# Vitamin D status and skeletal changes during reproduction

## A longitudinal study from late pregnancy through lactation

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*To Moa and Pontus*

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# ABSTRACT

Low vitamin D status has been associated with sub-optimal bone health. During both pregnancy and postpartum, it has been speculated that vitamin D status may affect maternal bone health, due to its importance in maintaining the calcium homeostasis in the body.

The overall aim of this thesis was to evaluate vitamin D status and bone changes during pregnancy and postpartum in women living in the vicinity of Gothenburg, Sweden. Ninety-five fair-skinned pregnant women and 21 non-pregnant and non-lactating controls were recruited. Blood samples, anthropometric data, information about sun exposure and lactation habits and four-day food diaries were collected in the third trimester of pregnancy and two weeks (baseline), four, 12 and 18 months postpartum. Serum concentrations of 25-hydroxyvitamin D (25OHD) were analyzed. Bone status was assessed postpartum with dual-energy X-ray absorptiometry (DXA) and high-resolution peripheral quantitative computed tomography (HR-pQCT).

In the third trimester, mean 25OHD concentration was 47±18 (mean±SD) nmol/L. During the first year postpartum, no change in mean 25OHD concentration was found and no association between duration of lactation and changes in 25OHD concentrations was observed. Estimates of sun exposure and use of vitamin D supplements were found to be major determinants both for 25OHD concentrations during pregnancy and for the variation in changes in 25OHD concentrations postpartum. During the first four months postpartum, bone decreases were observed at several skeletal sites in women lactating four months or longer. At 18 months postpartum, cortical volumetric bone mineral density and trabecular thickness at the ultradistal tibia were still significantly lower than baseline in women lactating nine months or longer. Calcium intake and 25OHD concentrations appear to have different influences on the cortical and trabecular bone changes postpartum.

In conclusion, a majority of the women were vitamin D insufficient in the third trimester of pregnancy. No change in mean 25OHD concentration was observed during the first year postpartum. Longer follow-up than 18 months is needed to confirm whether women with long lactation fully recover their bone minerals after weaning or whether the postpartum bone changes could potentially lead to an increased fracture risk in later life.

**Keywords**: Vitamin D, 25OHD, BMD, DXA, HR-pQCT, pregnancy, lactation, postpartum
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# SAMMANFATTNING

Det finns två källor till D-vitamin; via solljus och via kost och tillskott. D-vitamin är ett hormon vars viktigaste uppgift är att reglera kalciumbalansen i kroppen. Låga nivåer av D-vitamin har relaterats till en suboptimal benhälsa, men också till en ökad förekomst av många kroniska sjukdomar. Under både graviditet och amning finns teorier om att mammans D-vitaminnivåer kan påverka hennes benhälsa.

Avhandlingens övergripande frågeställning var att studera D-vitaminstatus och benförändringar under graviditet och postpartum. Nittiofem ljushyade gravida kvinnor och 21 icke-gravida och icke-ammande kontroller rekryterades. Blodprover, vikt och längd, information om solvanor och amningsstatus samt kostdagböcker samlades in i tredje trimestern av graviditeten, två veckor och fyra, 12 och 18 månader postpartum. D-vitaminstatus mättes som serumkoncentrationer av 25-hydroxyvitamin D (25OHD). Benförändringar postpartum analyserades med dual energy x-ray absorptiometry (DXA) och high resolution peripheral quantitative computed tomography (HR-pQCT).

I tredje trimestern av graviditeten var medelkoncentrationen av 25OHD 47 nmol/L. Vi fann ingen förändring i medelkoncentrationer av 25OHD under det första året postpartum och inget samband mellan amningslängd och variationen i förändring i 25OHD under det första året postpartum. De främsta determinanterna för både 25OHD koncentrationer under graviditet och förändring i 25OHD koncentrationer under amning var solexponering och användandet av vitamin D-tillskott. Under de första fyra månaderna postpartum fann vi att kvinnor som ammade minst fyra månader minskade i bentäthet. Fortfarande 18 månader efter förlossningen var den kortikala volumetriska bentätheten och den trabekulära tjockleken i det ultradistala skenbenet lägre än precis efter förlossningen hos kvinnor som ammade minst nio månader. Våra resultat antyder att kalciumintag och 25OHD koncentrationer har olika inverkan på kortikala och trabekulära benförändringar postpartum.

Sammanfattningsvis, så hade en majoritet av kvinnorna D-vitamininsufficiens (<50 nmol/L) i tredje trimestern av graviditeten. Vi fann ingen förändring i medelkoncentrationer av 25OHD under första året postpartum. Längre uppföljning än 1,5 år postpartum behövs för att ytterligare studera om kvinnor som ammar länge återhämtar sina benmineraler efter avslutad amning eller om benförändringarna postpartum på sikt kan leda till en ökad frakturrisk senare i livet.

# LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Brembeck P**, Winkvist A, Olausson H. Determinants of vitamin D status in pregnant fair-skinned women in Sweden. *British Journal of Nutrition 2013; 110: 856-864.*

II. **Brembeck P**, Winkvist A, Bååth M, Hedlund L, Augustin H. Determinants of changes in vitamin D status postpartum in Swedish women. *Submitted.*

III. **Brembeck P**, Lorentzon M, Ohlsson C, Winkvist A, Augustin H. Changes in cortical volumetric bone mineral density and thickness, and trabecular thickness in lactating women postpartum. *Journal of Clinical Endocrinology and Metabolism 2015; 100(2): 535-543.*

IV. **Brembeck P**, Winkvist A, Ohlsson C, Lorentzon M, Augustin H. Calcium intake and vitamin D status as determinants of microstructural, dimensional and bone mineral changes postpartum. *Manuscript.*

# RELATED PUBLICATIONS

Related publications with Petra Brembeck as a co-author, not included in this thesis.

1. Hedlund L, **Brembeck P**, Olausson H. Determinants of vitamin D status in fair-skinned women of childbearing age at northern latitudes. *PLoS One 2013; 8(4): 1-6.*

2. Hedlund L, Brekke H K, **Brembeck P**, Augustin H. A short questionnaire for assessment of dietary vitamin D intake. *European Journal of Nutrition and Food Safety 2014; (4)2: 150-156.*

# CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| $1.25OH_2D$ | 1.25-dihydroxyvitamin D |
| 25OHD | 25-hydroxyvitamin D |
| aBMD | Areal bone mineral density |
| BA | Bone area |
| BMC | Bone mineral content |
| BMD | Bone mineral density |
| BMI | Body mass index |
| CI | Confidence interval |
| CLIA | Chemiluminescence immunoassay |
| DXA | Dual energy X-ray absorptiometry |
| FFQ | Food frequency questionnaire |
| HR-pQCT | High-resolution peripheral quantitative computed tomography |
| IOM | Institute of Medicine |
| LC-MS/MS | Liquid chromatography tandem mass spectrometry |
| NNR | Nordic Nutrition Recommendations |
| PAL | Physical activity level |
| PTH | Parathyroid hormone |
| PTHrP | Parathyroid hormone related protein |
| Q1-Q3 | Quartile 1 - quartile 3 |
| SD | Standard deviation |

| | |
|---|---|
| UVB | Ultraviolet beta |
| vBMD | Volumetric bone mineral density |
| WHO | World Health Organization |

# 1 INTRODUCTION

Studies of vitamin D-related health issues are an increasing research field. The major function of vitamin D is to regulate the calcium homeostasis in the body by increasing intestinal calcium uptake. Vitamin D status is usually assessed by measuring serum or plasma concentrations of 25-hydroxyvitamin D (25OHD). Associations between vitamin D status and bone health are well studied. At low 25OHD concentrations, calcium resorption from the skeleton may occur to sustain the calcium balance. Children may then develop rickets and adults may develop osteomalacia (1). During lactation, decreases in bone minerals are known to occur, but the decreases are not thoroughly studied and the importance of vitamin D in relation to these decreases is yet to be evaluated. Relationships have also been found between lower vitamin D concentrations and higher frequencies of many chronic diseases, including cancers (2, 3), infectious diseases (2), cardiovascular diseases (4), autoimmune diseases, diabetes type 1 (5), multiple sclerosis (2, 6) and depression (7). During pregnancy, low vitamin D status has been associated with suboptimal pregnancy outcomes. For example, lower concentrations of 25OHD have been associated with maternal unhealth, such as higher risk for hypertensive disorders (8), gestational diabetes (6, 9), preeclampsia (8) and caesarian section (10). For the unborn child, low maternal vitamin D status is associated with fetal imprinting (11), low birth weight (12-14), small-for-gestational age (15, 16), lower bone mineral content at birth (14) and possibly also neonatal rickets (17). However, vitamin D status and changes in vitamin D status during pregnancy and lactation and its determinants are sparsely studied, especially among women living at northern latitudes. In short, vitamin D status may impact several different stages and diseases in life.

These are the background theories and questions for the concept of this thesis (**Figure 1**).

