.0	31.9	26.3	29.9	28.5	11.2	28.6	30.7	28.2	7.1
64	15.5	30.1	31.8	28.8	24.6	27.0	23.1	5.4	28.1	28.5	24.3	8.1
68	20.2	38.6	31.3	30.3	26.2	29.3	27.5	7.6	28.7	29.7	27.0	8.2
71	18.3	38.5	33.1	33.9	23.8	29.0	25.2	16.8	22.7	28.8	27.0	7.0
76	20.1	42.8	36.8	33.1	25.5	29.1	28.8	20.5	29.7	34.4	30.1	7.1
78	17.8	33.2	35.3	30.8	24.6	28.1	27.0	25.6	27.0	30.8	28.0	5.0
83	19.7	49.9	23.5	28.9	23.7	25.0	31.6	26.7	22.5	30.0	28.2	8.5

Table II-3: Rats' strontium intake from drinking water. The amount of strontium intake for each rat was estimated based on the strontium concentration in the drinking water and the amount of water consumed. The total amount of strontium intake was added up at the end of the study and the average daily intake was calculated. Empty cells show that no reading was taken on those days and the total amount of intake was based on the following monitoring periods. Numbers in the table header indicate individual rats (n=10) in each of the groups.

Day	Sr Conc.	Total Amount of Strontium Intake (mg)										
	(%)	1	2	3	4	5	6	7	8	9	10	Mean ± SD
Low Dose Group												
0	0.2	179	315	243	294	236	224	222	252	209	187	
4	0.2	186	279	231	283	239	236	216	225	210	184	
8	0.2	152	202	177	223	187	199	154	199	175	134	
11	0.2	214	281	241	273	264	212	205	271	240	178	
15	0.2	266	395	334.86	454	391	262	234	328	343	241	
20	0.2	269	351	333	402	402	293	254	141	145	106	
22	0.2	-	-	-	-	-	-	-	196	203	169	
25	0.2	112	252	237	227	251	254	296	248	172	179	
28	0.2	160	190	178	373	238	143	143	333	120	218	
32	0.2	159	219	181	228	215	165	160	198	260	165	
35	0.2	187	305	255	348	332	227	192	304	309	206	
39	0.2	164	240	196	252	229	148	156	239	259	158	
42	0.2	278	418	339	410	342	276	257	426	368	228	
47	0.2	113	174	127	171	128	111	100	139	178	104	

Table II-3 (cont'd): Rats' strontium intake from drinking water.

Day	Sr Conc. (%)	Total Amount of Strontium Intake (mg)											
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Mean ± SD**	
49	0.2	258	306	287	302	257	215	213	296	295	201		
53	0.2	179	238	196	267	216	170	167	222	204	152		
56	0.2	199	275	224	342	282	192	192	243	272	214		
60	0.2	161	228	188	289	197	176	155	205	207	165		
63	0.2	217	305	243	294	263	223	201	242	249	192		
67	0.2	171	224	161	178	216	178	148	228	218	155		
70	0.2	336	443	357	439	416	331	303	382	379	296		
76	0.2	228	335	337	365	349	262	239	318	339	232		
81	0.2	157	232	199	249	201	161	134	161	204	128		
Total		4344	6209	5262	6661	5849	4659	4337	5798	5557	4192		
Average / day		52	734	63	79	70	56	52	69	66	50		
Average / kg / day		**76**	**116**	**106**	**113**	**115**	**88**	**82**	**97**	**103**	**86**	**98**	**15**
High Dose Group													
0	1	578	569	481	746	554	525	610	639	560	586		
3	1	579	807	670	766	906	694	777	734	585	780		
6	1	1338	2020	1709	2007	1683	1698	1928	1554	1357	1873		

Table II-3 (cont'd): Rats' strontium intake from drinking water.

Day	Sr Conc. (%)	Total Amount of Strontium Intake (mg)										
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Mean ± SD**
13	1	1368	2141	1968	2006	1630	1873	1784	1771	1592	2019	
20	1	1654	2530	2270	2498	2036	2087	2295	1943	1873	2158	
28	1	1297	1745	1594	1883	1291	1022	1581	1410	1460	1746	
34	1	836	1259	1106	1200	894	1011	1085	947	1048	1066	
37	1	642	997	850	862	761	694	933	717	756	801	
40	1	847	1359	1219	1267	1003	1039	1150	1076	1074	1159	
44	1	1053	1758	1518	1514	1316	1263	1290	1394	1338	1426	
49	1	1077	1730	1551	1724	1330	1047	1472	1397	1389	1841	
54	1	909	743	848	1126	645	407	285	508	918	804	
57	1	1011	1462	1568	1059	1168	1453	1434	1981	1106	1158	
61	1	706	1101	1051	956	790	896	854	336	858	921	
64	1	619	1202	1272	1153	986	1081	924	214	1124	1139	
68	1	606	1159	937	910	786	880	826	229	861	890	
71	1	916	1926	1656	1696	1192	1449	1260	838	1134	1442	
76	1	403	856	736	663	511	582	576	411	593	688	
78	1	710	1329	1410	1233	983	1123	1079	1025	1080	1233	
83	1	197	499	235	289	237	250	316	267	225	300	

Table II-3 (cont'd): Rats' strontium intake from drinking water.

Day	Sr Conc. (%)	Total Amount of Strontium Intake (mg)											
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Mean ± SD**	
Total		17345	27190	24651	25555	20699	21073	22456	19391	20930	24030		
Average / day		207	324	294	304	246	251	267	231	249	286		
Average / kg / day		**321**	**421**	**419**	**414**	**346**	**411**	**371**	**345**	**378**	**402**	**382.79**	**35.91**

Table II-4: Rats' feed consumption. The feed intake for each rat was monitored regularly. The total amount of feed intake was added up at the end of the study and the average daily intake was calculated. Empty cells show that no reading was taken on those days and the average was based on the following monitoring periods. Numbers in the table header indicate individual rats (n=10) in each of the groups.

Day	Average Feed Intake (g/day)											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Mean ± SD**	
Control Group												
1	24	29	26	29	21	27	25	26	31	26	26	3
4	24	27	26	31	22	29	24	32	31	26	27	3
11	27	31	29	33	25	34	27	21	34	29	29	4
18	25	31	27	31	25	31	26	26	33	27	28	3
32	25	31	27	31	27	30	25	27	32	27	28	2
56	26	30	27	29	25	30	25	27	-	28	27	2
Average	**25**	**30**	**27**	**30**	**25**	**30**	**25**	**27**	**31**	**27**	**28**	**2**
Low Dose Group												
4	23	30	30	26	25	27	25	27	25	23	26	2
15	26	29	24	29	28	28	27	30	26	24	27	2
49	27	30	23	27	28	27	26	29	26	24	27	2
83	-	25	18	-	-	-	-	-	-	-	-	-
Average	**25**	**28**	**24**	**26**	**26**	**26**	**25**	**27**	**25**	**23**	**26**	**2**

Table II-4 (cont'd): Rats' feed consumption.

Day	Average Feed Intake (g/day)											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Mean ± SD**	
High Dose Group												
0	26	29	25	29	27	24	29	28	29	35	28	3
6	23	29	24	26	23	23	28	23	21	32	25	3
13	23	30	24	28	26	23	28	25	23	33	26	3
23	25	31	25	30	26	21	29	25	24	32	27	4
55	24	29	26	29	26	23	27	14	23	33	25	5
77	-	-	-	-	-	-	-	-	-	32	32	-
83	-	38	-	29	-	-	-	-	-	38	35	-
84	-	-	-	-	-	-	31	-	-	35	33	-
Average	**24**	**30**	**25**	**29**	**26**	**22**	**28**	**21**	**24**	**33**	**26**	**4**

Table II-5: Strontium concentration in rat serum samples. The serum samples were collected one day before the sacrifice. Strontium spike ($n(Sr_{spike})$) was added into the sample for quantification. The $^{86}Sr/^{88}Sr$ measurements ($n(^{86}Sr)/n(^{88}Sr)$) were obtained from single measurements using TIMS (relative measurement uncertainty of ~10 ppm, *k*=1). Number of moles strontium in the sample ($n(Sr_{sample})$) was calculated following IDMS principles. Concentration of strontium in the sample ($c(Sr_{sample})$) was calculated based on the number of moles strontium in the sample and the mass of the serum sample (m_{serum}). Column numbers are indicated in square brackets.

Group	**m_{serum} (g)**	**$n(Sr_{spike})$ ($x10^{-6}$ mol)**	**$n(^{86}Sr)/n(^{88}Sr)$**	**$n(Sr_{sample})$ ($x10^{-6}$ mol)[a]**	**$c(Sr_{sample})$ (µg/g)[b]**
[1]	**[2]**	**[3]**	**[4]**	**[5]**	**[6]**
Baseline					
1	0.20549	0.00527	13.80674	0.00020	0.084
2	0.20886	0.00530	13.59199	0.00021	0.086
3	0.20301	0.00524	13.14927	0.00022	0.094
4	0.20943	0.00526	13.54903	0.00021	0.086
5	0.19944	0.00524	13.19258	0.00022	0.095
6	0.20214	0.00528	13.21627	0.00022	0.094
7	0.20640	0.00522	13.36808	0.00021	0.089
8	0.20611	0.00528	12.71088	0.00024	0.100
9	0.20415	0.00528	13.61613	0.00020	0.087
10	0.20080	0.00534	13.68012	0.00020	0.089
Average					**0.091**
SD					**0.005**
%RSD					**6%**
Control Group					
1	0.19581	0.00512	13.68779	0.00020	0.087
2	0.20131	0.00517	13.77199	0.00019	0.085
3	0.20482	0.00522	13.65571	0.00020	0.086
4	0.20000	0.00518	13.81659	0.00019	0.085
5	0.20087	0.00525	13.83975	0.00020	0.085
6	0.20463	0.00527	13.67686	0.00020	0.086
7	0.20890	0.00526	13.58284	0.00020	0.086
8	0.20768	0.00523	13.13229	0.00022	0.092
10	0.20092	0.00523	13.67553	0.00020	0.087
Average					**0.086**
SD					**0.002**
%RSD					**3%**

Table II-5 (cont'd): Strontium concentration in rat serum samples.

Group	m_{serum} (g)	$n(Sr_{spike})$ ($x10^{-6}$ mol)	$n(^{86}Sr)/n(^{88}Sr)$	$n(Sr_{sample})$ ($x10^{-6}$ mol)	$c(Sr_{sample})$ (µg/g)
Low Dose Group					
1	0.10312	0.00517	0.73594	0.00938	8.0
2	0.10281	0.00518	0.62337	0.01156	9.9
3	0.10462	0.00518	0.56206	0.01320	11.1
4	0.10708	0.00519	0.47979	0.01630	13.3
5	0.10227	0.00512	0.73857	0.00925	7.9
6	0.10184	0.00523	0.70176	0.01008	8.7
7	0.10179	0.00524	0.94650	0.00703	6.1
8	0.10428	0.00527	0.68408	0.01047	8.8
9	0.09909	0.00533	0.49901	0.01587	14.0
10	0.105	0.00511	0.99273	0.00648	5.4
Average					**9.3**
SD					**2.8**
%RSD					**30%**
High Dose Group					
1	0.09976	0.00509	0.25909	0.04160	36.5
2	0.10429	0.00523	0.23784	0.05049	42.4
3	0.09968	0.00520	0.25582	0.04355	38.3
4	0.10894	0.00523	0.24860	0.04625	37.2
5	0.10108	0.00521	0.26217	0.04169	36.1
6	0.10158	0.00508	0.28470	0.03503	30.2
7	0.09093	0.00523	0.29053	0.03484	33.6
8	0.10142	0.00517	0.29287	0.03401	29.4
9	0.10508	0.00518	0.25664	0.04314	36.0
10	0.10229	0.00518	0.25987	0.04214	36.1
Average					**35.6**
SD					**3.8**
%RSD					**11%**

[a] Number of moles of strontium in the sample was calculated following IDMS principles (Eqn. 2).

[b] Concentration of strontium in the sample was calculated by multiplying the number of mol of strontium in the sample (column [5]) with the standard atomic weight of strontium in the sample (87.61681) and dividing it by the mass of the serum (column [2]).

Table II-6: Strontium concentration in rat bone samples. After being subjected to three-point bending measurement, the whole right tibia was weighed and subjected to microwave-assisted acid digestion. The digest was evaporated and a portion of it was used for strontium analysis. Strontium spike ($n(Sr_{spike})$) was added and equilibrated with the strontium from the sample ($n(Sr_{sample})$). The $^{86}Sr/^{88}Sr$ measurements ($n(^{86}Sr)/n(^{88}Sr)$) were obtained from single measurements using TIMS (relative measurement uncertainty of ~10 ppm, k=1). Number of moles strontium in the sample ($n(Sr_{sample})$) was calculated following IDMS principles. Concentration of strontium in the sample ($c(Sr_{bone})$) was calculated based on the number of moles strontium in the sample, the total mass of digest (m_{digest}), the dilution factor (m_{total}/m_{dilute}), the mass of the sample used for measurement ($m_{analyze}$) and the mass of the bone sample (m_{bone}). Column numbers are indicated in square brackets.

Rat No.	m_{bone} (g)	m_{digest} (g)	m_{dilute} (g)	m_{total} (g)	$m_{analyze}$ (g)	$m(Sr_{spike})$ (µg)	$n(Sr_{spike})$ ($x10^{-6}$ mol)	$\frac{n(^{86}Sr)}{n(^{88}Sr)}$	$n(Sr_{sample})$ ($x10^{-6}$ mol)[a]	$c(Sr_{sample})$ (µg/g)[b]	$m(Sr_{bone})$ (µg)[c]	$c(Sr_{bone})$ (µg/g)[d]
[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]
Baseline												
1	0.66138	4.99794			0.25226	0.50612	0.00589	0.69937	0.0114	3.95	19.8	29.9
2	0.72278	5.00551			0.25006	0.50108	0.00583	0.62560	0.0130	4.54	22.7	31.4
3	0.76378	4.99660			0.25478	0.50085	0.00582	0.65501	0.0122	4.20	21.0	27.5
4	0.71218	5.00341			0.25622	0.50069	0.00582	0.60958	0.0134	4.57	22.9	32.1
5	0.82414	5.01645			0.26300	0.50948	0.00592	0.59821	0.0139	4.64	23.3	28.3
6	0.66256	5.00003			0.25858	0.50185	0.00584	0.65557	0.0122	4.14	20.7	31.3
7	0.66734	4.99332			0.25353	0.50879	0.00592	0.70952	0.0112	3.89	19.4	29.1
8	0.70748	5.00039			0.24967	0.50127	0.00583	0.64396	0.0125	4.38	21.9	31.0
9	0.66412	5.01405			0.26119	0.50347	0.00585	0.67797	0.0118	3.95	19.8	29.8
10	0.73086	5.43505			0.25492	0.49901	0.00580	0.67656	0.0117	4.02	21.8	29.9
Average												**30.0**
SD												**1.5**
%RSD												**4.9%**

Table II-6 (cont'd): Strontium concentration in rat bone samples.

Rat No.	m_{bone} (g)	m_{digest} (g)	m_{dilute} (g)	m_{total} (g)	$m_{analyze}$ (g)	$m(Sr_{spike})$ (μg)	$n(Sr_{spike})$ ($x10^{-6}$ mol)	$\frac{n(^{86}Sr)}{n(^{88}Sr)}$	$n(Sr_{sample})$ ($x10^{-6}$ mol)[a]	$c(Sr_{sample})$ (μg/g)[b]	$m(Sr_{bone})$ (μg)[c]	$c(Sr_{bone})$ (μg/g)[d]
Control Group												
1	0.6448	5.00598			0.12901	0.50346	0.00585	1.42237	0.00489	3.32	16.6	25.8
2	0.6812	5.00086			0.13908	0.50016	0.00582	1.16036	0.00615	3.87	19.4	28.4
3	0.6913	5.14605			0.13450	0.50208	0.00584	1.26331	0.00559	3.64	18.7	27.1
4	0.7517	4.92476			0.13240	0.50096	0.00583	1.30824	0.00536	3.55	17.5	23.2
5	0.7474	5.08199			0.13192	0.50132	0.00583	1.22871	0.00576	3.83	19.5	26.0
6	0.7179	4.98104			0.12854	0.50082	0.00582	1.20166	0.00591	4.03	20.1	28.0
7	0.7418	5.09413			0.13113	0.49978	0.00581	1.17088	0.00608	4.06	20.7	27.9
8	0.6562	5.08588			0.13090	0.50324	0.00585	1.27593	0.00554	3.71	18.9	28.7
9	0.7024	5.04293			0.13477	0.50002	0.00581	1.24283	0.00567	3.69	18.6	26.5
10	0.7388	4.97617			0.13028	0.57532	0.00669	1.27092	0.00636	4.28	21.3	28.8
Average												**27.0**
SD												**1.7**
%RSD												**6.4%**

Table II-6 (cont'd): Strontium concentration in rat bone samples.

Rat No.	m_{bone} (g)	m_{digest} (g)	m_{dilute} (g)	m_{total} (g)	$m_{analyze}$ (g)	$m(Sr_{spike})$ (µg)	$n(Sr_{spike})$ ($x10^{-6}$ mol)	$\frac{n(^{86}Sr)}{n(^{88}Sr)}$	$n(Sr_{sample})$ ($x10^{-6}$ mol)[a]	$c(Sr_{sample})$ (µg/g)[b]	$m(Sr_{bone})$ (µg)[c]	$c(Sr_{bone})$ (µg/g)[d]
Low Dose Group												
1	0.7086	5.81047	0.21389	50.00389	0.20009	0.50580	0.00588	2.18475	0.00300	307	1783	2516
2	0.7087	5.79609	0.20461	50.00289	0.20153	0.50391	0.00586	1.31620	0.00535	569	3295	4650
3	0.5627	5.59289	0.20883	50.00122	0.20844	0.50039	0.00582	2.67360	0.00235	236	1321	2347
4	0.7100	5.72480	0.20174	50.02046	0.20256	0.49945	0.00581	1.89745	0.00348	373	2138	3011
5	0.6571	5.69569	0.20720	50.01701	0.20226	0.49960	0.00581	2.91632	0.00212	221	1260	1918
6	0.6721	5.74194	0.20036	50.00645	0.20045	0.49891	0.00580	2.18460	0.00296	323	1852	2756
7	0.6638	5.81372	0.20467	49.99905	0.20112	0.50148	0.00583	3.41162	0.00176	188	1091	1644
8	0.7912	5.91495	0.20811	50.00426	0.19939	0.50181	0.00584	2.09564	0.00312	329	1949	2463
9	0.6469	5.82604	0.20972	49.99530	0.20041	0.50499	0.00587	2.07865	0.00317	330	1925	2975
10	0.7042	5.82727	0.20622	50.01032	0.20006	0.50801	0.00591	3.87486	0.00153	163	948	1346
Average												**2563**
SD												**917**
%RSD												**36%**

Table II-6 (cont'd): Strontium concentration in rat bone samples.

Rat No.	m_{bone} (g)	m_{digest} (g)	m_{dilute} (g)	m_{total} (g)	$m_{analyze}$ (g)	$m(Sr_{spike})$ (µg)	$n(Sr_{spike})$ ($x10^{-6}$ mol)	$\frac{n(^{86}Sr)}{n(^{88}Sr)}$	$n(Sr_{sample})$ ($x10^{-6}$ mol)[a]	$c(Sr_{sample})$ (µg/g)[b]	$m(Sr_{bone})$ (µg)[c]	$c(Sr_{bone})$ (µg/g)[d]
High Dose Group												
1	0.6816	5.00052	0.10644	49.99925	0.10426	0.50431	0.00586	2.44647	0.00262	1035	5176	7593
2	0.7602	5.00477	0.10387	50.00827	0.10630	0.49815	0.00579	1.79681	0.00370	1467	7343	9659
3	0.7797	4.98981	0.11358	50.00671	0.10027	0.51351	0.00597	1.76343	0.00389	1498	7476	9588
4	0.7640	5.00734	0.10725	49.99820	0.10715	0.50754	0.00590	1.77923	0.00381	1452	7272	9518
5	0.7732	5.00713	0.11379	50.00749	0.10011	0.49852	0.00580	1.96195	0.00334	1286	6441	8330
6	0.7372	4.99425	0.10209	50.00363	0.10203	0.50137	0.00583	3.69183	0.00160	674	3368	4569
7	0.7062	4.99972	0.10886	49.99795	0.10202	0.50062	0.00582	1.86156	0.00357	1407	7035	9962
8	0.7818	4.00774	0.10631	50.01851	0.10008	0.50137	0.00583	3.21890	0.00189	778	3119	3990
9	0.7047	5.02209	0.10208	50.00205	0.10026	0.49963	0.00581	3.03912	0.00202	863	4333	6148
10	0.6961	5.01058	0.10158	50.00150	0.10021	0.50037	0.00582	1.98200	0.00332	1428	7154	10277
Average												**7963**
SD												**2313**
%RSD												**29%**

[a] Number of moles of strontium in the sample was calculated following IDMS principles (Eqn. 2).

[b] Concentration of strontium in the sample was calculated by multiplying the number of moles of strontium in the sample (column [10]) with the standard atomic weight of strontium in the sample (87.61681) and dividing it by the mass of the bone digest used for strontium analysis (column [6]).

[c] Amount of strontium in the sample was calculated by multiplying the concentration of strontium in the sample (column [11]) with the dilution factor.

[d] The concentration of strontium in the sample was calculated by dividing the amount of strontium in the sample (column [12]) with the mass of the sample (column [2]).

Table II-7: Strontium to calcium amount ratio (Sr/Ca) of bone samples obtained from PIXE. Mid-shaft femur and femoral head of rats from control, low dose and high dose groups were analyzed to study the distribution of strontium in cortical and trabecular bone. Background areas were those areas with no increase of strontium deposition, whereas strontium rich areas were those with strontium deposition higher than the other parts of the bone.

n(Sr)/n(Ca)	Relative MU[a]	n(Sr)/n(Ca)	Relative MU[a]	n(Sr)/n(Ca)	Relative MU[a]
Control - Mid-shaft Femur		**Control - Femoral Head**			
Background					
0.00012	0.00013	0.00014	0.00001		
Low Dose - Mid-shaft Femur					
Background		Sr Rich Area			
0.0036	0.0001	0.071	0.001		
High Dose - Mid-shaft Femur					
Background		Sr Rich Area-1		Sr Rich Area-2	
0.0097	0.0002	0.123	0.001	0.223	0.002
Low Dose - Femoral Head					
Background		Sr Rich Area			
0.0038	0.0001	0.074	0.001		
High Dose - Femoral Head					
Background		Sr Rich Area-1		Sr Rich Area-2	
0.0089	0.0002	0.257	0.004	0.245	0.003

[a] Relative measurement uncertainty (k=1).

Table II-8: Bone mechanical properties. Maximum load (Max. Load), maximum stress (Max. Stress) and flexure modulus (Flex. Modulus) were obtained directly from the three-point bending measurement. Stiffness, yield load and failure load were calculated based on the earlier parameters.

Rat No.	Max. Load (N)	Max. Stress (MPa)	Flex. Modulus (MPa)	Stiffness (N/mm)	Yield Load (N)	Failure Load (N)
Baseline						
1	85	2176	61791	146	37	72.3
2	92	2355	46182	79	68	78.5
3	71	1797	50383	101	59	65.9
4	74	1872	72098	186	45	69.4
5	105	2677	57296	180	86	88.2
6	70	1780	58371	101	59	63.0
7	86	2188	70130	151	64	80.9
8	73	1851	65555	161	40	63.0
9	83	2118	87515	207	70	71.4
10	77	1962	48888	105	64	70.0
Average	**82**	**2078**	**61821**	**142**	**59**	**72.3**
SD	**11**	**286**	**12578**	**43**	**15**	**8.1**
%RSD	**14%**	**14%**	**20%**	**31%**	**25%**	**11%**
Control Group						
1	65.9	1679	58282	134	43.4	65.2
2	70.9	1804	32587	0.00	0.00	0.00
3	60.3	1534	61406	0.00	0.00	0.00
4	72.5	1846	52478	105	60.4	72.5
5	79.2	2015	70035	179	50.9	75.1
6	85.0	2164	64515	156	65.7	82.4
7	84.8	2159	42973	165	65.0	81.6
8	67.5	1719	48855	108	53.1	64.3
9	66.7	1699	73706	186	53.3	65.0
10	66.6	1695	47239	88	63.1	59.5
Average	**71.9**	**1831**	**55208**	**140**	**56.8**	**70.7**
SD	**8.4**	**214**	**12777**	**37**	**7.9**	**8.5**
%RSD	**12%**	**12%**	**23%**	**26%**	**14%**	**12%**

Table II-8 (cont'd): Bone mechanical properties.

Rat No.	Max. Load (N)	Max. Stress (MPa)	Flex. Modulus (MPa)	Stiffness (N/mm)	Yield Load (N)	Failure Load (N)
Low Dose Group						
1	84.6	2155	78750	190	61.5	83.9
2	86.2			163	64.2	71.8
3	73.4	1869	77413	177	62.9	71.8
4	86.7	2209	97084	250	56.3	82.9
5	91.0	2317	32986	172	77.7	80.4
6	79.7	2030	79001	187	53.0	79.3
7	94.1	2396	70526	169	65.3	91.9
8	78.6	2002	101023	206	62.5	75.3
10	84.0	2139	75367	203	67.6	74.9
Average	**84.3**	**2139**	**76519**	**191**	**63.5**	**79.1**
SD	**6.4**	**172**	**20590**	**26**	**7.0**	**6.5**
%RSD	**7.5%**	**8.0%**	**27%**	**14%**	**11%**	**8.2%**
High Dose Group						
1	69	1769	81620	178	48	66
2	56	1435	57502	134	35	41
3	76	1926	48931	112	57	74
4	133	3385	73060	168	110	128
5	85	2165	67547	152	65	82
6	74	1893	52101	112	57	68
7	79	2014	84578	157	55	79
8	54	1381	41702	84	39	54
9	84	2133	85705	206	63	77
10	85	2172	78067	177	58	84
Average	**80**	**2027**	**67081**	**148**	**59**	**75**
SD	**22**	**553**	**16023**	**37**	**21**	**23**
%RSD	**27%**	**27%**	**24%**	**25%**	**35%**	**30%**

Table II-9: Serum biomarkers for bone resorption (CTX) and bone formation (P1NP). Serum samples were collected one day before the sacrifice. The levels of these biomarkers were determined by using ELISA kits and calculated based on UV-vis absorption against a standard calibration curve. Each sample was measured in duplicates.

Rat No.	c(CTX) (ng/mL)	c(P1NP) (ng/mL)
Baseline		
1	19.5	1.50
2	22.9	1.25
3	33.8	2.00
4	22.9	1.40
5	15.3	1.35
6	35.2	0.75
7	27.0	1.25
8	24.1	1.00
9	23.5	1.00
10	29.3	1.20
Average	**25.4**	**1.27**
SD	**6.1**	**0.34**
%RSD	**24%**	**27%**
Control Group		
1	10.7	0.20
2	27.9	0.25
3	8.3	0.30
4	24.5	0.40
5	20.3	0.50
6	19.2	0.00
7	19.6	0.50
8	27.8	0.25
10	8.9	0.25
Average	**18.6**	**0.39**
SD	**7.6**	**0.33**
%RSD	**41%**	**84%**

Table II-9: Serum biomarkers for bone resorption (CTX) and bone formation (P1NP).

Rat No.	c(CTX) (ng/mL)	c(P1NP) (ng/mL)
Low Dose Group		
1	32.3	0.30
2	24.2	0.25
3	13.5	0.20
4	11.5	0.40
5	11.3	0.00
6	16.7	0.00
7	14.8	0.25
8	13.7	0.50
9	17.6	0.00
10	31.7	0.00
Average	**18.7**	**0.19**
SD	**7.9**	**0.18**
%RSD	**42%**	**97%**
High Dose Group		
1	11.5	0.00
2	12.8	0.00
3	15.5	0.00
4	12.3	0.25
5	36.4	0.00
6	22.2	0.00
7	18.1	0.00
8	13.3	0.00
9	35.3	0.00
10	20.2	0.40
Average	**19.8**	**0.07**
SD	**9.1**	**0.15**
%RSD	**46%**	**220%**

Table II-10: Strontium concentration in rat soft tissues. Freeze-dried organs from the rats were weighed and digested using microwave-assisted acid digestion. A known amount of strontium spike ($n(Sr_{spike})$) was added to each sample for quantification. Measurements of $^{86}Sr/^{88}Sr$ ($n(^{86}Sr)/n(^{8}Sr)$) were obtained from single measurements using TIMS (relative measurement uncertainty of ~10 ppm, k=1). Measured amount of strontium in the sample ($n(Sr_{sample})$) was calculated following IDMS principles and corrected for strontium blank contribution (corr. $n(Sr_{sample})$), including the blank contribution from the calcium spike ($n(Sr_{blank})$). Concentration of strontium in the sample ($c(Sr_{sample})$) was calculated based on the corrected measured amount of strontium in the sample and the mass of the organ (m_{organ}). Column numbers are indicated in square brackets.