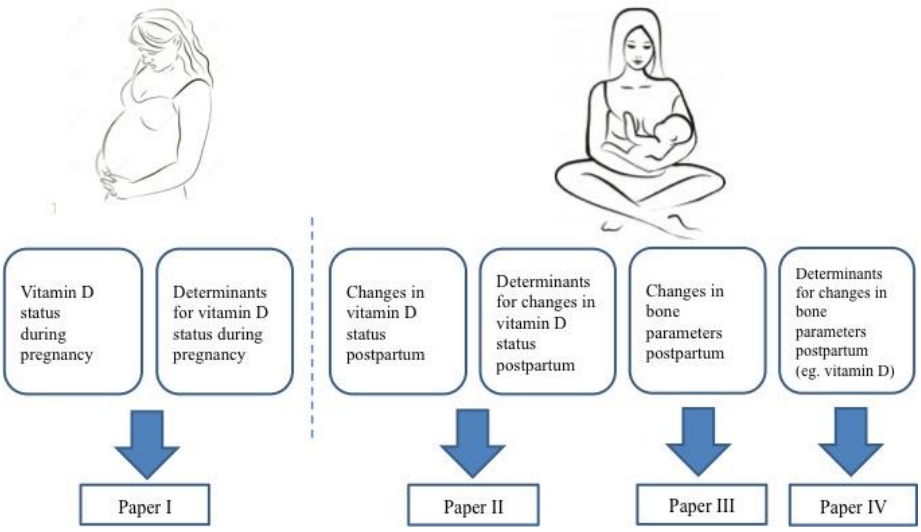


*Figure 1. The basic concept of this thesis.*

# 1.1 Vitamin D metabolism

Vitamin D is a fat-soluble steroid-hormone obtained via sunlight exposure or ingested via diet and supplements (18). It is estimated that 90-95% of the human vitamin D requirements can be mediated via skin production (19). Ultraviolet B (UVB) radiation of wavelengths 290-315 nm converts 7-dehydrocholesterol in the skin to pre-vitamin $D_3$, which in turn is converted to vitamin $D_3$ (cholecalciferol) in a heat-demanding process (**Figure 2**) (18). Vitamin $D_3$ from sun exposure, diet and supplements is either stored in fat cells or transported bound to vitamin D-binding protein in the circulation to the liver, where it is metabolized to 25-hydroxyvitamin D (25OHD), also known as calcidiol or 25-hydroxycholecalciferol. This is the main circulating form of vitamin D and also the vitamin D metabolite that is usually used to estimate vitamin D status. From the liver, 25OHD is transported to the kidneys where the enzyme 25-hydroxyvitamin D-1-α-hydroxylase converts 25OHD to the active vitamin D metabolite, 1.25-dihydroxyvitamin D ($1.25OH_2D$), also known as calcitriol or 1.25-dihydroxycholecalciferol (18).

The metabolite $1.25OH_2D$, together with parathyroid hormone (PTH) and calcitonin, regulate the concentrations of calcium and phosphorous in the serum (1). The main function of $1.25OH_2D$ is to increase the calcium uptake from the intestines (1). Further, $1.25OH_2D$ increases calcium reabsorption from the skeleton and calcium reabsorption from the kidneys (1, 18).

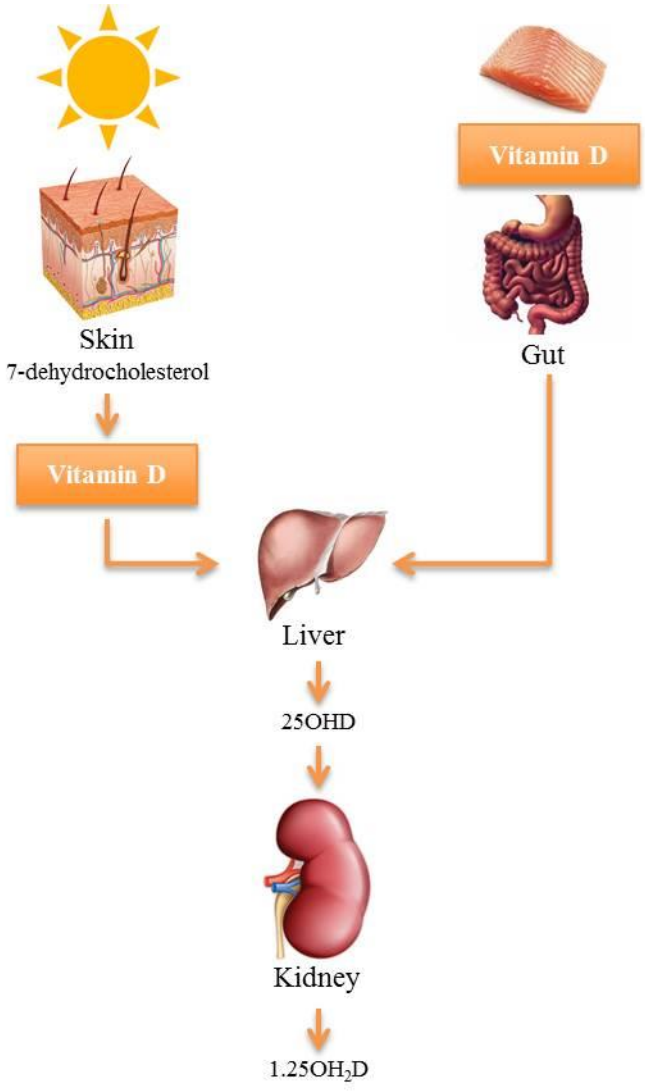


*Figure 2. Vitamin D metabolism from UVB exposure and from diet.*

In the absence of vitamin D, only 10-15% of dietary calcium is absorbed from the intestine (18). The interaction with $1.25OH_2D$, however, increases the intestinal calcium uptake to 30-40% (18). There is a negative feedback mechanism between 25OHD and PTH concentrations, which means that at decreasing concentrations of 25OHD, PTH concentrations increase (18, 20). The synthesis of $1.25OH_2D$ in the kidneys is regulated by PTH, calcium and phosphorous concentrations in the serum. PTH increases the conversion of

25OHD to 1.25$OH_2$D (18) and hence plays a major role in maintaining the calcium balance. Yet during pregnancy and lactation, the relationship between decreasing calcium concentrations and increasing PTH concentrations may differ from non-pregnant state, since some studies have suggested that PTH may be supressed during pregnancy (21, 22). Therefore, the conversion of 25OHD to 1.25OH2D in the presence of PTH may be weaker in pregnant and lactating women (21-23). Instead, the PTH-related protein (PTHrP) has been observed to increase during pregnancy. It is speculated that PTHrP may contribute both to the rise in 1.25$OH_2$D and to the suppression of PTH during pregnancy (22).

## 1.2 Vitamin D status and health

Associations between vitamin D status and bone health are well known. At low vitamin D concentrations, children may develop rickets and adults may develop osteomalacia (1). Further, relationships have been found between lower 25OHD concentrations and higher frequencies of colon, prostate and breast cancers (2, 3), infectious diseases (2), cardiovascular diseases (4), autoimmune diseases, diabetes type 1 (5), multiple sclerosis (2, 6), depression (7) and lower muscle strength (24). Vitamin D intoxication is uncommon. Constantly high vitamin D concentrations can lead to hypercalcemia, nephrocalcinosis and kidney failure (1).

There is currently no consensus on the optimal concentrations of 25OHD. Both the Nordic Nutrition Recommendations (NNR) (1) and the Institute of Medicine (IOM) (25) from Canada and the US have made systematic literature reviews to evaluate the evidence of 25OHD concentrations on different health aspects. They both define vitamin D deficiency as serum 25OHD <30 nmol/L and vitamin D insufficiency as serum 25OHD 30-50 nmol/L. Concentrations of 25OHD above 50 nmol/L are regarded as sufficient by both IOM and NNR, to optimize calcium absorption and bone mineral density, and to avoid rickets and osteomalacia (1, 25).

Other researchers, however, suggest higher serum 25OHD, of between 70-80 nmol/L, to reduce the risk of fracture (26). There is an inverse association between 25OHD and PTH until serum 25OHD reaches concentrations of 75-100 nmol/L, where PTH begins to level off (18). Heaney et al. observed that the intestinal calcium uptake increased by 65% when 25OHD concentrations increased from 50 to 86 nmol/L (27). Given this, some researchers speculate that 25OHD concentrations between 52 and 72 nmol/L can be considered vitamin D insufficiency, whereas levels ≥75 nmol/L can be considered sufficient (26). Vitamin D intoxication has been observed at 25OHD

concentrations above 374 nmol/L, according to Holick et al. (18) Excessive exposure to sunlight cannot cause vitamin D intoxication, since the vitamin D is then converted to inactive products (18).

## 1.2.1 Vitamin D and pregnancy

### Health outcomes

The role of vitamin D during reproduction is one focus of current attention. Low vitamin D status during pregnancy has been associated with unfavorable health outcomes for both infants and mothers. For pregnant women, low 25OHD concentrations have been associated with an increased risk of pre-eclampsia (8), hypertensive disorders (8), gestational diabetes (6), cesarean section (10) and preterm birth (28). For infants, low maternal serum concentrations of 25OHD during pregnancy may affect fetal imprinting (11) and has been associated with an increased risk of low birth weight (12-14), small-for-gestational age (15, 16), low bone mineral content at birth (14), osteopenia (29), neonatal hypocalcemia (29), neonatal rickets (17), enamel hypoplasia in the infant (29) and slow statural growth during the first year of life (29). Also, studies have found positive relations between maternal vitamin D concentrations during pregnancy and the child's bone health at nine years of age (30), as well as the adolescencent's bone mass at 20 years of age (31).

### Vitamin D status

There are a few population-based studies on 25OHD concentrations during pregnancy. These have reported mean 25OHD concentrations between 51 and 57 nmol/L among pregnant women in Australia (13), The Netherlands (15) and Belgium (32). Fifteen percent of the pregnant women in the Australian study had 25OHD concentrations <25 nmol/L (13), whereas 23% of the pregnant women in the Dutch study had 25OHD concentrations <30 nmol/L (15). In minority groups, mean (±SD) 25OHD concentrations of 15±12 to 26±26 nmol/L have been observed in pregnant immigrant women in The Netherlands, whereas a higher mean 25OHD concentration of 53±22 nmol/L was observed in pregnant western women in the same study (33). Similar results were found in a Belgian study, where a mean 25OHD concentration of 28±30 nmol/L was reported among completely covered women and a mean 25OHD concentrations of 59±28 nmol/L was reported among uncovered women during pregnancy (34). In the US, lower 25OHD concentrations have been observed among African-American pregnant women (69±33 nmol/L) than non-African-American women (79±35 nmol/L) (35).

Generally, 25OHD concentrations have in most studies been reported to be unchanged during pregnancy (36). Concentrations of $1.25OH_2D$ have, however, been found to increase in gestational week 10-12 and vitamin D-binding protein somewhat earlier, in gestational week 8-10 (36). Only one study have previously reported vitamin D status and it´s determinants in pregnant fair-skinned women at northern latitudes (32). Hence, little is known about vitamin D status in pregnant women living at northern latitudes, where cutaneous production of vitamin D is not possible all year around.

## 1.2.2 Vitamin D and lactation

### Health outcomes

At birth, the infant's vitamin D status is totally dependent on the maternal serum concentrations of 25OHD (1, 29). The cord blood 25OHD concentrations are approximately 75% of the maternal 25OHD concentrations and correlate with them, whereas the cord blood $1.25OH_2D$ concentrations are, on average, 52% of the maternal $1.25OH_2D$ concentrations and do not correlate (36). This has led to the suggestion that 25OHD is the primary vitamin D metabolite that is transferred to the fetus, although both 25OHD and $1.25OH_2D$ may cross the placenta (36). The vitamin D content in breast milk is also dependent on the maternal 25OHD concentrations (1). The vitamin D content in breast milk is low, between 0.1-3.4 µg/L, depending on the season (37). During the first six months of life, breast milk contains all nutrients a healthy infant needs, except for vitamin D (1, 38, 39). Vitamin D supplements are thus recommended for all infants in Sweden from the first week of life until the age of two (1).

The international recommendation from the World Health Organization (WHO) is for women to exclusively breastfeed their infant for the first six months postpartum, and to continue breastfeeding as a complement to solid foods until the child is two years of age or older (39). The Nordic Nutrition Recommendations (NNR) are consistent with WHO and recommends exclusive breastfeeding of the infant for the first six months postpartum and thereafter breastfeeding as a complement to solid foods until the child is one year or older (1). The definition of exclusive breastfeeding is that the infant is given no other food or liquids than breast milk, with the exception of additional vitamins, minerals and medications (39). According to Butte et al., mean production of breast milk is 749 g/day during the first five months postpartum among women who are exclusively breastfeeding (40). For partial breastfeeding, the mean production of breast milk is 492 g/day during the first two years postpartum (40). Laskey et al. observed a somewhat higher

mean breast milk production at six to eight weeks postpartum of 890 ml/day (range 607-1500 ml/day) in fully breastfeeding women (41).

Given that women who are exclusively breastfeeding produce around 800 ml/day of breast milk and given that they are breastfeeding at least six months, the amount of vitamin D transferred from mother to child through breast milk during lactation may theoretically reach substantial amounts. Thus, breastfeeding may have an impact on maternal vitamin D status. In line with this, it has been suggested that there is an increased maternal need for vitamin D during breastfeeding (42).

### Vitamin D status

There is a dearth of studies on maternal vitamin D status during lactation. In the studies that do exist, conducted among lactating mothers in Greece (43), Turkey (44), Poland (45), Shanghai (46), Mexico (46) and the United States (46) in the early postpartum period, these have found mean concentrations of 25OHD between 27-70 nmol/L. In addition, a Swedish study conducted at 6-12 months postpartum observed mean serum 25OHD of 53 nmol/L in Swedish-born women and mean serum 25OHD of 29 nmol/L in immigrant women (47). Studies of changes in maternal vitamin D status postpartum are very rare. One such study from the United Arab Emirates, where cutaneous production of vitamin D is possible all year round, observed a decrease in mean 25OHD concentration during the first six months postpartum (42). However, in a Danish study, where cutaneous production is only possible during the summer months, mean serum 25OHD were around 60 nmol/L at both 2 weeks and 9 months postpartum and did not differ depending on breastfeeding status (48). A study conducted among lactating American women also observed no change in serum 25OHD during the first six months postpartum (49). These few studies make it clear that changes in vitamin D status postpartum are not thoroughly investigated and results are inconsistent.

## 1.2.3 Methods for measuring 25-hydroxyvitamin D

The metabolite 25OHD is considered a good marker for vitamin D (measured in serum or plasma) and is usually used as a proxy for vitamin D status (1). It is also relatively stable with a half-life of approximately 15 to 50 days (50). However, different vitamin D assays give different results (51, 52). It is important to keep this in mind when comparing results of 25OHD concentrations from studies that have used different methods or when comparing results with, e.g., the IOM or NNR cut-offs for vitamin D deficiency and insufficiency. What is categorized as vitamin D deficiency by

one method may be categorized as vitamin D insufficiency or sufficiency by another method (51, 52).

Available assays for measuring 25OHD concentrations include different immunoassays such as radioimmunoassay (RIA), enzyme immunoassays (EIA) and chemiluminescence immunoassay (CLIA), as well as different types of high-pressure liquid chromatography (HPLC) and mass spectrometry (MS) (51). Other alternatives include competitive protein-binding assays and automated chemiluminescence protein-binding assays (51).

A study investigating three different 25OHD methods found high inter-assay disagreements (51). Of the three investigated methods, the highest mean 25OHD concentration was found for high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI-MS) (85 nmol/L, 95% CI 81-89). An intermediate mean was found for RIA (70 nmol/L 95% CI 66-74), while lowest mean was fond for CLIA (60 nmol/L, 95% CI 56-64) (51). Using the 50 nmol/L cut-off for vitamin D sufficiency/insufficiency by IOM (25), 8% of the subjects were insufficient using HPLC-APCI-MS, 22% with RIA and 43% with CLIA. The most valid method in the study was HPLC-APCI-MS, intermediate was RIA and lowest validity was observed for CLIA. The greatest inter-seasonal difference was also observed by the HPLC-APCI-MS assay (51).

More recently, liquid chromatography tandem mass spectrometry (LC-MS/MS) has been developed to evaluate 25OHD concentrations. Still, there is no golden standard for measuring 25OHD concentrations, but LC-MS/MS is considered as a candidate due to its improved sensibility and specificity compared with immunoassays and competitive binding assays (1, 53, 54). Just like HPLC, LC-MS/MS generally gives lower mean 25OHD concentrations compared to immunoassays (1, 54).

Black et al. compared 25OHD concentrations from three laboratories using either a LC-MS/MS method or chemiluminescence by a DiaSorin Liaison kit, to 25OHD concentrations from a laboratory using an LC-MS/MS assay that was certified to a standard reference method developed by the National Institute of Standards and Technology (52). Serum 25OHD was 12.4 (95% CI -17.8-42.6) nmol/L to 12.8 (95% CI 0.8-24.8) nmol/L higher in the laboratories using LC-MS/MS compared to the certified laboratory. In the laboratory using chemiluminiscence assay, serum 25OHD was instead 10.6 (95% CI -48.4-27.1) nmol/L lower compared to the certified laboratory (52). Mean (SD) serum 25OHD was 65.5±22.7 nmol/L at the certified laboratory, 82.0±35.0 nmol/L and 82.4±29.1 nmol/L at the LC-MS/MS laboratories, but

only 54.4±25.6 nmol/L at the laboratory using the chemiluminiscence assay. Using the results from the certified laboratory, 24% of the subjects had serum 25OHD <50 nmol/L. Using the results from the LC-MS/MS laboratories, only 12-16% of the subjects had serum 25OHD <50 nmol/L, while using the results from the chemiluminiscence assay, serum 25OHD <50 nmol/L were found in 41% of the subjects (52). Lai et al. found similar results when comparing the Diasorin Liaison chemiluminiscence assays and LC-MS/MS and even suggests that due to the considerable variation between assays, defining vitamin D status according to a single universal cut-off may be inappropriate; instead assay-specific definitions may be required (55).

The Vitamin D External Quality Assessment Scheme (DEQAS) was incorporated in 1989. Its objective is to ensure the analytical reliability of 25OHD and 1.25OH2D assays (56). It has 1200 participants in 54 counties and it awards certificates to laboratories that reach the performance targets. Both the laboratories that analyzed the 25OHD concentrations within this thesis (the Central Laboratory at the Sahlgrenska University Hospital and the Central Laboratory in Malmö) are affiliated with DEQAS.

## 1.3 Determinants of vitamin D status

### Sun exposure

At northern latitudes, cutaneous production of vitamin $D_3$ is not possible all year round (57). At latitude 57º North, where Gothenburg is situated, cutaneous production of vitamin $D_3$ is only possible between April and September, whereas between latitudes 35º North and South, cutaneous vitamin $D_3$ production is possible all year round (57, 58). In Rome, at latitude 42º North, skin production of vitamin D is possible between March and October (57). Besides latitude and season, determinants of vitamin D status in non-pregnant and non-lactating women include other estimates of sun exposure, such as whether sunscreen is used, amount of clothing worn and whether there has been any travel to sunny climates (18, 59). Skin pigmentation is another determinant of vitamin D status (18). Sunscreen reduces the absorption of UVB radiation, as does a high melanin content in the skin (18). During the summer months (June-July), it is estimated that at latitude 60º North, sun exposure of face, arms and hands for 6-8 minutes a day, 2-3 times a week is sufficient to produce 5-10 µg/day of vitamin $D_3$ in fair-skinned adults. For individuals with darker pigmentation, sun exposure for 10-15 minutes a day would be necessary to produce the same amount of vitamin $D_3$ (1, 60). Some studies have reported that women who often use sunscreen have higher 25OHD concentrations than women who do not or

who rarely use sunscreen, which might be explained by the probability of sunscreen users spending more time in the sun (32).