Sample	m_{organ} (g)	$n(Sr_{spike})$ ($x10^{-6}$ mol)	$\frac{n(^{86}Sr)}{n(^{88}Sr)}$	$n(Sr_{sample})$ ($x10^{-6}$ mol)[a]	$n(Sr_{blank})$ ($x10^{-6}$ mol)[b]	Corr. $n(Sr_{sample})$ ($x10^{-6}$ mol)[c]	$c(Sr_{sample})$ (µg/g)[d]
[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]
Kidney							
Control Group							
1	0.3478	0.00176	0.31659	0.01016	0.00764	0.00251	0.632
2	0.3931	0.00177	0.31664	0.01022	0.00766	0.00255	0.568
3	0.3458	0.00178	0.31841	0.01018	0.00767	0.00250	0.634
4	0.3847	0.00178	0.31708	0.01026	0.00769	0.00257	0.584
5	0.3150	0.00178	0.32195	0.00100	0.00765	0.00234	0.651
6	0.4692	0.00174	0.31244	0.01028	0.00765	0.00262	0.489
7	0.3484	0.00189	0.34083	0.00973	0.00765	0.00207	0.520
8	0.3861	0.00175	0.31443	0.01025	0.00768	0.00257	0.583
9	0.5755	0.00174	0.29701	0.01115	0.00747	0.00368	0.560
10	0.4762	0.00176	0.32463	0.00977	0.00761	0.00216	0.397
Average							**0.562**
SD							**0.077**
%RSD							**14%**
Low Dose Group							
1	0.3297	0.00347	0.41934	0.01311	0.00010	0.01301	3.457
2	0.4230	0.00351	0.28375	0.02433	0.00010	0.02423	5.018
3	0.2905	0.00343	0.34556	0.01727	0.00010	0.01716	5.177
4	0.3876	0.00349	0.26942	0.02657	0.00010	0.02647	5.983
5	0.4209	0.00364	0.29139	0.02411	0.00010	0.02400	4.997

Table II-10 (cont'd): Strontium concentration in rat soft tissues.

Sample	m_{organ} (g)	$n(Sr_{spike})$ ($x10^{-6}$ mol)	$\frac{n(^{86}Sr)}{n(^{88}Sr)}$	$n(Sr_{sample})$ ($x10^{-6}$ mol)[a]	$n(Sr_{blank})$ ($x10^{-6}$ mol)[b]	Corr. $n(Sr_{sample})$ ($x10^{-6}$ mol)[c]	$c(Sr_{sample})$ (µg/g)[d]
6	0.3987	0.00353	0.39439	0.01459	0.00011	0.01449	3.183
7	0.3304	0.00354	0.58310	0.00860	0.00010	0.00849	2.252
8	0.3975	0.00355	0.39068	0.01485	0.00010	0.01475	3.251
9	0.3735	0.00342	0.44928	0.01175	0.00010	0.01165	2.733
10	0.3234	0.00344	0.74299	0.00618	0.00010	0.00607	1.645
Average							**3.8**
SD							**1.4**
%RSD							**38%**
High Dose Group							
1	0.3151	0.00174	0.12411	0.42514	0.00774	0.41739	116.060
2	0.3555	0.00176	0.12478	0.37595	0.00757	0.36837	90.789
3	0.3263	0.00174	0.12639	0.28625	0.00762	0.27862	74.814
4	0.3433	0.00177	0.12832	0.22745	0.00764	0.21980	56.097
5	0.3280	0.00175	0.12661	0.27918	0.00761	0.27157	72.543
6	0.3105	0.00175	0.14438	0.08062	0.00763	0.07298	20.593
7	0.4037	0.00176	0.13063	0.17990	0.00765	0.17224	37.382
8	0.3322	0.00179	0.13675	0.11841	0.00762	0.11078	29.217
9	0.3214	0.00180	0.13221	0.16097	0.00762	0.15334	41.803
10	0.3846	0.00179	0.13916	0.10389	0.00764	0.09625	21.926
Average							**56**
SD							**32**
%RSD							**57%**
Heart							
Control Group							
1	0.3290	0.00357	0.63945	0.00772	0.00761	0.00010	0.027
2	0.3380	0.00358	0.63526	0.00780	0.00756	0.00023	0.059
3	0.3337	0.00354	0.63018	0.00780	0.00768	0.00012	0.030

Table II-10 (cont'd): Strontium concentration in rat soft tissues.

Sample	m_{organ} (g)	$n(Sr_{spike})$ ($x10^{-6}$ mol)	$\frac{n(^{86}Sr)}{n(^{88}Sr)}$	$n(Sr_{sample})$ ($x10^{-6}$ mol)[a]	$n(Sr_{blank})$ ($x10^{-6}$ mol)[b]	Corr. $n(Sr_{sample})$ ($x10^{-6}$ mol)[c]	$c(Sr_{sample})$ (µg/g)[d]
4	0.3507	0.00355	0.63660	0.00772	0.00759	0.00012	0.030
5	0.2835	0.00354	0.63952	0.00766	0.00758	0.00007	0.022
6	0.3502	0.00372	0.65300	0.00784	0.00759	0.00024	0.060
8	0.3427	0.00361	0.63003	0.00795	0.00768	0.00027	0.069
9	0.4128	0.00357	0.62289	0.00798	0.00761	0.00036	0.077
10	0.3595	0.00356	0.63978	0.00769	0.00760	0.00008	0.020
Average							**0.044**
SD							**0.022**
%RSD							**50%**
Low Dose Group							
1	0.3054	0.00345	1.88906	0.00208	0.00010	0.00198	0.567
2	0.3823	0.00346	1.49688	0.00272	0.00010	0.00262	0.600
3	0.2787	0.00341	1.22766	0.00338	0.00010	0.00328	1.030
4	0.3308	0.00346	1.78194	0.00223	0.00010	0.00213	0.564
5	0.3708	0.00345	1.87898	0.00209	0.00010	0.00199	0.471
6	0.3058	0.00346	2.75734	0.00135	0.00010	0.00124	0.356
7	0.3142	0.00339	3.22564	0.00110	0.00010	0.00100	0.278
8	0.3443	0.00345	1.45860	0.00280	0.00010	0.00269	0.686
9	0.3061	0.00341	1.61373	0.00246	0.00010	0.00236	0.675
10	0.2984	0.00349	1.95627	0.00202	0.00010	0.00192	0.563
Average							**0.58**
SD							**0.21**
%RSD							**35%**
High Dose Group							
1	0.3151	0.00357	0.30237	0.02223	0.00771	0.01452	4.036
2	0.3555	0.00358	0.23798	0.03448	0.00760	0.02687	6.623
3	0.3263	0.00359	0.23146	0.03665	0.00760	0.02905	7.800

Table II-10 (cont'd): Strontium concentration in rat soft tissues.

Sample	m_{organ} (g)	$n(Sr_{spike})$ ($x10^{-6}$ mol)	$\frac{n(^{86}Sr)}{n(^{88}Sr)}$	$n(Sr_{sample})$ ($x10^{-6}$ mol)[a]	$n(Sr_{blank})$ ($x10^{-6}$ mol)[b]	Corr. $n(Sr_{sample})$ ($x10^{-6}$ mol)[c]	$c(Sr_{sample})$ (μg/g)[d]
4	0.3433	0.00358	0.30383	0.02217	0.00760	0.01456	3.715
5	0.2997	0.00360	0.29740	0.02306	0.00764	0.01541	4.504
6	0.2720	0.00359	0.43290	0.01297	0.00764	0.00533	1.718
7	0.3902	0.00362	0.35893	0.01718	0.00767	0.00950	2.133
8	0.2846	0.00354	0.41519	0.01359	0.00758	0.00600	1.848
9	0.2956	0.00351	0.27966	0.02504	0.00754	0.01749	5.184
10	0.3686	0.00352	0.39286	0.01461	0.00762	0.00699	1.661
Average							**3.9**
SD							**2.2**
%RSD							**55%**
Lungs							
Control Group							
1	0.2894	0.00355	0.61242	0.00811	0.00761	0.00049	0.149
2	0.3191	0.00354	0.60880	0.00815	0.00758	0.00056	0.154
3	0.3062	0.00355	0.62277	0.00794	0.00758	0.00035	0.099
4	0.3164	0.00357	0.61091	0.00818	0.00763	0.00054	0.150
5	0.2991	0.00358	0.60780	0.00826	0.00765	0.00060	0.177
6	0.3169	0.00362	0.61591	0.00821	0.00769	0.00051	0.142
7	0.3268	0.00360	0.59639	0.00850	0.00773	0.00077	0.206
8	0.2934	0.00361	0.60796	0.00833	0.00767	0.00065	0.195
9	0.4891	0.00359	0.61245	0.00820	0.00766	0.00054	0.096
10	0.2942	0.00361	0.61559	0.00819	0.00764	0.00054	0.161
Average							**0.153**
SD							**0.036**
%RSD							**23%**
Low Dose Group							
1	0.3276	0.00346	0.37669	0.01530	0.00010	0.01520	4.093

Table II-10 (cont'd): Strontium concentration in rat soft tissues.

Sample	m_{organ} (g)	$n(Sr_{spike})$ ($x10^{-6}$ mol)	$\frac{n(^{86}Sr)}{n(^{88}Sr)}$	$n(Sr_{sample})$ ($x10^{-6}$ mol)[a]	$n(Sr_{blank})$ ($x10^{-6}$ mol)[b]	Corr. $n(Sr_{sample})$ ($x10^{-6}$ mol)[c]	$c(Sr_{sample})$ (µg/g)[d]
2	0.3185	0.00348	0.35744	0.01664	0.00010	0.01653	4.576
3	0.2740	0.00354	0.31572	0.02057	0.00010	0.02046	6.577
4	0.3092	0.00350	0.40905	0.01371	0.00010	0.01361	3.886
5	0.3052	0.00347	0.36007	0.01639	0.00010	0.01629	4.707
6	0.2958	0.00344	0.36801	0.01571	0.00010	0.01561	4.653
7	0.3244	0.00345	0.45016	0.01181	0.00010	0.01170	3.189
8	0.5031	0.00357	0.27815	0.02564	0.00010	0.02553	4.465
9	0.2804	0.00350	0.38206	0.01514	0.00010	0.01504	4.732
10	0.2898	0.00351	0.77403	0.00600	0.00010	0.00590	1.813
Average							**4.3**
SD							**1.2**
%RSD							**29%**
High Dose Group							
1	0.2698	0.00361	0.16619	0.08855	0.00782	0.08072	26.213
2	0.3201	0.00359	0.15705	0.10946	0.00762	0.10184	27.874
3	0.3184	0.00361	0.14696	0.15036	0.00773	0.14262	39.246
4	0.3087	0.00360	0.15250	0.12467	0.00771	0.11695	33.194
5	0.2884	0.00362	0.14929	0.13901	0.00765	0.13136	39.906
6	0.2609	0.00360	0.19259	0.05635	0.00775	0.04859	16.317
7	0.3270	0.00362	0.18890	0.05968	0.00772	0.05196	13.922
8	0.2994	0.00352	0.17203	0.07678	0.00770	0.06907	20.214
9	0.2878	0.00364	0.15881	0.10589	0.00771	0.09818	29.889
10	0.3083	0.00363	0.20512	0.04850	0.00779	0.04071	11.569
Average							**26**
SD							**10**
%RSD							**39%**

[a] Number of moles of strontium in the sample was calculated following IDMS principles (Eqn. 2).

[b] Blank contribution of strontium from calcium spike was calculated based on the amount of ^{44}Ca spike added (refer to Table II-11) and the concentration was quantified in separate measurements.

[c] Corrected number of moles of strontium in the sample was calculated by subtracting the blank contribution of strontium from calcium spike (column [6]) from the total number of moles of strontium in the sample (column [5]).

[d] The concentration of strontium in the sample was calculated by multiplying the corrected number of moles of strontium in the sample (column [7]) with the standard atomic weight of strontium in the sample (87.61681) and then dividing it with the mass of sample (column [2]).

Table II-11: Calcium concentration in soft tissues. Freeze-dried left kidney, heart and lungs from the rats were weighed and digested using microwave-assisted acid digestion. A known amount of calcium spike (n_{spike}) was added to each sample for quantification. The measurements of $^{44}Ca/^{42}Ca$ ($(^{44}Ca/^{42}Ca)_{measured}$) were obtained from single measurements by using TIMS (relative measurement uncertainty of ~10 ppm, *k*=1). The measured amount of calcium in the sample (n_{sample}) was calculated based on IDMS equation and corrected for calcium blank contribution (Ca in sample - blank corrected). The concentration of calcium in the sample (conc. of Ca in sample) was calculated based on the corrected measured amount of calcium in the sample and the mass of the organ/soft tissue. Column numbers are indicated in square brackets.

Sample	**m_{organ} (g)**	**$n(Ca_{spike})$ ($x10^{-6}$ mol)**	**$\frac{n(^{44}Ca)}{n(^{42}Ca})$ ($x10^{2}$)**	**$n(Ca_{sample})$ ($x10^{-6}$ mol)[a]**	**Corr. $m(Ca_{sample})$ (µg)[b, c]**	**$c(Ca_{sample})$ (µg/g)[d]**
[1]	**[2]**	**[3]**	**[4]**	**[5]**	**[6]**	**[7]**
Kidney						
Control Group						
1	0.3478	4.50712	4.00954	1.604	63.89	183.7
2	0.3931	4.51955	3.38036	1.935	77.15	196.3
3	0.3458	4.52509	4.46053	1.433	57.04	165.0
4	0.3847	4.53359	3.96935	1.631	64.98	168.9
5	0.3150	4.51955	3.92342	1.647	65.60	208.3
6	0.4692	4.51249	2.48157	2.688	107.34	228.8
7	0.3484	4.51293	4.77623	1.326	52.73	151.4
8	0.3861	4.52903	3.25067	2.023	80.65	208.9
10	0.4762	4.48557	3.14854	2.073	82.67	173.6
Average						**187**
SD						**25**
%RSD						**13%**
Low Dose Group						
1	0.3297	2.27391	2.63001	1.274	50.63	153.6
2	0.4230	2.29706	1.82628	1.892	75.43	178.3
3	0.2905	2.27618	2.43710	1.382	54.99	189.3
4	0.3876	2.28435	2.06055	1.657	66.00	170.3
5	0.4209	2.27800	1.87722	1.823	72.65	172.6
6	0.3987	2.31295	2.10089	1.644	65.47	164.2
7	0.3304	2.26983	2.05993	1.647	65.60	198.5

Table II-11 (cont'd): Calcium concentration in soft tissues.

Sample	m_{organ} (g)	$n(Ca_{spike})$ ($x10^{-6}$ mol)	$\frac{n(^{44}Ca)}{n(^{42}Ca)}$ ($x10^{2}$)	$n(Ca_{sample})$ ($x10^{-6}$ mol)[a]	Corr. $m(Ca_{sample})$ (µg)[b, c]	$c(Ca_{sample})$ (µg/g)[d]
8	0.3975	2.26756	1.77124	1.929	76.91	193.5
9	0.3735	2.26983	2.28265	1.478	58.81	157.4
10	0.3234	2.25667	2.01021	1.680	66.93	207.0
Average						**178**
SD						**18**
%RSD						**10%**
High Dose Group						
1	0.3486	4.56363	3.98578	1.635	65.11	186.8
2	0.4294	4.46474	2.99403	2.178	86.87	202.3
3	0.3285	4.49246	2.99281	2.192	87.45	266.2
4	0.4088	4.50614	3.49987	1.859	74.08	181.2
5	0.3280	4.48548	3.26092	1.996	79.60	242.7
6	0.3105	4.50059	4.93545	1.275	50.70	163.3
7	0.4037	4.51463	2.89166	2.285	91.19	225.9
8	0.3322	4.49577	3.90127	1.649	65.66	197.7
9	0.3214	4.49532	3.31914	1.963	78.27	243.5
10	0.3846	4.50363	4.17768	1.533	61.02	158.7
Average						**207**
SD						**36**
%RSD						**18%**
Heart						
Control Group						
1	0.3290	4.49049	7.13907	0.838	33.18	100.9
2	0.3380	4.46063	6.98533	0.854	33.81	100.0
3	0.3337	4.52795	6.32771	0.971	38.49	115.4
4	0.3507	4.47824	6.76054	0.890	35.26	100.6
5	0.2835	4.47109	7.34713	0.807	31.94	112.7
6	0.3502	4.47878	5.25519	1.184	47.02	134.3

Table II-11 (cont'd): Calcium concentration in soft tissues.

Sample	m_{organ} (g)	$n(Ca_{spike})$ ($x10^{-6}$ mol)	$\frac{n(^{44}Ca)}{n(^{42}Ca)}$ ($x10^{2}$)	$n(Ca_{sample})$ ($x10^{-6}$ mol)[a]	Corr. $m(Ca_{sample})$ (µg)[b, c]	$c(Ca_{sample})$ (µg/g)[d]
7	0.3026	4.65044	7.32213	0.843	33.37	110.3
8	0.3427	4.52840	6.86686	0.884	35.02	102.2
10	0.3595	4.48522	6.41681	0.946	37.52	104.4
Average						**109**
SD						**11**
%RSD						**10%**
Low Dose Group						
1	0.3054	2.28290	4.36158	0.741	29.30	95.94
2	0.3823	2.25803	3.84769	0.841	33.28	87.04
3	0.2787	2.24673	4.35662	0.730	28.86	103.56
4	0.3308	2.27342	4.24438	0.760	30.07	90.91
5	0.3708	2.25562	3.46783	0.940	37.25	100.45
6	0.3058	2.23965	3.83433	0.837	33.13	108.35
7	0.3142	2.22476	4.02070	0.790	31.23	99.41
8	0.3443	2.22780	3.59108	0.894	35.41	102.84
9	0.3061	2.25930	3.66213	0.888	35.15	114.83
10	0.2984	2.23642	3.83633	0.835	33.06	110.82
Average						**101.4**
SD						**8.7**
%RSD						**9%**
High Dose Group						
1	0.3151	4.54771	7.32109	0.824	32.63	103.6
2	0.3555	4.48388	6.28132	0.969	38.44	108.1
3	0.3263	4.48003	6.53652	0.925	36.68	112.4
4	0.3433	4.48531	6.65904	0.907	35.94	104.7
5	0.2997	4.50819	7.53240	0.791	31.27	104.4
6	0.2720	4.50346	8.08244	0.727	28.73	105.6
7	0.3902	4.52652	6.70597	0.908	35.98	92.2

Table II-11 (cont'd): Calcium concentration in soft tissues.

Sample	m_{organ} (g)	$n(Ca_{spike})$ ($x10^{-6}$ mol)	$\frac{n(^{44}Ca)}{n(^{42}Ca)}$ ($x10^{2}$)	$n(Ca_{sample})$ ($x10^{-6}$ mol)[a]	Corr. $m(Ca_{sample})$ (µg)[b, c]	$c(Ca_{sample})$ (µg/g)[d]
8	0.2846	4.47234	7.86516	0.746	29.47	103.6
9	0.2956	4.44713	4.94743	1.257	49.96	169.0
10	0.3686	4.49299	6.35422	0.959	38.01	103.1
Average						**111**
SD						**21**
%RSD						**19%**
Lungs						
Control Group						
1	0.2894	4.49049	1.62885	4.172	166.82	576.4
2	0.3191	4.47046	1.41313	4.823	192.87	604.4
3	0.3062	4.47180	1.84162	3.652	145.94	476.6
4	0.3164	4.50131	1.48026	4.625	184.95	584.5
5	0.2991	4.51231	9.13761	7.698	308.12	1030.2
6	0.3169	4.53627	1.63852	4.189	167.47	528.5
7	0.3268	4.55746	8.21281	8.702	348.34	1065.9
8	0.2934	4.52259	1.18674	5.861	234.49	799.2
10	0.2942	4.50855	1.08611	6.413	256.61	872.2
Average						**726**
SD						**222**
%RSD						**31%**
Low Dose Group						
1	0.3276	2.26847	0.73946	4.840	193.57	590.9
2	0.3185	2.24487	0.86848	4.041	161.54	507.1
3	0.2740	2.28753	1.17444	2.997	119.71	436.9
4	0.3092	2.24850	0.92281	3.796	151.75	490.8
5	0.3052	2.25031	0.66920	5.338	213.52	699.6
6	0.2958	2.21945	0.74984	4.666	186.59	630.8
7	0.3244	2.25712	0.72895	4.889	195.55	602.8

Table II-11 (cont'd): Calcium concentration in soft tissues.

Sample	m_{organ} (g)	$n(Ca_{spike})$ ($x10^{-6}$ mol)	$\frac{n(^{44}Ca)}{n(^{42}Ca)}$ ($x10^{2}$)	$n(Ca_{sample})$ ($x10^{-6}$ mol)[a]	Corr. $m(Ca_{sample})$ (µg)[b, c]	$c(Ca_{sample})$ (µg/g)[d]
8	0.5031	2.26665	0.51132	7.170	286.94	570.4
9	0.2804	2.24124	0.69517	5.106	204.21	728.2
10	0.2898	2.27482	1.10493	3.178	126.95	438.0
Average						**570**
SD						**101**
%RSD						**18%**
High Dose Group						
1	0.2698	4.61379	1.75203	3.971	158.72	588.3
2	0.3201	4.49174	2.23443	2.991	119.45	373.2
3	0.3184	4.55817	1.44301	4.810	192.38	604.2
4	0.3087	4.54718	1.39504	4.972	198.87	644.2
5	0.2884	4.51114	1.63979	4.162	166.41	577.0
6	0.2609	4.57114	1.95103	3.512	140.36	538.0
7	0.3270	4.55031	2.29848	2.940	117.44	359.1
8	0.2994	4.53904	1.56167	4.409	176.28	588.8
9	0.2878	4.54646	1.29170	5.390	215.61	749.2
10	0.3083	4.59242	2.17850	3.141	125.47	407.0
Average						**543**
SD						**126**
%RSD						**23%**

[a] Number of moles of calcium in the sample was calculated following IDMS principles (Eqn. 2).

[b] Blank contribution of calcium was quantified in separate measurements.

[c] Corrected amount of calcium in the sample was calculated by subtracting the blank contribution of calcium from the total amount of calcium in the sample, which was calculated by multiplying the number of moles of calcium in the sample (column [5]) with the standard atomic weight of calcium in the sample (40.078).

[d] The concentration of calcium in the sample was calculated by dividing the corrected amount of calcium in the sample (column [6]) with the mass of sample (column [2]).

Appendix III

Supplementary Data for Age-Dependent Differences in Calcium and Magnesium Intake in Singaporean Chinese Women (Part B, Chapter 4)

Table III-1: Assigned calcium and magnesium content for food items. The assigned calcium and magnesium contents are estimated with reference to the calcium contents from FAAS analysis, food composition tables and labels.

Food Name	Serving Size (Serving weight)	Assigned Calcium Content (mg/100g)	Assigned Magnesium Content (mg/100g)
Staple foods/ carbohydrate sources			
Rice	1 rice-bowl (200g)	15	4.0
Glutinous rice	1 serving (100g)	15	4.0
Porridge	1 bowl (500g)	5.0	1.5
Noodles	1 serving (200g)	10	13
Pau	1 item (100g)	30	25
Breakfast cereals (Ca fortified)	1 serving (35g)	A.T.B. (175-667)	A.T.B. (16-73)
Breakfast cereals (non-fortified)	1 serving (35g)	30	A.T.B.
White bread, loaf	2 slices (57g)	170	27
Milk bread, loaf	2 slices (57g)	300	27
Wholemeal, grains and other flavoured loaves	2 slices (60g)	120	64
Cream roll (Ca fortified)	1 item (65g)	A.T.B. (185-951)	27
Cream roll (non-fortified)	1 item (60g)	40	27
Other breads, buns, Indian breads, burger (without cheese)	1 item (60g)	50	27
Other breads, buns, Indian breads, burger (with cheese)	1 item (60g)	120	27
Pasta (non cheese-based sauce)	1 serving (160g)	10	10
Pasta (cheese-based sauce)	1 serving (160g)	110	10
Parmesan cheese	1 tablespoon (4g)	1120	43
Pizza	1 slice (75g)	110	13
Western Baked Rice	1 serving (200g)	50	5.0
Potato and potato based products, without cheese	1 serving (50-100g)	15	20
Potato and potato based products, with cheese	1 serving (100g)	130	20

*A.T.B.: according to brand/label.

Table III-1 (cont'd): Assigned calcium and magnesium content for food items.

Food Name	Serving Size (Serving weight)	Assigned Calcium Content (mg/100g)	Assigned Magnesium Content (mg/100g)
Vegetable and vegetable products			
Spinach	1 serving (70g)	150	35
Chye Sim	1 serving (70g)	100	15
Kale/ Kai lan	1 serving (70g)	110	35
Watercress, Cassava leaves	1 serving (70g)	130	15
Other dark green leafy vegetables	1 serving (70g)	70	15
Dark green leafy vegetables, general	1 serving (70g)	100	25
Other vegetables, vegetable products and legumes	1 serving (70g)	30	60
Meat, Poultry, Seafood, Egg			
Canned sardines	1 fish (70g)	300	50
Canned mackerel	1 fish (70g)	190	40
Unagi, Stingray, other fishes eaten with bones	1 serving (70g)	130	25
Deep fried prawn eaten with shell	1 serving (70g)	750	55
Crab	1 serving (70g)	130	40
Frog leg, Beef stomach	1 serving (40g)	100	12
Other meats, fishes (not eaten with bones),seafood, egg and their products	1 serving (70g)	30	40
Soybean products			
Firm tofu (taukwa)	1/2 square (70g)	190	50
Soft tofu	1/3 block (100g)	60	32
Egg tofu	1 serving (60g)	25	14
Tempeh	I serving (30g)	80	60
Mock/ Vegetarian meat (beancurd or gluten)	1 serving (70g)	20	60

Table III-1 (cont'd): Assigned calcium and magnesium content for food items.

Food Name	Serving Size (Serving weight)	Assigned Calcium Content (mg/100g)	Assigned Magnesium Content (mg/100g)
Sauces, Toppings, Condiments			
Ikan bilis	1 tablespoon (4g)	900	240
Anchovy, canned	1 fish (4g)	180	240
Silver fish	1 tablespoon (4g)	500	240
Mermaid fish	1 serving (5g)	2150	240
Small dried prawn	1 tablespoon (4g)	500	240
Dried shrimp	1 tablespoon (1g)	2000	240
Chinchalok	1 tablespoon (10g)	600	-
Belacan, cooked with dish	1 serving (5g)	1600	-
Sambal belacan	1 tablespoon (20g)	150	-
Indian curry	1/2 rice-bowl (100g)	30	15
Soup, milk-based	1 rice-bowl (200g)	100	
Soup, Laksa lemak	1 rice-bowl (200g)	46	15
Gravy, Mee rebus	1 rice-bowl (200g)	42	15
Soup, Mee Siam	1 rice-bowl (200g)	20	15
Fruits			
Fruits, fresh	1 serving (120g)	20	15
Dried	1 serving (60g)	50	40
Preserved, processed	1 serving (60g)	15	15
Traditional desserts			
Black sesame paste, hawker	1 bowl (400g)	60	-
Black sesame paste, instant	1 sachet (30g)	200	-
Glutionous rice ball (tangyuen), sesame filling	3 pieces (60g)	90	-
Other desserts	1 bowl (380g)	15	20
Other desserts and snacks			
Snacks and desserts	1 serving (60g)	45	25
Almond nuts	2 tablespoons (20g)	220	180
Pistachio nut, Walnut	2 tablespoons (20g)	90	180
Other nuts	2 tablespoons (20g)	40	180

Table III-1 (cont'd): Assigned calcium and magnesium content for food items.

Food Name	Serving Size (Serving weight)	Assigned Calcium Content (mg/100g)	Assigned Magnesium Content (mg/100g)
Dairy products			
Cheese slices	1 slice (20g)	A.T.B. (367-1193)	30
Cheese spread	1 portion (15g)	A.T.B. (250-1265)	30
Yogurts	1 serving (150g)	A.T.B. (106-250)	15
Dairy-based ice cream, mousse, custard	1 serving (100g)	180	15
Milk chocolate	6 small blocks (25g)	160	70

*A.T.B.: according to brand/label.

Table III-2: Calcium and magnesium contents in food products with assigned calcium and magnesium contents according to brand/labels.

Food Name	Products on market	Calcium Content (mg/100g)	Magnesium Content (mg/100g)
Breakfast Cereal (calcium enriched)	K Special Original Bran flakes	667	16
	Nestle Fitnesse	600	60
	Nestle Fitnesse and fruit	560	60
	Nestle Cornflakes	600	25
	Nestle Honey Goldflakes	500	25
	Nestle Snow flakes	440	25
	Nestle Cookie Crisp	420	73
	Nestle Milo	360	73
	Nestle Clusters	267	73
	Nestle Duo Coco	267	73
	Nestle Koko Crunch	256	73
	Nestle Trix	260	73
	Nestle Honey Stars	240	25
	Kellogg's Coco Pops	580	25
	Kellogg's Balls	175	25
	Kellogg's Coco Chex	175	45
	F&N Alive Light and Tasty, various flavours	500	73
	F&N Alive Bixies, wildberry	343	114
Cream roll (calcium enriched)	Sunshine Butter roll	185	27
	Sunshine Chocolate cream roll	205	27
	Sunshine Coffee cream roll	206	27
	Sunshine Peanut cream roll	216	27
	Sunshine Strawberry cream roll	233	27
	Sunshine Apple cream roll	233	27
	Sunshine Blueberry cream roll	214	27
	Sunshine Vanilla cream roll	951	27
	Sunshine Butter sugar cream roll	691	27
Cheese Slices	Kraft	800	30
	Chesdale	1193	30
	Cowhead	618	30

Table III-2 (cont'd): Calcium and magnesium contents in food products with assigned calcium and magnesium contents according to brand/labels.