Age, obesity, physical activity, dietary intake and supplement use are other determinants for serum 25OHD (18, 57, 61-64). With increasing age, the precursor 7-dehydrocholesterol decreases in the skin, which reduces the vitamin D synthesis (18). Serum concentrations of 25OHD are known to be lower in obese individuals compared to leaner individuals (65, 66). One theory behind this observed relationship is that vitamin D is sequestered in adipose tissue, which would reduce the availability of vitamin D in the circulation (67). Also other physiological factors such as malabsorption, liver failure and chronic kidney disease may decrease serum concentrations of 25OHD (18). In addition, another determinant for serum 25OHD is the genetic component (51).

Defined risk groups for low vitamin D status among individuals living at northern latitudes are individuals with dark pigmentation who wear covering clothing or who avoid sun exposure, elderly individuals who seldom stay outside, individuals with hip fractures or osteoporosis, individuals with malabsorption such as untreated celiac disease and inflammatory bowel disease, individuals with renal or liver failure, obese individuals, individuals taking certain medications such as cortisone and pregnant women (especially dark-skinned pregnant women) (68, 69).

## Vitamin D intake

Dietary vitamin D intake and total vitamin D intake (including diet and supplements) have in some studies been found to be determinants for 25OHD concentrations (35, 62). However, data of vitamin D intake from natural sources are limited (1). Intake of vitamin D-fortified foods has been associated with increases in 25OHD concentrations among adults (70). In addition, intake of vitamin D supplements has been associated with serum 25OHD in several previous studies (7, 32, 71-73).

In Sweden, the most common dietary sources of vitamin D are fatty fish and fish dishes, and fortified dairy products and spreads, as shown in the national dietary survey Riksmaten in 2010 (74). Plant materials also contain some vitamin D, but in the form of vitamin $D_2$ (1). Vitamin $D_3$ is considered to be more efficiently metabolized to 25OHD than vitamin $D_2$ (75). Today, enrichment with vitamin D is mandatory for some dairy products in Sweden (76). Low and medium fat milk are fortified with 0.38-0.5 µg vitamin $D_3$/100 g in Sweden and margarines and spreads are fortified with 7.5-10 µg of vitamin $D_3$/100 g (76). In addition, several natural low and medium fat

yoghurts, sour milks and margarines are voluntarily enriched with vitamin $D_3$. Vitamin $D_3$ used for enrichment in Sweden and most European countries is extracted through UVB exposure of 7-dehydrocholesterol from lanolin, fat from wool (76). Vitamin $D_2$ used for enrichment is synthetized from UVB irradiation of ergosterol present in yeast (77). Riksmaten reported that 27% of the women in Sweden were using supplements, and of these, 29% were using supplements with multivitamins, vitamin D or calcium and vitamin D (74).

According to Riksmaten, mean daily dietary intake of vitamin D among women was 6.4 µg/day, and among women aged 31-44 years, 6.2 µg/day (74). The recommended daily intake according to the NNR is 10 µg/day of vitamin D for both children and adults, as well as pregnant and lactating women (25). The recommendation for elderly individuals (≥75 years) is somewhat higher at 20 µg/day. The average required intake of vitamin D is considered to be 7.5 µg/day, with a lower intake level of 2.5 µg/day and an upper intake level of 100 µg/day (1). The daily intake of vitamin D recommended by the IOM in the US and Canada is 15 µg/day for pregnant and lactating women (25). This higher recommendation is partly because the IOM did not include sun exposure in the calculation (25), whereas the NNR considered there was also some contribution of vitamin D from outdoor activities during the summer season (1).

In the NNR from 2004, the recommended daily intake was higher for pregnant and lactation women (10 µg/day), than for non-pregnant and non-lactating women (7.5 µg/day) (78). The background with regard to the higher recommendation for pregnant and lactating women at that time point was an observed increase in $1.25OH_2D$ during pregnancy and the close association between the vitamin D status of the mother and the new-born child (78, 79). In the new NNR from 2014, the recommended intake is, as mentioned, the same for all adults, including pregnant and lactating women (10 µg/day) (1). This is due to the fact that data concerning the association between vitamin D supplementation and health outcomes during pregnancy and lactation are limited and inconclusive, and because there are uncertainties in the clinical significance of recommending a higher intake among pregnant and lactating women (1). In addition, intervention studies in the Nordic countries have shown that an intake of 10 µg/day of vitamin D is required to maintain concentrations of 25OHD around 50 nmol/L in the majority of the population during winter (1, 71). In a review by Cashman et al., a daily vitamin D intake of 10.2 µg (95% CI 8.9-11.4) was found to be needed to maintain 25OHD concentrations of at least 50 nmol/L during winter in 50% of the population (71). However, to maintain serum 25OHD >50 nmol/L in 97.5% of the population during winter, a daily intake of 28.0 µg (95% CI 24.2-32.8) vitamin D would instead be needed (71). Lamberg-Allart et al. conclude that

for individuals older than three years, a daily vitamin D intake of 10 µg will be needed if the target 25OHD concentration is 50 nmol/L (80). That means that 50% of the population may need a higher daily vitamin D intake and 50% may need a lower vitamin D intake. They further conclude that if 97.5% of the population would have a 25OHD concentration of 50 nmol, a daily vitamin D intake of 15 µg would be needed, assuming minimal sun exposure (80). In a review by Cranney et al., vitamin D intake of 10-12 µg/day from vitamin D-fortified foods resulted in an increase in serum 25OHD by 16 nmol/L (70). Cranney et al., however, concluded that there is a lack of studies in premenopausal women, especially pregnant and lactating women, and that there is a need for studies within this group (70). On average, serum 25OHD is estimated to increase by 1.2 nmol/L for every µg vitamin $D_3$ given as a daily dose at low starting levels of serum 25OHD and by only 0.7 nmol/L or less at starting levels of serum 25OHD at 70 nmol/l or higher (27). According to an observational study by Andersen et al. among Danish adolescent girls and elderly women, 25OHD concentrations of 50 nmol/L during winter was achievable when the 25OHD concentrations during summer were approximately 100 nmol/L (59). If the 25OHD concentrations during summer were instead around 60 nmol/L, the 25OHD concentrations during winter would hardly be higher than 28 nmol/L (59).

The Swedish National Food Agency has presented a proposition to increase and extend the enrichment of dairy products with vitamin $D_3$, since this is considered to be of significant positive importance for the public health (76). The aim is that the general population should have sufficient serum concentrations of 25OHD, to decrease the risk for osteoporosis and total mortality (76).

### 1.3.1 Vitamin D determinants during pregnancy

Season and ethnicity have been observed to be determinants of 25OHD concentrations in pregnant women in the Netherlands, Australia, Canada and the US (13, 33, 35, 72, 81). Lifestyle factors that may affect 25OHD concentrations, such as other estimates of sun exposure, supplement use and dietary intake of vitamin D, have not been well studied. Total vitamin D intake (35) and use of vitamin D supplements have been associated with 25OHD concentrations in pregnant women in Norway, Belgium, Australia and the US (32, 72, 73). Sun exposure has been related to 25OHD concentrations during pregnancy in American, Australian and Belgian women (32, 34, 35, 72). Only one study conducted in Belgium has investigated determinants for 25OHD concentrations during pregnancy thoroughly (32). This large national study reported that travels to sunny climates, use of sunscreen, use of vitamin D supplements and alcohol were

all associated with higher 25OHD concentrations during pregnancy (32). Smoking, preference for shade, low education and non-Caucasian origin were associated with lower 25OHD concentrations (32).

### 1.3.2 Vitamin D determinants during lactation

Few studies have been conducted to evaluate determinants of vitamin D status postpartum. Even fewer report determinants of changes in vitamin D status postpartum. A study conducted among Turkish postpartum women found low socioeconomic status, wearing concealed clothing and low educational level to be risk factors for low 25OHD concentrations shortly after delivery (44). Dawodu et al. found vitamin D supplementation, season, obesity and geographical site to be determinants for maternal vitamin D status in the early postpartum period among women in China, Mexico and the US (46). Additionally, an intervention study observed higher serum 25OHD in lactating Polish women after six months of supplementation with 30 µg vitamin D/day, compared to women supplemented with a daily dose of 10 µg vitamin D (45). A decrease in mean serum 25OHD was observed during the first six months postpartum in a population of women in the United Arab Emirates (42), whereas others found no association between lactation and 25OHD concentrations (48, 49). Results are hence scarce and inconsistent.

## 1.4 Breastfeeding habits in Sweden

The breastfeeding prevalence in Sweden is high (1). The national survey in Sweden showed that at one week postpartum, 81% of women are exclusively breastfeeding and 96% are breastfeeding to some extent. Corresponding numbers at four months postpartum are 52% and 75% respectively (82). At one year postpartum, only 0.1% of women in Sweden are exclusively breastfeeding, but 18% are still breastfeeding to some extent (82). Aside from the nutritional, emotional and psychological aspects, breastfeeding has several positive health effects for both mother and child. For the child, breastfeeding (both exclusively and to some extent) may be a protective factor against the development of overweight and obesity (83) and may prevent infections; both overall infections as well as gastrointestinal and respiratory tract infections during childhood (83, 84). Further, breastfeeding may protect the child against adulthood high blood pressure (83, 85) and may also have a preventive effect against the development of celiac disease (83, 86), inflammatory bowel disease (83, 87) and type 1 and type 2 diabetes mellitus (83). For the mother, relationships have been observed between longer duration of lactation and a lower risk of developing diabetes type 2 (88), heart disease (89, 90) and breast and ovarian cancers (39, 91).

## 1.5 Bone structure and bone changes

As previously mentioned, the major function of vitamin D is to sustain the balance of calcium in the body by increasing the intestinal calcium uptake, and also by increasing calcium reabsorption from the kidneys and from the skeleton (18).

The skeleton is our largest calcium reservoir and consists of approximately 1000-1200 g calcium (92). The skeleton comprises both long bones, such as the radius, femur and tibia, and flat bones, such as the skull, sternum and scapula (93). There are two main histological types of bone, cortical and trabecular bone. Cortical or compact bone has a dense, ordered structure and is found primarily in the shaft of the long bones and the surface of the flat bones (93). Trabecular or cancellous bone has a lighter, less compact and irregular structure and is found primarily in the end of the long bones and the inner parts of the flat bones (93). Generally, each bone has a dense outer layer of cortical bone, overlaying trabecular bone (93). Cortical bone makes up 80% of the skeleton, but the proportion of cortical and trabecular bone varies at different skeletal sites (94). The femoral neck is composed to 75% of cortical bone and 25% of trabecular bone. The vertebrae, however, are composed to more than two-thirds of trabecular bone (95). Trabecular bone is better at withstanding compressive stress, and is the predominant bone found in the vertebrae (93). Trabecular bone is also more metabolically active than cortical bone and forms 65-70% of the total bone surface and therefore serves as a calcium reservoir (95).

Bone is highly dynamic and undergoes constant remodelling (93). It is estimated that it takes 10 years for an adult's skeleton to be totally regenerated through bone remodeling (96). The skeleton consists of three different types of bone cells: osteoblasts, osteocytes and osteoclasts (97). Osteoblasts are the bone formatting cells and have a lifetime of approximately three months (96, 98). After bone formation, some osteoblasts develop into osteocytes (98). These cells are long-lived and constitute about 95% of all bone cells. Osteocytes can also regulate bone remodeling (98). Osteoclasts are the bone resorbing cells (97). They are important for bone resorption during growth, for bone remodeling and for maintaining the calcium balance in the body (99). Aside from bone cells, the skeleton consists to 90% of extracellular matrix. This is composed of mineralized and organic matrix, lipids and water. Ninety-nine percent of the body's storage of calcium is found in the mineralized matrix (100).

Throughout childhood and adolescence, the skeletal mass continues to accumulate, from approximately 70-95 g at birth, to 2400 to 3330 g in young women and men respectively (101). Peak bone mass is the maximal bone mass attained during life (102). This is attained in young adulthood (102). The age for attaining peak bone mass has been found to differ depending on gender, skeletal site and measuring method (102-104), but is generally reached in the late teenage years or young adult years (105). Boot et al. reported peak bone mass to occur between 18 and 20 years for women and between 18 and 23 years for men (102). Peak bone mass is of vital importance for skeletal health throughout life and a high peak bone mass could delay the onset of osteoporosis and reduce the risk of fractures later in life (106). Heredity is the major determinant for peak bone mass and accounts for 60-80% of the variation in peak bone mass (107). After reaching peak bone mass, the bone mass steadily decreases throughout life. Substantial trabecular bone loss occurs after reached peak bone mass throughout life in both sexes. Among women, primarily the number of trabeculae is reduced (108). Previous studies have suggested that cortical bone remains fairly stable until mid-life. At menopause, estrogen deficiency begins to drive cortical bone loss (108). Changes in cortical porosity are an important marker for bone quality in both women and men, but this is not captured by all measuring methods (108). No previous study has investigated changes in cortical and trabecular bone separately during pregnancy and lactation, which is why such information has so far been unknown.

## 1.5.1 Methods for measuring bone changes

### Dual-energy X-ray absorptiometry

The most common technology to measure bone mineral density (BMD) is by dual-energy X-ray absorptiometry (DXA). This method is also the golden standard to assess bone mass in humans and is used clinically to diagnose osteoporosis (109). Dual-energy X-ray absorptiometry is based on X-ray technology and it gives a two-dimensional measurement of the bone (**Figure 3**). The DXA measures the bone area (BA) and the bone mineral content (BMC) of a given area. Areal bone mineral density (aBMD) is calculated by dividing BMC (g) by the area ($cm^2$) and gives an areal measurement in $g/cm^2$.

*Figure 3. Image of the hip, as assessed with dual-energy X-ray absorptiometry (Hologic Discovery W, Tromp Medical B.V.).*

Interpretations of the results are made by comparing with T-score and Z-score. The Z-score is used in younger individuals and compares the obtained value with an average value for the respective age and gender (110). The T-score is used in older individuals and compares the obtained value with an average value of a younger individual of the same sex (110). A T-score of -2.5 or less means that the obtained value is 2.5 standard deviations below the value of a younger individual of the same gender and indicates osteoporosis (109).

Briefly, the DXA scans the human body with two X-ray beams of different energy levels, one with low energy and one with high energy, as described by Rudäng et al. (111) This allows for separation of soft tissue and denser bone tissue. Sensors of the DXA detect the absorbed amount of energy in different tissues of the body and produce an image of the mineralized bone and the soft tissue at the site of interest of the body. A value of the density, expressed as $g/cm^2$, is also obtained. DXA had the advantage that it may measure many different skeletal sites, such as radius, lumbar spine, femur and whole-body. One weakness with the DXA, however, is that it only measures the areal BMD and therefore gives no information about the depth or volume of the bone.

## High-resolution peripheral quantitative computed tomography

Today, there is also a newer X-ray technology: high-resolution peripheral quantitative computed tomography (HR-pQCT). In contrast to DXA, HR-pQCT differentiates between cortical and trabecular bone and gives a three-dimensional measurement of the bone (**Figure 4**). It thus measures volumetric BMD (vBMD) in g/cm$^3$. HR-pQCT also gives a more detailed measurement with higher resolution and thus can also give information about microstructural changes, such as trabecular thickness and number and trabecular bone volume fraction, and dimensional changes, such as cortical thickness and area. However, no such data from the postpartum period have previously been published and so changes in vBMD, microstructural and dimensional parameters during the postpartum period are unknown. Such information would increase the understanding of skeletal changes during and after lactation. So far, a previous study among postmenopausal women has shown that cortical and trabecular vBMD and cortical thickness are significant determinants of fracture risk (112). The same study showed another advantage of HR-pQCT, in that it may be able to detect small changes in BMD that are not detectable by DXA (112).

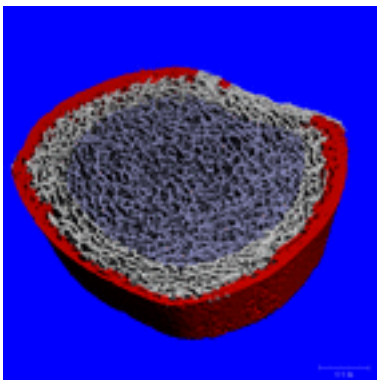

*Figure 4. Image of tibia segmentation, as assessed with high-resolution peripheral quantitative computed tomography (XtremeCT, Scanco Medical AG, Brüttisellen, Switzerland).*

## 1.5.2 Bone changes during pregnancy

During pregnancy, extra amounts of calcium are needed for the production of the fetal skeleton. One way to fulfil this extra calcium need is through resorption of calcium from the maternal skeleton to the fetal skeleton (22). A newborn's skeleton contains approximately 20-30 g calcium (113, 114). Most of this calcium is transferred towards the end of pregnancy (113, 114). It is estimated that approximately 50 mg calcium/day is transferred at 20 weeks of gestation and 330 mg calcium/day in gestational week 35 (114).

The increased demand for calcium during pregnancy is also partly met by an increased maternal intestinal calcium uptake (22). Studies have observed that calcium absorption increases from approximately 35% in non-pregnant women to approximately 60% in the third trimester of pregnancy (113). Serum concentrations of $1.25OH_2D$ have also been observed to be 50-100% higher during the second trimester and 100% higher during the third trimester, compared to the non-pregnant state (113). It is speculated that the kidneys account for most of this rise in $1.25OH_2D$ (22). The renal calcium excretion has also been observed to increase during pregnancy, which is probably a reflection of the increased intestinal calcium absorption (22). Some studies have found PTH to be unchanged during pregnancy (36), whereas others have found PTH to be suppressed during the first trimester followed by an increase to normal values by the end of pregnancy (22). Instead, the PTH-related protein (PTHrP) has been observed to increase during pregnancy (22). The PTHrP is produced by many tissues, in both the fetus and the mother, including the placenta, umbilical cord and breast tissue (22). It is suggested that PTHrP may contribute both to the rise in $1.25OH_2D$ and to the suppression of PTH during pregnancy (22). Bone markers have shown that bone turnover is increased during pregnancy, as early as in gestational week 10 (22).

Since both the DXA and the HR-pQCT are X-ray methods, it is difficult to measure BMD during pregnancy. The radiation from both DXA (115, 116) and HR-pQCT (manufacturer specifications) is low, but there is always a potential risk of harming the fetus. Decreases in aBMD of approximately 0.5-4% have been observed during pregnancy at the lumbar spine, total hip, radius and wholebody, as measured prepregnancy and shortly after delivery (117-120). Hypothetically, the mother's vitamin D status may be of importance for these skeletal changes. Whether the changes in bone minerals during pregnancy are vitamin D dependent, however, are not yet known. Women who are pregnant during winter - when UVB exposure and cutaneous production of vitamin D is low - have higher ultrasound indices of maternal

bone loss (30). This may indicate a role for maternal vitamin D in bone metabolism during pregnancy.