Food Name	Products on market	Calcium Content (mg/100g)	Magnesium Content (mg/100g)
	President	367	30
	Valumetric	450	30
	Lemnos	625	30
	SCS	695	30
Cheese Spreads	Chesdale	1265	30
	Laughing Cow	250	30
	President	434	30
Yogurt	Marigold	168	15
	Meiji	132	15
	Dutch Lady	175	15
	Dairy Farmers	180	15
	Yoplait	173	15
	F & N	106	15
	Anlene	250	15

Table III-3: Assigned calcium and magnesium content for items in liquid beverage categories. The assigned calcium and magnesium contents are estimated with reference to the calcium and magnesium contents from FAAS analysis, food composition tables and labels.

Food Name	Serving Size (Serving weight)	Assigned Calcium Content (mg/100 mL)	Assigned Calcium Content (mg/100 mL)
Water			
Tap water	1 cup (200 mL)	1.0	0.0
Bottled mineral water	1 bottle (500 mL)	A.T.B. (0.095-18)	A.T.B.
Coffee			
Coffee, brewed (homemade/ coffee shop)	1 cup (200 mL)	2.0	5.0
Coffee, brewed (café outlet)	1 cup (300 mL)	2.0	5.0
Coffee, brewed, with milk (café outlet)	1 cup (300 mL)	70	5.0
Canned coffee, without milk	1 can (240 mL)	2.0	5.0
Canned coffee, with milk	1 can (240 mL)	40	5.0
Tea			
Tea, brewed (homemade/ coffee shop)	1 cup (200 mL)	1.0	5.0
Tea, brewed, without milk (café outlet)	1 cup (300 mL)	1.0	5.0
Tea, brewed, with milk (café outlet)	1 cup (300 mL)	60	5.0
Malt beverage			
Milo, ready-to-drink	1 can/packet (240 mL)	60	23
Soya bean milk			
Soya bean milk, ready-to-drink	1 cup (200 mL)	10	10
Soya bean milk, commercially available, unfortified	1 cup (200 mL)	10	20
Soya bean milk, commercially available, fortified	1 cup (200 mL)	80	10
Milk			
Milk, unfortified	1 cup (200 mL)	120	13
Milk, fortified	1 cup (200 mL)	160	15
Milk, concentrated, Anlene	1 packet (125 mL)	520	60

***** A.T.B.: according to brand/label.

Table III-3 (cont'd): Assigned calcium and magnesium content for items in liquid beverage categories.

Food Name	Serving Size (Serving Weight)	Assigned Calcium Content (mg/100 mL)	Assigned Magnesium Content (mg/100 mL)
Condensed milk	1 tablespoon (15 mL)	250	28
Evaporated milk	1 tablespoon (15 mL)	250	26
Milk products			
Milk shake	1 cup (300 mL)	120	11
Bandong	1 cup (200 mL)	20	-
Yoghurt drinks	1 cup (200 mL)	50	11
Probiotic drinks	1 cup (75 mL)	40	5.0
Fruit juices			
Fruit juice, freshly prepared	1 cup (200 mL)	3.0	9.0
Syrup / cordial	1 tablespoon (15 mL)	6.0	17
Fruit juice, processed	1 cup (200 mL)	10	9.0
Soft drinks			
Carbonated drinks	1 cup (200 mL)	2.0	1.0
Non-carbonated drinks	1 cup (200 mL)	1.0	0
Isotonic drinks	1 cup (200 mL)	1.0	5.2
Energy drinks	1 can (250 mL)	5.0	-
Alcoholic drinks			
Beer	1 can (350 mL)	5.0	7.7
Wine	1 portion (120 mL)	7.0	7.5
Spirit	1 glass (250 mL)	1.0	0.12
Others			
Honey	1 tablespoon (15 mL)	7.0	2.0
Grass jelly drink	1 cup (200 mL)	8.0	3.0
Herbal tea, brewed	1 cup (200 mL)	2.0	-

Table III-4: Assigned calcium and magnesium content for items in powdered beverage categories. The assigned calcium and magnesium contents are estimated with reference to the calcium and magnesium contents from FAAS analysis, food composition tables and labels.

Food Name	Serving Size (Serving weight)	Assigned Calcium Content (mg/100 g)	Assigned Magnesium Content (mg/100 g)
Coffee			
Coffee, instant, powder	2 tablespoons (30g)	140	15
Coffee, 3-in-1, powder	1 sachet (30g)	15	15
Tea			
Tea, instant, powder	1 sachet (30 g)	10	15
Tea, 3-in-1, powder	1 sachet (30 g)	200	15
Malt beverages and instant cereals			
Milo, powder	2 tablespoons (30 g)	500	125
Milo, 3-in-1, powder	1 sachet (30 g)	450	125
Horlicks, powder	2 tablespoons (30g)	1800	50
Horlicks, 3-in-1, powder	1 sachet (30 g)	1800	-
Ovaltine, powder	2 tablespoons (30 g)	800	108
Ovaltine, 3-in-1, powder	1 sachet (30 g)	1200	-
Hot chocolate, powder	2 tablespoons (30 g)	40	483
Cereal, powder	2 tablespoons (30 g)	400	104
Cereal, 3-in-1, powder	1 sachet (30 g)	A.T.B. (0-210)	-
Cereal, 3-in-1, fortified, powder	1 sachet (30 g)	A.T.B. (505-1000)	-
Soya bean milk			
Soya bean milk powder	1 sachet (30 g)	610	30
Milk			
Milk powder, unfortified	2 tablespoons (30 g)	600	A.T.B. (50-87)
Milk powder, fortified	2 tablespoons (30 g)	1500	A.T.B. (75-160)
Creamer			
Creamer, powder	1 teaspoon (5 g)	6.0	-

* A.T.B.: according to brand/label.

Table III-5: Calcium and magnesium contents in beverage products with assigned calcium and magnesium contents according to brand/labels.

Food Name	Products on market	Assigned Calcium Content (mg/100 g)	Assigned Magnesium Content (mg/100 g)
Water			
Mineral water	Volvic Natural Mineral Water	1.2	0.8
	Danone Aqua Mountain Spring Water	1.6	1.0
	Polar Natural Mineral Water	1.8	0.4
	Pere Ocean Natural Mineral Water	3.0	0.1
	Ice Mountain Pure Drinking Water	3.4	0.7
	Evian Mineral Water	8.0	2.6
	Voda Voda	7.4	1.4
	Ron88 Elite Mini	0.87	0.29
	Team Alkaline Drinking water	1.4	0.074
	Aquarin Natural Mineral Water	5.4	2.1
	Vittel Natural Mineral Water	9.3	2.0
	Mountain H^2 Premium Natural Spring Water	<0.1	<0.1
	Virga Pure Tasmanian Water	0.30	0.07
	Fiji Natural Artesian Water	1.7	1.3
	Maniva Mineral Water	4.0	0.5
	Glinter Mineral Water	-	-
	Ice Berg Mineral Water	3.0	2.4
	Spritzer Natural Mineral Water	4.6	0.5
	Acqua Panna Natural Mineral Water	3.3	0.7
	San Benedetto Natural Mineral Water	4.8	2.9
	First Choice French Spring Water	5.8	2.4
	First Choice Natural Mineral Water	0.095	0.006
	Perrier Natural Mineral Water	-	-
	BreadTalk, pure mineralised water	0.16	0.016
Mineral water, carbonated	Highland Spring Sparkling Spring Water	4.1	1.2
	Gerolsteiner Sparkling Mineral Water	14	4.9
	San Benedetto Sparkling Mineral Water	4.8	2.9
	Apollinaris Classic Sparkling Mineral Water	9.0	12

Table III-5 (cont'd): Calcium and magnesium contents in beverage products with assigned calcium and magnesium contents according to brand/labels.

Food Name	Products on market	Assigned Calcium Content (mg/100 g)	Assigned Magnesium Content (mg/100 g)
	Sparkling Spritzer Carbonated Mineral Water	1.2	0.16
	San Pellegrino Sparkling Natural Mineral Water	18	5.4
	Perrier Sparkling Mineral Water, lime	-	-
	Perrier Sparkling Mineral Water, lemon	-	-
Powdered drinks			
Cereal, 3-in-1, powder	Nestle Nesvita 3-in-1 cereal drink	210	-
	Super Cereal, 3 in 1	0	-
	Gold Kili Cereal, 3 in 1	0	-
Cereal, 3-in-1, powder, fortified	Nestle Nesvita 3-in-1 oat cereal drink, calcium fortified	505	-
	Vita Quaker 3 in 1 cereal, calcium fortified, Chocolate	1000	-
	Vita Quaker 3 in 1 cereal, calcium fortified, Berry Burst	1000	-
	Vita Quaker 3 in 1 cereal, calcium fortified, Original	1000	-

Table III-6: Data from a duplicate diet study for validation of a method to estimate calcium intake from 3-day weighted food records. Measured calcium intake was determined based on the analysis of duplicate diet samples collected using AAS. Estimated calcium intake was calculated based on the assigned calcium contents in the in-house database (Table III-1 – III-5) and the reported amount of food consumed in the 3-day food record.

Subject No.	Calcium Intake (mg/ day)					
	Measured			Estimated		
	Food	Drinks	Total	Food	Drinks	Total
1	1090	96	1186	1033	117	1150
2	158	7	165	178	6	184
3	298	12	309	365	23	388
4	173	15	187	192	12	204
5	939	36	975	876	36	912
6	168	23	192	211	24	235
7	264	26	290	290	76	366
8	334	17	352	365	24	389
9	408	126	534	382	222	604
10	621	108	729	555	140	694
11	381	44	425	165	53	218
12	336	177	513	350	152	503
13	186	21	207	243	16	259
14	276	48	325	371	131	502
15	162	21	183	215	14	229
16	618	110	728	664	178	841
17	332	220	551	344	102	446
18	166	248	414	213	176	389
19	1075	67	1142	869	135	1004
20	349	488	837	555	382	936
21	487	93	579	472	212	684
22	237	165	402	301	108	409
23	148	189	337	241	158	399
24	180	55	235	171	126	297

Table III-7: Data from a duplicate diet study for validation of a method to estimate magnesium intake from 3-day weighted food records. Measured magnesium intake was determined based on the analysis of duplicate diet samples collected using AAS. Estimated magnesium intake was calculated based on the assigned magnesium contents in the in-house database (Table III-1 – III-5) and the reported amount of food consumed in the 3-day food record.

No.	Magnesium Intake (mg/day)					
	Measured			Estimated		
	Food	Drinks	Total	Food	Drinks	Total
1	186	12	198	190	6	196
2	143	0	143	100	2	101
3	162	9	170	181	27	208
4	129	5	135	120	5	125
5	129	7	136	145	10	155
6	375	23	398	395	26	420
7	189	58	246	174	44	218
8	202	37	239	194	42	236
9	153	22	176	173	10	183
10	161	19	180	184	30	214
11	111	2	113	142	6	148
12	76	11	86	88	19	107
13	123	15	138	116	45	161
14	200	50	250	192	34	225
15	151	3	154	155	20	174
16	138	44	182	168	33	201
17	66	42	107	91	29	120
18	121	0	122	122	1	123
19	302	0	302	246	1	247
20	250	17	267	270	26	296
21	195	79	274	186	66	253
22	167	2	169	117	11	128
23	330	15	344	196	27	223
24	107	7	114	135	7	143

Table III-8: Total calcium and magnesium dietary intake by female aged ≥ 55 years-old. The average calcium and magnesium intake was calculated based on the assigned calcium and magnesium contents in the in-house database (Table III-1 – III-5) and the reported amount of food consumed in the 3-day food record.

Subject No.	Average Calcium Intake (mg/day)	Average Magnesium Intake (mg/day)
1	208	128
2	1052	396
3	364	182
4	431	147
5	333	111
6	507	166
7	268	148
8	456	163
9	940	484
10	2397	487
11	574	213
12	477	223
13	1116	521
14	1109	308
15	467	208
16	200	125
17	418	292
18	876	297
19	932	276
20	468	210
21	342	152
22	483	230
23	1422	296
24	639	270
25	633	193
26	429	183
27	1134	381
28	420	254
29	837	152
30	370	192
31	569	312
32	331	194
33	245	178
34	312	203

Table III-8 (cont'd): Total calcium and magnesium dietary intake by female aged ≥ 55 years-old.

Subject No.	Average Calcium Intake (mg/day)	Average Magnesium Intake (mg/day)
35	349	160
36	615	202
37	1228	281
38	499	220
39	679	179
40	1387	598
41	916	264
42	3136	230
43	1020	244
44	1101	209
45	583	175
46	1436	172
47	1229	289
48	758	322
49	721	197
50	788	259
51	1378	169
52	982	302
53	875	204
54	650	177
55	436	244
56	225	99
57	358	182
58	407	181
59	1313	394
60	184	69
61	311	178
62	247	95
63	454	137
64	409	187
65	358	269
66	206	106
67	1131	218
68	621	161
69	306	175

Table III-8 (cont'd): Total calcium and magnesium dietary intake by female aged ≥ 55 years-old.

Subject No.	Average Calcium Intake (mg/day)	Average Magnesium Intake (mg/day)
70	279	133
71	274	154
72	371	181
73	997	185
74	473	161
75	329	224
Mean	**653**	**225**
SD	**407**	**98**
SE	**47**	**11**

Table III-9: Total calcium and magnesium dietary intake by female aged 18-30 years-old. The average calcium and magnesium intake was calculated based on the assigned calcium and magnesium contents in the in-house database (Table III-1 – III-5) and the reported amount of food consumed in the 3-day food record.

Subject No.	Average Calcium Intake (mg/day)	Average Magnesium Intake (mg/day)
1	243	92
2	193	80
3	265	125
4	579	339
5	540	261
6	379	163
7	1035	185
8	343	90
9	506	164
10	431	144
11	406	109
12	192	85
13	288	113
14	234	119
15	446	337
16	446	346
17	1127	231
18	1582	296
19	586	190
20	380	167
21	452	213
22	539	149
23	281	151
24	355	147
25	332	133
26	863	193
27	689	161
28	418	150
29	380	189
30	433	174
31	525	302
32	510	203
33	439	334

Table III-9 (cont'd): Total calcium and magnesium dietary intake by female aged 18-30 years-old.