### 1.5.3 Bone changes during lactation

During lactation, it is estimated that mothers who are fully lactating secrete 200-300 mg calcium/day to the breast milk during the first months postpartum, and some women secrete as much as 400 mg calcium/day (41, 121, 122). In women who are breastfeeding twins, the calcium losses may be as large as 1000 mg/day (22). The maternal calcium losses during lactation depend on the duration of lactation, the calcium concentration in the breast milk and the amount of breast milk produced (123). During lactation, maternal intestinal calcium absorption and serum $1.25OH_2D$ are no longer increased. Shortly after delivery, $1.25OH_2D$ falls back to normal and remains there throughout lactation (22). In contrast to pregnancy, it is suggested that renal calcium absorption is increased during lactation (22). Levels of PTHrP are still increased, as during pregnancy, and estrogen levels are low. The increase in PTHrP levels continues through lactation and levels off first during weaning (22).

To meet the extra calcium demand during lactation, calcium may be mobilized from the maternal skeleton (22). It is speculated that the skeletal calcium resorption is mainly mediated via the increased PTHrP, released via the breast tissue, and the low estrogen levels (22). The child's suckling during breastfeeding induces the release of prolactin. The suckling and the prolactin suppress the gonadotropins luteinizing hormone and follicle-stimulating hormone, which leads to low levels of the sex hormones estradiol and progesterone. The production of PTHrP and its release from the breast is regulated by factors such as the child's suckling, prolactin levels and calcium receptors. The PTHrP together with low estradiol levels is supposed to increase bone resorption. This releases calcium in the circulation, which then reaches the breast and the breast milk (22). The total skeletal calcium losses during six months of lactation for a women who is exclusively breastfeeding is approximately 40 g (123). Together with the calcium loss of a pregnancy approximating 30 g, this would constitute about 7% of the maternal skeletal calcium reservoir, if the skeleton was the only calcium source (123).

Several longitudinal studies have observed decreases in maternal aBMD during lactation (41, 118, 119, 122, 124, 125), as assessed with DXA. Changes are found to be highest in femoral neck and lumbar spine aBMD, with decreases of 2-6% during the first months of lactation (41, 118, 119, 122, 124, 125). Greater decreases in aBMD postpartum are observed in

women with longer duration of lactation, compared to women with shorter duration of lactation or formula-feeding mothers (41, 118, 120, 122, 124, 126, 127). Several studies indicate that the influence of lactation on maternal aBMD differ depending on skeletal site, with an initial decrease at skeletal sites consisting mainly of trabecular bone (118, 122, 124, 128). During extended lactation, bone loss is suggested to be mostly located to cortical bone (118, 124, 128). However, since previous studies of changes in aBMD postpartum mostly have used DXA, which is not able to separate between cortical and trabecular bone, this have only been speculations.

Most previous studies of changes in aBMD during pregnancy and lactation have indicated that the decreases in maternal aBMD during reproduction are only transient. Replenishment of bone minerals occurs at the end of a period of long lactation or after ceased lactation (92, 114, 118, 129). However, Affinito et al. reported an incomplete recovery at lumbar spine at six months after weaning (130). Hypothetically, repeated pregnancies and/or extended lactations may lead to residual decreases in bone minerals and an increased risk for fractures in later life (41, 131). However, previous studies have found no or even an inverse relation between parity, lactation and fractures (126, 132-134). Theoretically, vitamin D may play a role in the decreases in bone minerals during lactation and in the replenishment of bone minerals after ceased lactation (135).

Long duration of lactation is common in Sweden and the Nordic countries (1) and studies among women with extended lactation require a long follow-up period. Only a few previous studies have investigated changes in aBMD postpartum in women with extended lactation and a follow-up period longer than 12 months. Only one of these also included a control group (48). No study has previously investigated postpartum changes in cortical and trabecular bone separately or changes in microstructural and dimensional bone parameters postpartum. Consequently, previous suggestions are only speculations.

## 1.6 Determinants of bone changes during pregnancy and lactation

During pregnancy, body weight change and prepregnancy BMI have been observed as positive predictors for bone changes, as measured prepregnancy to shortly after delivery (117, 136). A higher gestational body weight gain and a higher prepregnancy BMI has been associated with smaller decreases in bone minerals during pregnancy (117, 136). Lactation has, as previously

mentioned, been associated with decreases in aBMD postpartum (41, 118, 119, 122, 126, 127). Further, longer duration of lactation has been related to larger decreases in aBMD (41, 118, 120, 122, 126, 127). Besides lactation, determinants for changes in aBMD are not well studied. Studies investigating the relationship between vitamin D status and decreases in aBMD postpartum are very rare. Krebs et al. found no association between dietary vitamin D intake and lumbar spine or mid-radius aBMD postpartum (92). Relations between serum 25OHD and changes in aBMD postpartum have not been studied before. Studies investigating the relationship between calcium intake and changes in aBMD postpartum have mostly found no such association (41, 122, 123). However, Krebs et al. found a positive association between total dietary calcium intake and lumbar spine aBMD postpartum (92). Serum estradiol have also been positively related to lumbar spine aBMD postpartum (92). Further, positive associations have been observed between body weight or body weight change and postpartum decreases in femoral neck and femoral trochanter aBMD by some (137), but not by others (122, 123). Negative associations have been found between parity (92, 128), maternal height (41) and maternal age (128, 137), and changes in aBMD at different skeletal sites postpartum. No previous study has investigated changes in vBMD, microstructural or dimensional parameters or its determinants before.

# 2 AIMS

The overall aim of this thesis was to study vitamin D status and skeletal changes during pregnancy and postpartum in a population of women living in Sweden.

## 2.1 Paper I

Aims were:

- To study serum concentrations of 25-hydroxyvitamin D (25OHD) in the third trimester of pregnancy in women living in Sweden.
- To study determinants of serum concentrations of 25OHD in the third trimester of pregnancy in women living in Sweden.

## 2.2 Paper II

Aims were:

- To study changes in serum concentrations of 25OHD between two weeks and 12 months postpartum in women living in Sweden.
- To specifically evaluate lactation as a determinant of changes in serum 25OHD during the first year postpartum in women living in Sweden.

## 2.3 Paper III

Aims were:

- To study changes in areal bone mineral density (aBMD), volumetric BMD (vBMD), microstructural and dimensional bone parameters between two weeks and 18 months postpartum in women living in Sweden.
- To study associations between lactation and changes in bone parameters during the first 18 months postpartum in women living in Sweden.

## 2.4 Paper IV

Aims were:

- To study determinants of changes in aBMD, vBMD, microstructural and dimensional bone parameters postpartum in women living in Sweden.
- To specifically evaluate calcium intake and vitamin D status as determinants of changes in bone parameters during the first 18 months postpartum in women living in Sweden.

# 3 SUBJECTS AND METHODS

## 3.1 Subjects

The study, with the acronym BUGA (Benmetabolism Under Graviditet och Amning: Bone metabolism During Pregnancy and Lactation), is a longitudinal observational study. Women were recruited between July 2008 and July 2011 through advertisement on a webpage addressing pregnant women (Gravid.se) and through posters at 11 maternity health care clinics and at public places in the vicinity of Gothenburg, Sweden. Recruitment was spread evenly throughout the year, to account for the seasonal variation in concentrations of 25OHD (57). In total, 95 pregnant women (called "pregnant" and after delivery "postpartum" women) and 26 non-pregnant and non-lactating women ("controls") agreed to participate. Inclusion criteria for all women were that they were aged 25-40 years and that they declared themselves healthy. For the pregnant women, that their pregnancy was in gestational week 35-37 when starting the study was also an inclusion criterion. Exclusion criteria were prescribed intake of medicine known to effect the calcium or bone metabolism, recent bone fracture, pregnancy during the last 1.5 years before the start of the present pregnancy or before entering the study as a control, breastfeeding during the last year before the start of the present pregnancy or before entering the study as a control, twin pregnancy or and development of gestational diabetes or preeclampsia. Oral and written information about the study was given to all women before recruitment. The study was conducted according to the guidelines laid down in the Declaration of Helsinki (138) and all procedures involving the subjects were approved by the Regional Ethical Review Board in Gothenburg and the Swedish Radiation Safety Authority. Written informed consent was obtained from all women.

## 3.2 Study design

The pregnant women first visited the Department of Internal Medicine and Clinical Nutrition, University of Gothenburg, Sweden, in gestational week 35-37. All women thereafter visited the department at baseline (two weeks after delivery for the "postpartum" women), and four, 12 and 18 months thereafter (**Figure 5**). At all visits, venous blood was drawn in the morning after overnight fast. Body weight in underwear (Tanita, BWB-800MA, Rex Frederiksbergs Vaegtfabrik) and height (standardized wall stadiometer) were

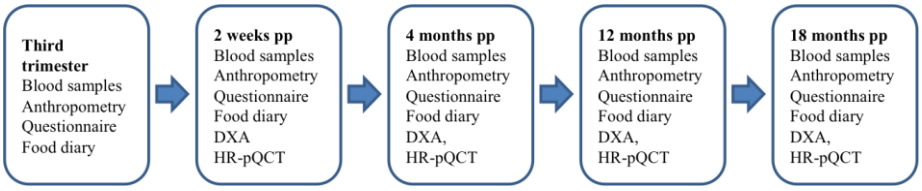


*Figure 5. Study design of the BUGA-study. Months pp; Months postpartum, DXA; dual-energy X-ray absorptiometry, HR-pQCT; high-resolution peripheral quantitative computed tomography.*

measured. At all visits, women were asked questions from a questionnaire concerning medical history, medical intake, physical activity level (PAL), smoking habits, dietary and supplement intake, sun exposure, skin type, use of hormonal contraceptives and current lactation. After delivery, women were asked to report date of birth and birth weight and length of the baby. At all visits, women were instructed to complete a four-day food diary. At each visit except during pregnancy, bone variables were measured at the Osteoporotic Laboratory and the Osteoporotic unit at the geriatric medicine clinic, Sahlgrenska University Hospital, Gothenburg (**Table 1**).

*Table 1. Data included in the different papers.*

| **Paper** | **I** | **II** | **III** | **IV** |
|---|---|---|---|---|
| Data | Cross-sectional | Longitudinal | Longitudinal | Longitudinal |
| Study visits | Third trimester | Two weeks postpartum, 4 months postpartum, 12 months postpartum | Two weeks postpartum, 4 months postpartum, 12 months postpartum, 18 months postpartum | Two weeks postpartum, 4 months postpartum, 12 months postpartum, 18 months postpartum |
| Participants (n) | 95 pregnant women | 78 postpartum women[a] | 81 postpartum women[b], 21 controls[b] | 81 postpartum women[b] |
| Measurements | Blood samples Anthropometry Lifestyle questionnaire Food diary | Blood samples Anthropometry Lifestyle questionnaire Food diary | Blood samples Anthropometry Lifestyle questionnaire | Blood samples Anthropometry Lifestyle questionnaire Food diary |
| Bone measurements | | | DXA HR-pQCT | DXA HR-pQCT |

[a]Women with complete set of data at both baseline and 12 months postpartum.
[b]Women with at least two repeated bone measurements.

Number of pregnant women and controls participating at the different study visits are shown in **Figure 6**.

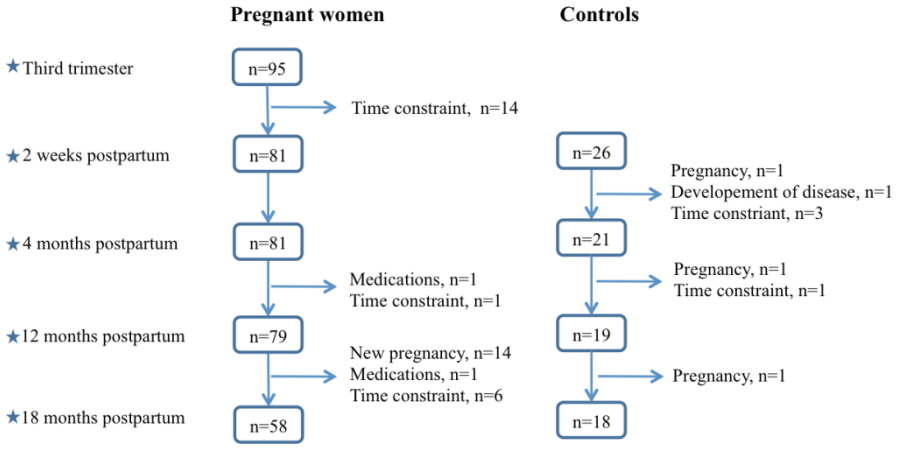


*Figure 6. Numbers of pregnant women and controls participating at the different study visits. Reasons for drop-out are specified.*

# 3.3 Methods

## 3.3.1 Laboratory analyses

Blood samples were protected from UVB light and centrifuged maximum 45 minutes after blood sampling, at 5°C, 3800g, for 9 minutes (Centrifuge CR3i, Jouan Quality System). Serum was then aliquoted and stored at -70°C until analyzed. In *Paper I*, analyses of intact PTH were performed with an immunological two-step analysis of sandwich type, using chemiluminescent microparticle immunoassay technology (Abbott Laboratory Diagnostics Division) at the Central Laboratory at the Sahlgrenska University Hospital, Gothenburg, Sweden. Coefficients of variations for PTH were 3.7, 4.5 and 3.5% for serum PTH of 10, 40 and 730 ng/L, respectively. Serum concentrations of total 25OHD and of 25-hydroxyvitamin $D_3$ in *Paper I* were analyzed with LIASON® 25OHD chemiluminescence immunoassay (CLIA, DiaSorin) at the Central Laboratory at the Sahlgrenska University Hospital, Gothenburg, Sweden. Intra-assay coefficients of variations for serum 25OHD as assessed with CLIA were 7.3, 5.7 and 5.3% for serum 25OHD of 22, 50 and 150 nmol/L. In *Papers II* and *IV*, serum concentrations of 25OHD were analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS; Mass spectrometer API 4000) at the Central Laboratory in Malmö,

Sweden. The method has a measuring range of 6-450 nmol/L for $25OHD_3$ and an inter-coefficient of variation of 6% at 40 nmol/L.

## 3.3.2 Bone changes

Bone variables were measured at the baseline visit and four, 12 and 18 months thereafter. The same bone variables were measured at all occasions.

### Dual-energy X-ray absorptiometry

DXA (Lunar Prodigy, GE Lunar Corp., Madison, WI, software version 11.400.004) was used to measure aBMD at ultradistal radius at the non-dominant arm, femoral neck, femoral shaft, femoral trochanter, total femur, lumbar spine (L1-L4) and whole-body. Briefly, the DXA scans the human body with two X-ray beams of different energy levels, as described by Rudäng et al (111). The amount of energy that passes through the body is measured for each beam, which gives information about the different tissues in the body. The DXA also produces an image of the mineralized bone and the soft tissue at the site of interest of the body and gives a value of the areal bone density, expressed as $g/cm^2$. The coefficients of variance for the DXA measurements of aBMD were 0.5 to 3%, depending on measuring sites. The effective radiation dose for a hip and spine scan is low, ranging from 1-10 µSv. This is comparable to the natural background radiation from the ground and space of approximately 7 µSv/day (115, 116)

### High-resolution peripheral quantitative computed tomography

HR-pQCT (XtremeCT, Scanco Medical AB, Brüttisellen, Switzerland, software version 5.3) was used to measure cortical vBMD, cortical thickness, cortical area, trabecular vBMD, trabecular thickness, trabecular bone volume fraction and trabecular number at ultradistal tibia, on the same side as the non-dominant upper limb. Briefly, a reference line was manually placed at the center of the scan of the end plate of the distal tibia. The first computed tomography slice started 22.5 mm proximal of the reference line for the tibia, as described by Rudäng et al (111). From each skeletal site, 110 parallel computed tomography slices were obtained, with a resolution of 82 µm. These delivered a 3D image of an approximately 9 mm section of the skeletal site. Standard evaluations were used. The coefficients of variance were determined from three repeated measurements according to a standardized protocol from two subjects, as previously described (139). The CVs for the HR-pQCT measurements were: cortical vBMD (0.1%), cortical thickness (0.3%), cortical area (0.4%), trabecular vBMD (0.2%), trabecular thickness (0.7%), trabecular bone volume fraction (0.3%) and trabecular number

(1.6%). The effective radiation dose from one scan is approximately 5 µSv and is restricted to the scanned region (manufacturer specifications). All measurements were evaluated according to a five-graded scale, where 1 corresponded to highest quality, 2 and 3 to acceptable quality and 4 and 5 to unacceptable quality. Measurements graded 4 and 5 were excluded from the analysis.

### 3.3.3 Breastfeeding habits

Information about current lactation habits was collected at all study visits postpartum, including number of lactation sessions per day, number and amount of formula feedings per day, date of introduction of solid foods and amount of solid foods given. Women were asked to record the last date of lactation. Duration of total lactation refers to duration of any type of lactation (full and partial) in months. Duration of full lactation (months) refers to the periods when ≥90% of the child's energy intake came from breast milk. In *Paper II*, the unit of lactation used was duration of total lactation (months) and duration of full lactation (months). In *Paper III*, the postpartum women were categorized into three different lactation groups depending on duration of total lactation: 0-3.9 months, 4-8.9 months or ≥9 months. In *Paper IV*, units of lactation used were at four months postpartum if the woman was fully lactating or not (yes or no), at 12 months postpartum duration of full lactation (months) and at 18 months postpartum duration of total lactation (months).

### 3.3.4 Sun exposure

Sun exposure was estimated using questions constructed by Burgaz et al (62). These included use of sunscreen (always, sometimes or never) and preference for sun or shade when outdoors in the summer (always in the sun, both sun and shade, always in the shade). Women were asked whether they had used a sunbed during the previous six months and whether they had been travelling to southern latitudes during the previous six months. Southern latitudes were defined as a location below 35° North and above 35º South, where cutaneous production of vitamin D is possible all year round (57, 58). Skin types were defined using the Fitzpatrick scale (I=always burns, never tans, II=usually burns, tans with difficulty, III=sometimes burns mildly, tans gradually, IV=rarely burns, tans easily) (140). Women were asked to estimate the numbers of hours spent outdoors on weekdays and weekends, in summer and winter, respectively. Weekdays correspond to working days and weekends to non-working days. Summer was defined as May-October and Winter as November-April. This was based on the cutaneous synthesis of vitamin D

being possible between April and September in Gothenburg (latitude 57°North) (57).

### 3.3.5 Dietary intake of vitamin D and calcium

Dietary intake of vitamin D and calcium was estimated using four-day food diaries. Women were asked to register all food and drinks consumed during four consecutive days, including at least one non-working day, as precisely as possible, preferably starting no later than one week after the study visit. Both oral and written information on how to fill in the food diaries was given. Women were asked to register the amount of food consumed, using household measurements, kitchen scale or using photographs of different portion sizes using the Swedish portion guide "Matmallen" (141). Women were asked not to change their diet during the food registration. Food diaries were directly checked after completion and if any ambiguities were noted in the food diaries, the women were promptly contacted. Dietary intake was calculated using DietistXP, version 3.1 (The National Food Agency food database, Kost- och näringsdata, Bromma, version 2009-11-10). To study frequency and quantity of intake of vitamin D-rich food items, such as fatty fish and dairy products, a short complementary FFQ (food frequency questionnaire) was also used. Details of use, frequency, amounts and brand of dietary supplements were also collected.