Subject No.	Average Calcium Intake (mg/day)	Average Magnesium Intake (mg/day)
34	542	169
35	532	213
36	365	215
37	633	197
38	539	210
39	782	241
40	543	310
41	504	194
42	477	171
43	365	351
44	541	217
45	314	128
46	291	141
47	449	161
48	332	175
49	801	208
50	426	185
51	506	252
52	626	320
53	1028	205
54	354	165
55	570	177
56	350	165
57	373	186
58	670	257
59	215	106
60	453	127
61	249	128
62	416	158
63	335	184
64	684	240
65	327	158
66	513	201
67	644	294
68	179	102

Table III-9 (cont'd): Total calcium and magnesium dietary intake by female aged 18-30 years-old.

Subject No.	Average Calcium Intake (mg/day)	Average Magnesium Intake (mg/day)
69	378	142
70	253	148
71	168	96
72	434	147
73	445	216
74	389	217
75	224	106
76	738	228
77	786	311
78	390	191
79	477	129
80	275	163
81	393	159
82	317	150
83	548	212
84	498	142
85	392	242
86	200	144
87	362	137
88	837	192
89	329	155
90	655	186
91	711	164
92	1037	288
93	539	174
94	309	149
Mean	**483**	**187.6**
SD	**230**	**65**
SE	**24**	**6.7**

Table III-10: Example of calcium and magnesium assessment table. The serving sizes, calcium and magnesium contents were based on the in-house database (Table III-1-III-5). The numbers of servings were based on the 3-day food record collected from the study participants. Total daily intake was calculated by summing up the individual's mineral intake in a day. Total weekly intake was estimated by assigning different weightage for the total daily intake (2.5 for each weekday and 2 for weekend) and summing them up. The average daily intake was calculated by dividing the total weekly intake by seven.

No.	Food / Beverage	Serving size Food (g) / Drinks (ml)	Ca content (mg/100g) / (mg/100ml)	Mg content (mg/100g) / (mg/100ml)	1								
					Weekday 1			Weekday 2			Weekend		
					No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)
A	**FOOD**												
1	**Staple foods**												
a	White rice	200	7	4		0	0	1.5	21	12	1	14	8
	Brown rice	200	14	49		0	0		0	0		0	0
	Glutinous rice	100	7	4		0	0		0	0		0	0
	Porridge	500	5	1.5		0	0		0	0		0	0
b	Pau (steamed bun)	100	30	25		0	0		0	0		0	0
c	Loaf bread												
	White bread	57	170	27		0	0		0	0		0	0
	Milk bread	57	341	27		0	0		0	0		0	0
	Wholemeal bread	60	170	64		0	0		0	0		0	0
	Gardenia Enriched White	57	139	27		0	0		0	0		0	0
	Gardenia High Fibre White	57	153	27		0	0		0	0		0	0
	Gardenia Wholemeal	60	167	64		0	0		0	0		0	0
	Gardenia Fruit & Nut	60	199	64		0	0		0	0		0	0
	Gardenia Banana Walnut	60	128	64		0	0		0	0		0	0
	Gardenia Cranberry Fruity Loaf	60	170	27		0	0		0	0		0	0

Table III-10 (cont'd): Example of calcium and magnesium assessment table.

No.	Food / Beverage	Serving size Food (g) / Drinks (ml)	Ca content (mg/100g) / (mg/100ml)	Mg content (mg/100g) / (mg/100ml)	1								
					Weekday 1			Weekday 2			Weekend		
					No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)
	Bonjour Wholemeal	60	103	64		0	0		0	0		0	0
	Bonjour Raisin Loaf	40	75	27		0	0		0	0		0	0
	Bonjour Grain Loaf with Flax Seed	57	160	64		0	0		0	0		0	0
	Fairprice Enriched White	57	180	27		0	0		0	0		0	0
	Fairprice Wholemeal	53	192	64		0	0		0	0		0	0
	Sure Value White	57	196	27		0	0		0	0		0	0
	Sunshine Enriched Soft White	57	220	27		0	0		0	0		0	0
	Sunshine Fruit & Grain	80	166	64		0	0		0	0		0	0
	Sunshine Softmeal	57	116	64		0	0		0	0		0	0
	Sunshine Milk Toast	57	370	27		0	0		0	0		0	0
	Top one Enriched White	57	273	27		0	0		0	0		0	0
	Top one Wholemeal	60	158	64		0	0		0	0		0	0
	Top one High Fiber	53	272	27		0	0		0	0		0	0
	7-11 Quickbites Wholemeal	53	192	64		0	0		0	0		0	0
d	Cream-filled bread roll	65	205	27		0	0		0	0		0	0
	Other breads & buns	60	50	27	1	30	16		0	0		0	0
	Other breads & buns w/ cheese	60	120	27		0	0		0	0		0	0
	Other breads & buns wholemeal	60	50	64		0	0		0	0		0	0
e	Noodles (egg, rice vermicelli, mungbean)	200	10	13		0	0		0	0	0.2	4	5
	Noodles with milk	200	60	33		0	0		0	0		0	0
f	Pasta	160	10	10	1.5	24	24		0	0		0	0

Table III-10 (cont'd): Example of calcium and magnesium assessment table.

No.	Food / Beverage	Serving size Food (g) / Drinks (ml)	Ca content (mg/100g) / (mg/100ml)	Mg content (mg/100g) / (mg/100ml)	1								
					Weekday 1			Weekday 2			Weekend		
					No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)
	Pasta, cream-based	160	110	10		0	0		0	0		0	0
g	Potato (whole, salad, mashed, fries)	100	15	20	1	15	20		0	0		0	0
	Hash brown	50	24	20		0	0	1	12	10		0	0
	Yam	100	30	20		0	0		0	0		0	0
h	Breakfast cereal												
	Whole-wheat	35	48	115		0	0		0	0		0	0
	Nestle Koko Krunch Duo	35	266	73		0	0		0	0		0	0
	Nestle Koko Krunch	35	200	73		0	0		0	0		0	0
	Nestle Honey Stars	35	267	25		0	0		0	0		0	0
	Nestle Corn Flakes	35	600	25		0	0		0	0		0	0
	Nestle Milo	35	360	73		0	0		0	0		0	0
	Kelloggs Corn Flakes	35	16	25		0	0		0	0		0	0
	Kelloggs Special K	35	16	54		0	0		0	0		0	0
	Kelloggs Coco Chex	35	175	45		0	0		0	0		0	0
	Weetabix	35	16	73		0	0		0	0		0	0
	Quaker Oatmeal	35	80	130		0	0		0	0		0	0
	Vita Quaker 3-in-1 Oat Cereal	19	1000	130		0	0		0	0		0	0
	Organic Instant Baby Oats	35	57	130		0	0		0	0		0	0
	Uncle Toby's Muesli Bar	31	16	50		0	0		0	0		0	0
	F&N Alive Light 'n' Tasty - Triple Berry	35	500	73		0	0		0	0		0	0
	Alpen Light Prebiotic Mixed Cereal Bar	21	16	38		0	0		0	0		0	0

Table III-10 (cont'd): Example of calcium and magnesium assessment table.

No.	Food / Beverage	Serving size Food (g) / Drinks (ml)	Ca content (mg/100g) / (mg/100ml)	Mg content (mg/100g) / (mg/100ml)	1								
					Weekday 1			Weekday 2			Weekend		
					No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)
	Fairprice 3in1 Instant Cereal	35	16	73		0	0		0	0		0	0
	Post Blueberries Cereal	55	36	47									
	Post Great Grains Raisins, Dates & Pecans	35	16	111		0	0		0	0		0	0
	Post Honey Bunches of Oats with Almonds	35											
	Nature Valley Roasted Almond Bar	35	91	180		0	0		0	0		0	0
	Suiss cereal bar	23	16	73		0	0		0	0		0	0
	Rolled oats	35	16	130		0	0		0	0		0	0
	Ca-fortified	35	507			0	0		0	0		0	0
	Non Ca-fortified	35	16			0	0		0	0		0	0
	Wheat	35	38	73		0	0		0	0		0	0
	Corn meal	35	16	25		0	0		0	0		0	0
	Rice	35	18	38		0	0		0	0		0	0
	Muesli	35	40	50		0	0		0	0		0	0
	Oat	35	16	130		0	0		0	0		0	0
	Bran	35	50	210		0	0		0	0		0	0

Table III-10 (cont'd): Example of calcium and magnesium assessment table.

No.	Food / Beverage	Serving size Food (g) / Drinks (ml)	Ca content (mg/100g) / (mg/100ml)	Mg content (mg/100g) / (mg/100ml)	1								
					Weekday 1			Weekday 2			Weekend		
					No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)
2	**Vegetables**												
a	Kale	70	110	35		0	0		0	0		0	0
	Spinach, amaranth	70	150	35		0	0		0	0		0	0
	Chye sim	70	100	15		0	0	0.3	23	3.5		0	0
	Watercress, cassava leaves	70	130	15		0	0		0	0		0	0
	All other green leafy veg	70	100	15	0.3	18	2.6		0	0		0	0
b	Okra	70	80	60		0	0		0	0		0	0
	Beans	40	30	60		0	0		0	0		0	0
	All other non-leafy veg	70	30	12		0	0		0	0		0	0
3	**Meat & meat pdts**												
a	Chicken, pork, beef, mutton	70	30	20	1.5	32	21	2	42	28	1.3	28	19
b	Egg	60	60	15		0	0		0	0		0	0
4	**Seafood**												
a	Anchovy	4	900	240		0	0		0	0		0	0
	Silverfish	4	500	240		0	0		0	0		0	0
	Dried baby prawn	4	500	240		0	0		0	0		0	0
	Dried baby shrimp	4	2000	240		0	0		0	0		0	0
	Dried scallop	4	77	40		0	0		0	0		0	0
b	Indian mackerel	40	130	40		0	0		0	0		0	0
	Eel (unagi), salmon	70	130	25		0	0		0	0		0	0

Table III-10 (cont'd): Example of calcium and magnesium assessment table.

No.	Food / Beverage	Serving size Food (g) / Drinks (ml)	Ca content (mg/100g) / (mg/100ml)	Mg content (mg/100g) / (mg/100ml)	1								
					Weekday 1			Weekday 2			Weekend		
					No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)
	Sardine	70	280	50		0	0		0	0		0	0
	Prawn with shell	70	750	55		0	0		0	0		0	0
	Crab	70	130	40		0	0		0	0		0	0
c	Other fish, seafood & pdts	70	30	40		0	0		0	0	1	21	28
5	**Snacks & desserts**												
a	Tart, kuey	60	35	25		0	0		0	0		0	0
b	Puff	90	30	25	1	27	23		0	0		0	0
c	Muffin, cake, pastry, pancake	60	45	25	0.5	14	7.5		0	0		0	0
d	Biscuits, cookies	30	50	30		0	0		0	0		0	0
e	Almond nuts	20	220	180		0	0		0	0		0	0
	Pistachio nuts and walnuts	20	90	180		0	0		0	0		0	0
	All other nuts	20	40	180		0	0		0	0		0	0
f	Seeds	40	30	350		0	0		0	0		0	0
g	Chips, crackers	30	80	25		0	0		0	0		0	0
h	Milk chocolate	25	160	70		0	0		0	0		0	0
i	Dried seaweed					0	0		0	0		0	0
	Tao Kae Noi	20	80	135		0	0		0	0		0	0
	Other brands	20	400	350		0	0		0	0		0	0
j	Jelly, pudding, dessert soup/paste	380	15	20		0	0		0	0		0	0

Table III-10 (cont'd): Example of calcium and magnesium assessment table.

No.	Food / Beverage	Serving size Food (g) / Drinks (ml)	Ca content (mg/100g) / (mg/100ml)	Mg content (mg/100g) / (mg/100ml)	1								
					Weekday 1			Weekday 2			Weekend		
					No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)
6	**Soy or vegetarian products**												
a	Egg tofu	60	25	14		0	0		0	0		0	0
	Soft tofu, bean curd dessert	100	60	32		0	0		0	0		0	0
	Firm tofu (tau kwa)	70	190	50		0	0		0	0	0.5	67	18
	Chinese tofu	70	60	28		0	0		0	0		0	0
	Tofu (exact type unknown)	70	60	25		0	0		0	0		0	0
b	Tempeh	30	80	60		0	0		0	0	0.5	12	9
c	Mock meat	70	20	60		0	0		0	0		0	0
7	**Fruits**												
	Fresh fruit	120	20	15		0	0		0	0		0	0
	Dried fruit	60	50	40		0	0		0	0		0	0
	Preserved fruit	120	15	15		0	0		0	0		0	0
8	**Dairy pdts**												
a	Cheese slice	20	700	30		0	0		0	0		0	0
	Fairprice cheese slice	20	510	30		0	0		0	0		0	0
	Fairprice Reduced Fat cheese slice	20	510	30		0	0		0	0		0	0
	Chesdale Original cheese slice	20	1193	30		0	0		0	0		0	0
	Kraft Singles Hi-Cal	20	800	30		0	0		0	0		0	0
	SCS Reduced Fat cheese slice	20	880	30		0	0		0	0		0	0

Table III-10 (cont'd): Example of calcium and magnesium assessment table.

No.	Food / Beverage	Serving size Food (g) / Drinks (ml)	Ca content (mg/100g) / (mg/100ml)	Mg content (mg/100g) / (mg/100ml)	1								
					Weekday 1			Weekday 2			Weekend		
					No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)
	Cheese spread (Laughing cow, creamy)	15	600	30		0	0		0	0		0	0
	Cheese cube (Laughing cow)	5	290	30		0	0		0	0		0	0
	Cheddar cheese	25	700	30		0	0		0	0		0	0
	Parmesan cheese	20	1120	43	0.5	112	4.3		0	0		0	0
	Cottage cheese	25	70	10		0	0		0	0		0	0
	Mozzarella cheese (President)	20	300	30		0	0		0	0		0	0
b	Yogurt	150	140	15		0	0		0	0		0	0
	Meiji (flavoured)	150	131.7	15		0	0		0	0		0	0
	F&N (flavoured)	135	106	15		0	0		0	0		0	0
	Emmi (flavoured)	100	90	15		0	0		0	0		0	0
	Marigold (non-fat)	125	183	15		0	0		0	0		0	0
c	Dairy based ice cream	100	180	15		0	0		0	0	0.8	144	12
8	**Others**												
	Curry, mee siam gravy	200	30	15		0	0		0	0		0	0
9	**Supplements (1 pill = 100 g)**												
	Cetrum Multivitamin & Multimineral	100	200	100		0	0		0	0		0	0
	GNC Women's Ultra Mega Bone Density	100	250	62.5		0	0		0	0		0	0
	GNC Calcimate Plus 800	100	200	15		0	0		0	0		0	0

Table III-10 (cont'd): Example of calcium and magnesium assessment table.

No.	Food / Beverage	Serving size Food (g) / Drinks (ml)	Ca content (mg/100g) / (mg/100ml)	Mg content (mg/100g) / (mg/100ml)	1								
					Weekday 1			Weekday 2			Weekend		
					No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)
	GNC Country Life Liquid Multi-vitamin & Multi-complex	100	50	50									
	21st Century Hair, Skin & Nails (Advanced Formula)	100	70	20		0	0		0	0		0	0
	Total Swiss Fit Solution (Cell Mineral)	6.7	4050	2000		0	0		0	0		0	0
	Nature's Farm Calcium Citramate	100	400	50		0	0		0	0		0	0
	Dr Hagiwara Barleygreen	6	583	200		0	0		0	0		0	0
	Suncal Calcium with Vitamin D	100	450	0		0	0		0	0		0	0
	Caltrate 600+D Plus Minerals	100	600	50		0	0		0	0		0	0
	Caltrate 600+D	100	600	0		0	0		0	0		0	0
	Brands Calcium Plus	100	600	0		0	0		0	0		0	0
	Berroca (with zinc, magnesim and calcium)	100	100	100		0	0		0	0		0	0
	CD-R Redoxon (calcium-d)	100	250	0		0	0		0	0		0	0
	CNI Ester-C Plus (with calcium)	100	50	0		0	0		0	0		0	0
	Scott's Emulsion Cod Liver Oil	100	414	0		0	0		0	0		0	0
	Multivitamin (brand unknown)	100	250	100		0	0		0	0		0	0
	Nature's Way Total Calcium Plus	100	600	0		0	0		0	0		0	0
	Calcium pill (prescribed)	100	300	0		0	0		0	0		0	0
	GNC Multivitamis for Teens	100	100	50		0	0		0	0		0	0
	Guardian calcium	100	180	0		0	0		0	0		0	0
	Equate-Women's Multivitamin	100	450	50		0	0		0	0		0	0
	Minibee Pollen Tablet	100	41	0.17		0	0		0	0		0	0

Table III-10 (cont'd): Example of calcium and magnesium assessment table.

No.	Food / Beverage	Serving size Food (g) / Drinks (ml)	Ca content (mg/100g) / (mg/100ml)	Mg content (mg/100g) / (mg/100ml)	1								
					Weekday 1			Weekday 2			Weekend		
					No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)
	Ocen Health Multivitamins and minerals	100	162	100		0	0		0	0		0	0
	Vitahealth multivitamin	100	162	100		0	0		0	0		0	0
B	**BEVERAGES**												
1	**Water**												
	Tap water	200	1	0	11	22	0	10.5	21	0	9	18	0
	Polar Natural Mineral Water	500	3	1.8		0	0		0	0		0	0
	Ice Mountain Pure Drinking Water	500	3.4	0.7		0	0		0	0		0	0
	Evian	500	8	2.6									
	Dasani	500	0	0									
2	**Coffee & Tea**												
a	Coffee with milk, canned	240	40	5		0	0		0	0		0	0
	Coffee without milk, canned	240	2	5		0	0		0	0		0	0
b	Tea with milk, brewed	200	31	5		0	0		0	0		0	0
	Tea without milk, brewed	200	1	5		0	0		0	0		0	0
	Coffee with milk, brewed	200	70	5		0	0		0	0		0	0
	Coffee without milk, brewed	200	2	5		0	0		0	0		0	0
c	Coffee, 3-in-1, powder	30	15	15		0	0		0	0		0	0
	White coffee, powder	30	24	15		0	0		0	0		0	0

Table III-10 (cont'd): Example of calcium and magnesium assessment table.

No.	Food / Beverage	Serving size Food (g) / Drinks (ml)	Ca content (mg/100g) / (mg/100ml)	Mg content (mg/100g) / (mg/100ml)	1								
					Weekday 1			Weekday 2			Weekend		
					No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)
	Tea, 3-in-1, powder	30	200	15		0	0		0	0		0	0
3	**Instant beverages**												
	Milo, powder	30	500	125.3		0	0		0	0		0	0
	Milo, 3-in-1, powder	30	450	125.3		0	0		0	0		0	0
	Milo, 3-in-1, easy cool	26	577	150									
	Ovaltine, chocolate flavour, 3-in-1 powder	30	1175	207									
	Horlicks	28	1800	50		0	0		0	0		0	0
	Chocolate drink	30	40	130		0	0		0	0		0	0
	Tehh tarik (pulled tea), powder	25	128	15		0	0		0	0		0	0
	Jing Huangbao Wheat Germ Soybean Phosphatide	10	125	59		0	0		0	0		0	0
	Nestle Nestum Original	250	390	29		0	0		0	0		0	0
	Nestle Nesvita 3 in 1 cereal drink	26	210	73		0	0		0	0		0	0
	Greenmax Black Sesame Cereal	30	240	350		0	0		0	0		0	0
	Infocafe Ginseng Cereal Drink	35											
4	**Milk & milk pdts**												
a	Milk, unfortified	200	120	13		0	0		0	0		0	0
	Marigold HL Original/Banana/Strawberry/Chocolate	200	127	15		0	0		0	0		0	0
	Magnolia UHT Fresh	250	125	15		0	0		0	0		0	0

Table III-10 (cont'd): Example of calcium and magnesium assessment table.

No.	Food / Beverage	Serving size Food (g) / Drinks (ml)	Ca content (mg/100g) / (mg/100ml)	Mg content (mg/100g) / (mg/100ml)	1								
					Weekday 1			Weekday 2			Weekend		
					No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)
	Magnolia High Ca Low Fat	250	175	15		0	0		0	0		0	0
	Meiji Fresh Original	200	109	15		0	0		0	0		0	0
	Meiji Fresh Strawberry	200	107	15		0	0		0	0		0	0
	Meiji Fresh Coffee	200	98	15		0	0		0	0		0	0
	Meiji Fresh Cocoa	200	106	15		0	0		0	0		0	0
	Meiji Fresh Low Fat	200	122	15		0	0		0	0		0	0
	F&N Daisy UHT Fresh	250	120	15		0	0		0	0		0	0
	F&N Daisy High Calcium	200	150	15		0	0		0	0		0	0
	Anlene Concentrate High Calcim Low Fat	125	520	60		0	0		0	0		0	0
	Greenfields Low Fat	250	163.2	14.4		0	0		0	0		0	0
	Meadow Fresh NZ Low Fat	200	150	15		0	0		0	0		0	0
	Farmhouse Fresh	200	120	15		0	0		0	0		0	0
	Farmhouse Low Fat	200	110	15		0	0		0	0		0	0
b	Condensed milk	15	250	28		0	0		0	0		0	0
	Evaporated milk	15	250	26		0	0		0	0		0	0
	Carnation Low Fat Evaporated Milk	15	250	26		0	0		0	0		0	0
c	Milk powder	30	970	100		0	0		0	0		0	0
	Nestle Nesvita Omega Plus	31	1600	100		0	0		0	0		0	0
	Fernleaf Full Cream	32	850	87		0	0		0	0		0	0
	Anlene Gold High Calcium 51+	30	2000	160		0	0		0	0		0	0
	Anlene Milk powder 19-50	30	1670	160		0	0		0	0		0	0

Table III-10 (cont'd): Example of calcium and magnesium assessment table.

No.	Food / Beverage	Serving size Food (g) / Drinks (ml)	Ca content (mg/100g) / (mg/100ml)	Mg content (mg/100g) / (mg/100ml)	1								
					Weekday 1			Weekday 2			Weekend		
					No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)
d	Yogurt drink (Marigold)	250	67	11		0	0		0	0		0	0
	F&N Magnolia Yoghurt Smoothie, Strawberry with Nata De Coco	200	68	11		0			0			0	
e	Milkshake	200	120	11		0	0		0	0		0	0
f	Cultured milk	100	40	5		0	0		0	0		0	0
g	Nestle Coffeemate Coffee Creamer	5	6	0		0	0		0	0		0	0
5	**Soy pdts**												
	Fortified soy milk	200	80	10		0	0		0	0		0	0
	Vitasoy (fortified soy milk)	375	67	10		0	0		0	0		0	0
	Sobe Fresh Soya Milk (Pasteurised), Trim	200	68	10		0	0		0	0		0	0
	Marigold Hi Cal low Sugar	200	180	23									
	Fresh soy milk	200	10	20		0	0		0	0		0	0
	Packaged soy milk/drink	200	10	10		0	0		0	0		0	0
6	**Fruit juice**												
	Fresh	200	3	9		0	0		0	0		0	0
	Processed	200	10	9		0	0		0	0		0	0

Table III-10 (cont'd): Example of calcium and magnesium assessment table.

No.	Food / Beverage	Serving size Food (g) / Drinks (ml)	Ca content (mg/100g) / (mg/100ml)	Mg content (mg/100g) / (mg/100ml)	1								
					Weekday 1			Weekday 2			Weekend		
					No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)	No. of servings	Ca intake (mg)	Mg intake (mg)
7	**Canned/packet beverages**												
	Carbonated soft drink	330	2	1	1.6	10	5		0	0		0	0
	Non-carbonated soft drink	330	1	0		0	0		0	0		0	0
	Isotonic drink	330	5	5.2		0	0		0	0		0	0
	Nestle Milo	240	60	23		0	0		0	0		0	0
8	**Alcoholic drinks**												
	Beer	330	5	7.7		0	0		0	0		0	0
	Wine	120	7	7.5		0	0		0	0		0	0
	Spirit	250	1	0.12		0	0		0	0		0	0
9	**Others**												
	Honey	15	7	2		0	0		0	0		0	0
	Grass jelly	200	8	3		0	0		0	0		0	0
				Total intake (mg/day)		303	123		119	53		307	98
				Total intake (mg/week)					1670	639			
				Ave intake (mg/day)					239	91			

PARTICIPANT NUMBER _____

Assessment of Intake of Calcium and Magnesium
by Female Singapore Residents

PARTICIPANT'S PARTICULARS

*Please delete where applicable

PARTICIPANT NUMBER _____

Participant Details

Name: (*Ms/Mrs)

_____

Address:

_____

Contact Number – Home: _____ Mobile:

_____

E-mail Address:

_____

Date of Birth: _____ (DD/MM/YYYY)

PARTICIPANT NUMBER _____

GENERAL INFORMATION

Marital Status: *Single/Married/Separated/Divorced/Widowed

Country of birth: *Singapore/Others: _____ (please specify)

Race: □ Chinese □ Caucasian □ Others: _____

Cultural Background: □ Chinese □ Others: _____

Diet: □ Vegetarian □ Halal □ No restriction
□ Others: _____

Religion: □ Buddhist or Taoist □ Christian □ Catholic □ Muslim □ Hindu □Others: _____

Citizenship: □ Singapore Citizen □ Singapore Permanent Resident
□ Others: _____

Number of Years Resided in Singapore: ____

Highest Education Qualification Attained: □ No Qualification □ Primary School □ Secondary School □ Technical Education □ Diploma Course □ University Degree /higher

Income Range (Individual or Family): □ 0 - <$1000 □ $1000 - $2000

□ $2000 - $3000 □ $3000 - $4000 □ >$4000

Occupation/Field of Work or Study:

_____

Location/Region of Stay in Singapore:_____

HEALTH STATUS

Height (m): _____ Weight (kg): _____

Body Mass Index (to be completed by interviewer)

For Chinese: □ *18.5 – 22.9* □ *<18.5 or >22.9*

For Caucasian: □ *18.5 – 24.9* □ *<18.5 or >24.9*

Which of these best describes your physical activity status:

□ 45 minutes of moderate-intensity physical activity 5 days per week or 30 minutes of vigorous-intensity physical activity 3 days per week or equivalent

□ 30 minutes of moderate-intensity physical activity 5 days per week or 20 minutes of vigorous-intensity physical activity 3 days per week or equivalent

□ 20 minutes of moderate-intensity physical activity 5 days per week or 10 minutes of vigorous-intensity physical activity 3 days per week or equivalent

□ None

How many standard drinks of alcohol do you consume on average per day? *Standard drink = 1 glass of beer, 1 glass of wine, 1 shot of spirits or one small bottle of wine cooler/alco-pops*

Number of drinks _____

Are you pregnant or lactating at the moment?

□ Yes □ No

Have you been diagnosed with any bone-, kidney-, thyroid- or liver-related disease(s)?

□ Yes □ No

If yes, please specify:_____

Have you been diagnosed with hypertension?

□ Yes □ No

Have you been diagnosed with diabetes?

□ Yes □ No

Are you suffering from gastrointestinal disorders or any metabolic disease?

□ Yes □ No

If yes, please specify:_____

Are you suffering from any chronic disease?

□ Yes □ No

If yes, please specify:_____

Are you taking any medication regularly except oral contraceptives?

□ Yes □ No

If yes, please specify:_____

PARTICIPANT NUMBER _____

EATING BEHAVIOUR

Have you changed your diet (e.g. eating less, consuming less meat, etc) in the past 1 month/
6 months / 2 years / 5 years?

□ No □ Yes, 1 month □ Yes, 6 months
□ Yes, 2 years □ Yes, 5 years

If yes, why did you change your diet and what were the changes made?

_____
_____
_____
_____

Please tick the boxes of which meals are regularly taken:

	Weekdays	Saturdays	Sundays
Breakfast			
Lunch			
Dinner			
Supper			

What type of breakfast do you usually eat?

□ Cereals (e.g. cereals, oat)
□ Western without cereals (e.g. bread, pasta, potato)
□ Asian, Chinese (e.g. rice, noodles, steamed bun/包)

Where do you usually get your meals from?

Breakfast:	□ Home	□ Outside (e.g. school canteen, hawker, restaurant)
Lunch:	□ Home	□ Outside (e.g. school canteen, hawker, restaurant)
Dinner:	□ Home	□ Outside (e.g. school canteen, hawker, restaurant)
Supper:	□ Home	□ Outside (e.g. school canteen, hawker, restaurant)

How many portions of snacks or desserts on average do you eat per day?

□ 0 □ 1-2 □ 3 or more (please specify:)

How would you consider your appetite/portion sizes in relation to others?

□ Small/less □ Medium/normal □ Large/add/extra

Are you taking any dietary supplements?

□ Yes □ No

If yes, please specify (brand, product name, frequency of intake):

_____

_____

Food Record Sheet 饮食记录

Please record down all food & beverages consumed in 24 hours in detail 请记下您在这24小时所吃和喝的食品和饮料

Name 姓名:

Age
年龄:

Day and date 日期: Weekday 1 工作日 1 / Weekday 2 工作日 2 / Weekend 周末 (please indicate as appropriate)

Time 时间	Food & Beverage Item 食品和饮料	Description 描述	Amount consumed (in servings) 份量

Version 1

Date of revision: 15th December 2011

Food Frequency Questionnaire

Food Item	**Portion**	**Servings**			
How often do you eat the following:		**Per day**	**Per week**	**Per month**	**Rarely/ Never**
(1) STAPLE FOODS / CARBOHYDRATE SOURCES					
Rice, glutinous rice, and porridge	1 serving (200 gr or 500 gr for porridge)				
Noodles	1 serving (200 gr)				
With milk added to soup, eg. fish soup noodles	1 serving (40 mL)				
Pau / bao	1 serving (100 gr)				
Breakfast cereals (calcium enriched) Please specify type and brand(s):_____ _____ _____	1 serving (35 gr)				
Types of milk taken with breakfast cereals					
Unfortified with calcium	*1 cup (200 mL)*				
Calcium fortified	*1 cup (200 mL)*				
Calcium fortified soybean milk	*1 cup (200 mL)*				
Breakfast cereals (others)	1 serving (35 gr)				
Types of milk taken with breakfast cereals					
Unfortified with calcium	*1 cup (200 mL)*				
Calcium fortified	*1 cup (200 mL)*				
Calcium fortified soybean milk	*1 cup (200 mL)*				

Food Item	**Portion**	**Servings**			
How often do you eat the following:		**Per day**	**Per week**	**Per month**	**Rarely / Never**
Bread (loaf)					
White bread	1 serving (2 slices)				
Milk bread	1 serving (2 slices)				
Wholemeal bread, grain bread, other flavoured loaves	1 serving (2 slices)				
Indian breads					
Without cheese	1 serving (120 gr)				
With cheese	1 serving (120 gr)				
Other breads, buns, burger					
Cream-filled rolls	1 serving (65 gr)				
Without cheese	1 serving (60 gr)				
With cheese	1 serving (60 gr)				
Pasta (dairy-based sauce)	1 serving				
With parmesan cheese topping	*1 tablespoon (4 gr)*				
Pasta (non dairy-based sauce)	1 serving (160 gr)				
With parmesan cheese topping	*1 tablespoon (4 gr)*				
Pizza, western baked rice	1 serving (160 gr)				
With parmesan cheese topping	*1 tablespoon (4 gr)*				

Food Item	**Portion**	**Servings**			
How often do you eat the following:		**Per day**	**Per week**	**Per month**	**Rarely / Never**
Potato and potato based products					
Without cheese	1 serving (100 gr)				
With cheese	1 serving (100 gr)				
(2) SIDE DISHES					
Vegetables, vegetable products					
Dark leafy green vegetables	1 serving (70 gr)				
Other vegetables, vegetable products and legumes	1 serving (70 gr)				
Meat, poultry, fish, seafood and their products					
Canned sardines	1 serving (70 gr)				
Canned mackerel	1 serving (70 gr)				
Canned salmon (with bones)	1 serving (70 gr)				
Unagi, stingray, other fishes eaten with bones	1 serving (70 gr)				
Deep fried prawn eaten with shell	1 serving (70 gr)				
Crab and soft-shell crab	1 serving (70 gr)				
Frog leg	1 serving (40 gr)				
Beef tripe	1 serving (40 gr)				
Chicken, duck, pork, beef, veal, mutton, chevron, other fish and fish products (without bones), other seafood, turkey, and eggs	1 serving (70 gr)				

Food Item How often do you eat the following:	Portion	Servings Per day	Per week	Per month	Rarely / Never
Soybean products					
Tofu (firm) eg. taukwa	1 serving (70 gr)				
Tofu (soft) eg. silken tofu, chinese tofu	1 serving (100 gr)				
Tempeh	1 serving (30 gr)				
Mock / vegetarian meat (beancurd or gluten)	1 serving (70 gr)				
(3) TOPPING, SAUCES, AND CONDIMENTS					
Dried anchovy (ikan bilis)	1 tablespoon (4 gr)				
Canned anchovy	1 tablespoon (4 gr)				
Silver fish	1 tablespoon (4 gr)				
Mermaid fish	1 serving (5 gr)				
Dried baby prawn	1 tablespoon (4 gr)				
Dried baby shrimp	1 tablespoon (1 gr)				
Sambal belacan	1 tablespoon (20 gr)				
Chin cha lok (fermented baby shrimp)	1 tablespoon (10 gr)				
Belacan dishes (eg. stingray, kangkung, rojak)	1 serving (5 gr of belacan)				
Indian Curry	1 serving (100 gr)				

Food Item **How often do you eat the following:**	**Portion**	**Servings**			
		Per day	**Per week**	**Per month**	**Rarely / Never**
Gravy/ Soup					
Laksa Lemak ☐ Drink all ☐ Drink half ☐ Drink a little only	1 serving (100 gr)				
Mee Rebus ☐ Drink all ☐ Drink half ☐ Drink a little only	1 serving (100 gr)				
Mee Siam ☐ Drink all ☐ Drink half ☐ Drink a little only	1 serving (100 gr)				
(4) FRUITS					
Fresh fruits	1 serving (120 gr)				
Canned fruits and dried fruits	1 serving (60 gr)				
(5) DESSERTS AND SNACKS					
Desserts in 'soup'					
Black sesame paste (hawker)	1 serving (400 gr)				
Black sesame paste (instant)	1 sachet				
Black sesame glutinous rice balls (tangyuen)/ mochi	1 serving (3 pieces)				
Others (eg. tauhuay, green bean soup, red bean soup, ice kacang)	1 serving (400 gr)				
Dried seaweed (snack)	1 serving (20 gr)				

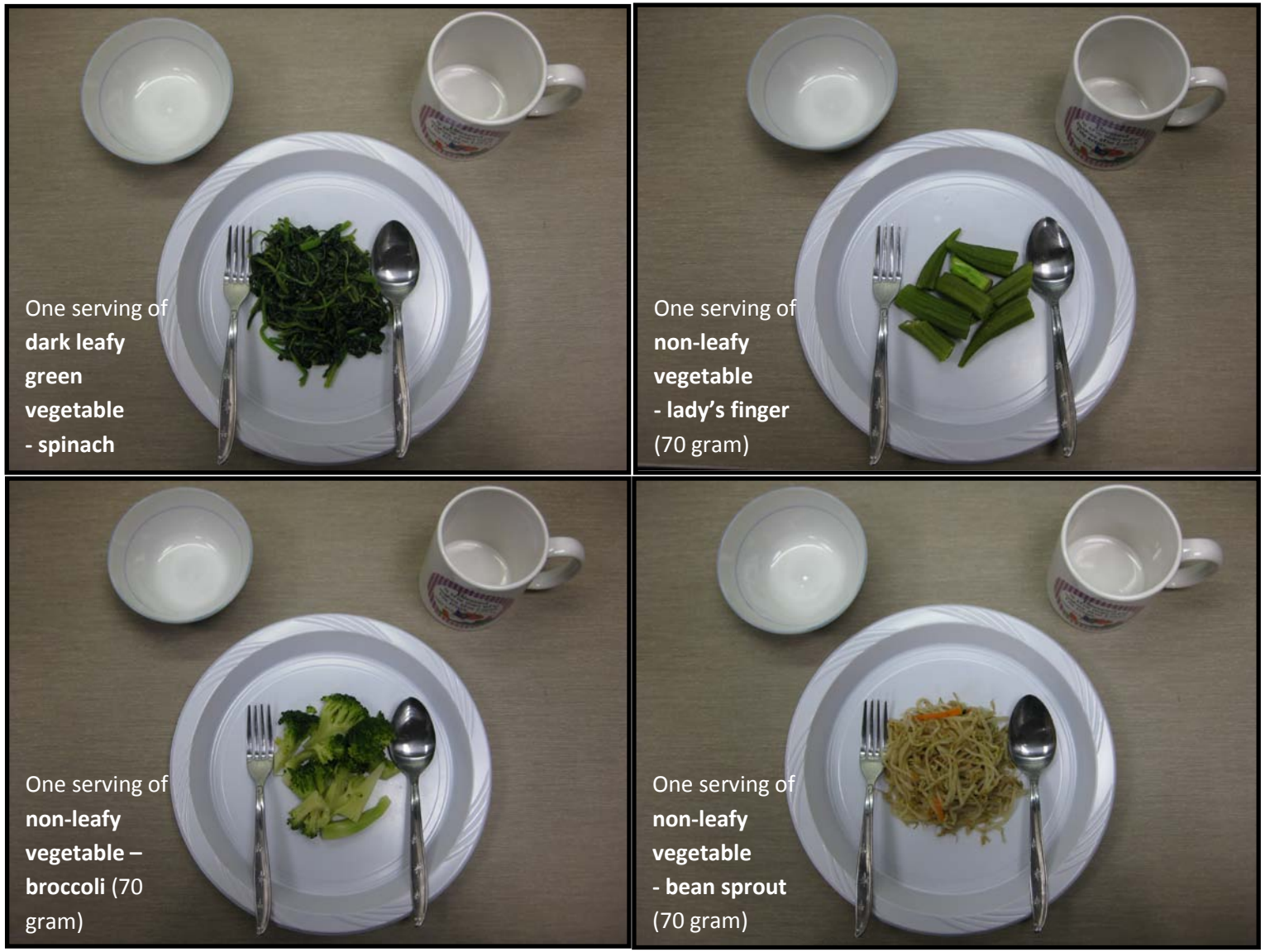
Food Item **How often do you eat the following:**	**Portion**	**Servings**			
		Per day	**Per week**	**Per month**	**Rarely/ Never**
Seaweed strands (ready to eat)	1 serving (60 gr)				
Other seaweed containing dishes e.g. soup, sushi	1 serving				
Nuts					
Almond nut	1 serving (20 gr)				
Pistachio nut and walnut	1 serving (20 gr)				
Others	1 serving (20 gr)				
Biscuits, pastries, cakes and other snacks e.g. croissants, danish, pies, puff, samosas, muffin, pudding, eclairs and sweet puff, tart, crispie cakes, getuk, dodol, waffles, chips, popcorn, seeds	1 serving				
(6) DAIRY PRODUCTS					
Cheeses (excluding those recorded previously together with staple foods)					
Cheese slices Please indicate type and brand:_____	1 slice (20 gr)				
Cheese spreads	1 serving (15 gr)				
Other cheeses e.g. Swiss, Edam, Brie, Feta, Roquefort Please indicate type and brand:_____	1 serving (25 gr)				
Yoghurts Please indicate type and brand :_____	1 serving (160 gr or around 1 small cup)				

Food Item	**Portion**	**Servings**			
How often do you eat the following:		**Per day**	**Per week**	**Per month**	**Rarely/ Never**
Dairy-based ice cream, Mousse and Custard e.g. gelato, kulfi (Indian ice-cream) (excluding sherbet, ice popsicle and ice potong)	1 serving (150 gr)				
Milk chocolate Chocolate brand(s): _____ _____ _____	1 serving (25 gr or around 6 blocks)				


One serving of **breakfast cereals** (35 gram)


One serving of **breakfast cereals** (35 gram)


Sunshine
Butter Sugar Roll
Sunshine
Vanilla Cream Roll
Sunshine cream-filled rolls


One serving of **dark leafy green vegetable - spinach**
One serving of **non-leafy vegetable - lady's finger** (70 gram)
One serving of **non-leafy vegetable – broccoli** (70 gram)
One serving of **non-leafy vegetable - bean sprout** (70 gram)


One serving of
meat – chicken
(70 gram)


One serving of **beef tripe** (40 gram)


Beef tripe


One serving of **firm tofu** (half a cube / 70 gram)


One serving of **firm tofu** (70 gram)


Firm tofu


One serving of **soft tofu** (1/3 tub / 100 gram)


One serving of **soft tofu** (100 gram)


Soft tofu


One serving of
tempeh (30 gram)


One serving of
tempeh (30 gram)


Tempeh


One serving of
dried anchovy
(1 tbsp / 4 gram)


Dried anchovy


One serving of
silver fish
(1 tbsp / 4 gram)


Silver fish

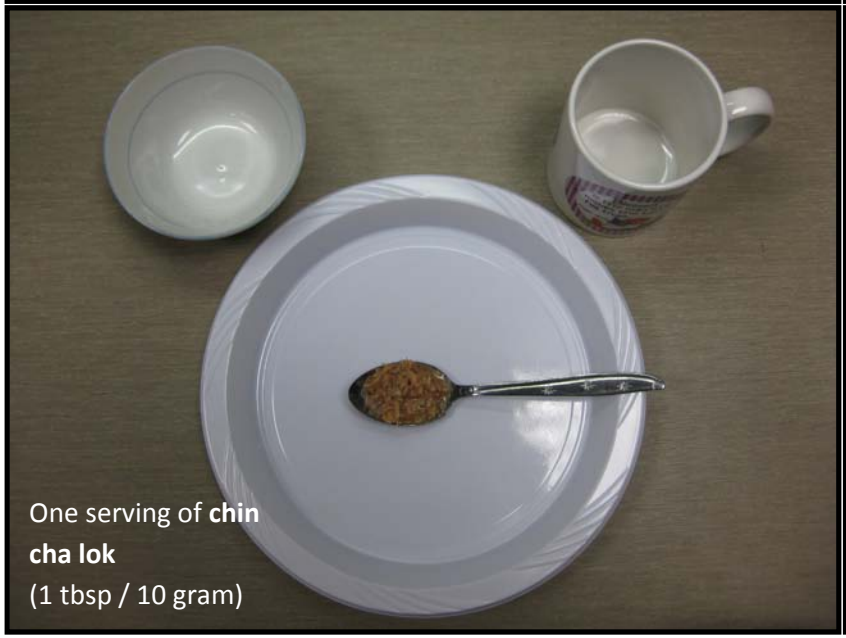

One serving of
mermaid fish
(5 gram)


One serving of
dried baby prawn
(1 tbsp / 4 gram)


Dried baby prawn


One serving of
dried baby shrimp
(1 tbsp / 4 gram)


Dried baby shrimp


One serving of **chin cha lok**
(1 tbsp / 10 gram)


Chin cha lok


One serving of **black sesame paste** (400 gram)
One serving of **black sesame paste** (400 gram)
Blessed is the man who makes the Lord his trust.
Psalm 40:4
One serving of **seaweed strands** (60 gram)
One serving of **milk chocolate** (6 blocks / 25 gram)


Lo-Fat Hi-Cal
Yeo's
ICE LEMON TEA
天与地
Coca-Cola
MARIGOLD
Yoghurt Drink
0% Fat
25% MORE
Life
200 mL packet
250 mL
330 mL can
500 mL
1 L packet
1.5 L bottle