### 3.3.6 Physical activity level

Physical activity level (PAL) was estimated through a VAS-scale (visual analog scale) where each woman rated her physical activity between 1 and 10. One indicated a sedentary life style, 5 a few long walks every week and 10 exercise several times per week. The number was converted to PAL where 1 corresponded to PAL 1.3 and 10 to PAL 2.2. Each step between them corresponded to a 0.1 increase. In a validation study by Löf et al., PAL assessed using this scale was correlated ($r=0.54$, $P=0.008$) with corresponding estimates obtained using criterion methods (i.e., the doubly labeled water method with indirect caliometry) in 22 healthy pregnant Swedish women (M Löf, personal communication). In *Paper I*, the individual self-estimated PAL was used for validating energy intake from the four-day food diary registrations and thus for identifying possible under reporters according to Goldberg et al. (142) and Black (143).

## 3.4 Statistical analyses

In all papers, a two-tailed p-value <0.05 was considered statistically significant. All statistical analyses except for *Paper III* were conducted with SPSS Statistics software (versions 19.0, 21.0, 22.0; IBM, Somers , NY). In *Paper III*, the mixed-procedure repeated measure ANOVA analyses were conducted with SAS for Windows (version 9.2; SAS Institute Inc). All values are presented as mean±SD, if not otherwise specified. Data that were not normally distributed were log-transformed before being analyzed.

In *Paper I*, independent sample t-tests and ANOVA were used to evaluate the difference in the mean concentration of 25OHD depending on lifestyle and other factors, such as parity, estimates of sun exposure, estimates of vitamin D intake and PTH. Possible determinants for serum concentrations of 25OHD were analyzed using univariable regression analyses. The variables found to be significant in the univariable regression analyses were included in the multivariable regression analysis. The effects of interactions between factors on 25OHD concentrations were modelled by the inclusion of combinations of sun exposure estimates and vitamin D intake estimates.

In *Paper II*, differences in means of serum 25OHD at baseline and at 12 months postpartum were analyzed with paired sample t-test. Individual changes in serum 25OHD between baseline and 12 months postpartum were calculated as the value at 12 months postpartum minus the value at baseline. Possible determinants for the changes in serum 25OHD between baseline and 12 months postpartum were analyzed with univariable linear regression. Paired sample t-test was used to evaluate and visualize differences in means of serum 25OHD at baseline and 12 months postpartum depending on each one of the significant determinants in the univariable regression model. Different durations of lactation were analyzed in a similar way, since lactation was the main determinant to be investigated.

Duration of lactation and the variables found to be significant in the univariable linear regression analysis were entered into multivariable regression analyses. Due to significant colinarity between season at baseline and serum 25OHD at baseline, only season was used in the multivariable regression analysis, because season was assumed to be the underlying predictor. Further, since season is known to be one of the major determinants of serum 25OHD (57), interactions were analyzed between season and each of the determinants found to be significant in the univariable regression analyses.

In *Paper III*, comparisons between postpartum women and controls at baseline were made using independent sample t-test. Data in this paper are presented as mean±SE, if not otherwise described. Because the data from the DXA and HR-pQCT were not normally distributed, they were log transformed before analyzed. The geometric mean (quartile 1-quartile 3) was used when presenting the means for postpartum women and controls at each time point. Repeated measures were analyzed by linear longitudinal models to study both between and within group differences over time. We assumed fixed effects, i.e., that the model holds true across the sample, and with the same intercept and slope, we could estimate the outcome. Unstructured covariance structure of our longitudinal data was used. The longitudinal analysis yielded estimates of least square means with SE and P-values. The repeated measures ANOVA from mixed procedure yielded the differences of these least square means and whether these differences were statistically significant. Longitudinal analyses were adjusted for body weight, PAL, age, and use of hormonal contraceptives when significant in the models. Percentage changes in aBMD, vBMD, and microstructural and dimensional parameters were calculated by the difference in log-transformed data between two time points and multiplied by 100. This approximates the percentage change as shown by Cole (144). The data from the lactation category 0-3.9 months at 18 months postpartum was not used in the longitudinal analyses, due to the fact that at that point in time only two women constituted that group.

In *Paper IV*, univariable linear regression analyses were first used to evaluate the relationships between the percentage change in each of the investigated bone parameters (lumbar spine aBMD, cortical vBMD, cortical thickness and trabecular thickness) at four, 12 and 18 months postpartum respectively compared to baseline, and possible determinants. The variables found to be significant in the univariable linear regression analyses were entered into a multivariable regression analysis also adjusted for lactation, body weight at baseline and change in body weight at the time of measurement minus baseline. At four months, lactation was coded as full lactation (yes=1) or not (no=0). At 12 and 18 months, respectively, duration of full lactation (months) and duration of total lactation (months) were used.

Since bone variables were not normally distributed, these variables were log-transformed before further analysis, as described for *Paper III*. Because of significant colinearity between calcium intake at four months postpartum and postpartum changes in calcium intake, as well as between baseline serum 25OHD and postpartum changes in serum 25OHD, only the calcium intake at

four months postpartum and the baseline serum 25OHD were included in the analyses.

In both *Paper II* and *IV*, dietary intake of vitamin D/calcium, use of hormonal contraceptives containing estrogen, PAL and sun preference (*Paper II*) at four months postpartum instead of baseline were included in the regression analyses, since this time point was regarded to more accurately reflect the actual situation during the first four months postpartum than do the values at baseline.

# 4 RESULTS

## 4.1 Descriptive characteristics

Descriptive characteristics of the participating women are shown in **Table 2**.

*Table 2. Characteristics of the women at the first study visit[a], included in Papers I-IV. Median (Q1-Q3) shown.*

| Characteristics | Pregnant women (n=95) | Controls (n=21) |
|---|---|---|
| Age (years) | 32.0 (30.0-35.3) | 33.6 (27.6-36.8) |
| Height (cm) | 168.5 (164.8-173.0) | 168.0 (163.5-171.9) |
| Body weight before pregnancy (kg) | 63.0 (59.0-69.0) | 63.0 (55.3-68.0) |
| Gestational weight gain (kg)[b] | 12 (10.0-15.3) | |
| Postpartum body weight reduction (kg)[c] | -5.1 (-7.2- -2.8) | |
| BMI before pregnancy (kg/m$^2$) | 22.2 (20.7-23.7) | 21.9 (20.7-24.6) |
| Child's birth weight (g) | 3505 (3208-3953) | |
| Gender of the baby (% girls) | 47 | |
| Parity | 0.7 (0-1) | 0.0 (0-2) |
| PAL[d] | 1.6 (1.5-1.7) | 1.8 (1.7-2.0) |
| Smoking (%) | 0 | 17 |
| 3 years of university education (%) | 80 | 71 |

[a]The first study visit took place in gestational week 35-37 for the pregnant women and at baseline for controls.
[b]Body weight gain at first study visit in gestational week 35-37.
[c]Body weight reduction was calculated as body weight at baseline minus 12 months postpartum, n=79 pregnant women, 19 controls.
[d]PAL; Physical acitivity level.

Information about breastfeeding habits, vitamin D intake, calcium intake and sun exposure among the postpartum women are specified below.

### 4.1.1 Breastfeeding habits

Median duration (Q1-Q3) of full lactation was 5.0 (3.0-6.1) months and median duration of total lactation was 8.1 (6.8-10.4) months. Percentages of women lactating at the different study visits postpartum are shown in **Table 3**. Median duration (Q1-Q3) of full and total lactation among women who were fully lactating at four months postpartum was 6.0 (5.0-6.1) months and 8.9 (8.0-11.6) months, respectively. Corresponding figures of full and total lactation for women who were not fully lactating at four months postpartum were 1.0 (0.0-3.0) months and 5.5 (3.4-7.3) months, respectively. Only one

woman did not breastfeed at all and one woman was breastfeeding for longer than 18 months.

*Table 3. Percentages of women who were fully lactating and who were lactating to some extent at the different study visits.*

| | Duration of lactation | |
|---|---|---|
| | **Full lactation** | **Total lactation** |
| 2 weeks postpartum | 91% | 99% |
| 4 months postpartum | 69% | 87% |
| 12 months postpartum | 0% | 17% |
| 18 months postpartum | 0% | 2% |

Full lactation; ≥90% of the child's energy intake came from lactation.
Total lactation; lactation to some extent.

Postpartum women were further categorized according to duration of total lactation: 0-3.9 months, 4-8.9 months and ≥9 months. Median duration of lactation within each lactation category is shown in **Table 4**.

*Table 4. Median duration (quartile 1-quartile 3) of full and total lactation within the different lactation categories.*

| **Lactation category**[a] | **Full lactation (months)** | **Total lactation (months)** |
|---|---|---|
| All women | 5.0 (3.0-6.1) | 8.1 (6.8-10.4) |
| 0-3.9 months postpartum | 0.5 (0.0-1.5) | 2.7 (1.0-3.7) |
| 4-8.9 months postpartum | 4.6 (3.5-6.0) | 7.6 (6.8-8.2) |
| ≥9 months postpartum | 6.0 (5.0-6.8) | 11.9 (10.1-12.6) |

[a]Duration of total lactation.

The lactation category 0-3.9 months captures those women who were both fully and totally breastfeeding for a short period of time. For the women with extended lactation (lactation category ≥9 months), median duration of full and total lactation was longer than the median duration of full and total lactation for the whole group.

### 4.1.2 Vitamin D intake

Mean dietary intake of vitamin D in the third trimester of pregnancy was 6.1±3.1 µg/day and mean total intake of vitamin D (from diet and supplements) was 9.3±4.9 µg/day. More than half of the women (56%) were using supplements containing vitamin D in the third trimester. Mean dietary intake of vitamin D at four months postpartum was 6.7±4.1 µg/day and at 12

months postpartum 6.0±3.1 µg/day. Mean total intake of vitamin D was 8.1±5.1 µg/day at four months postpartum and 7.4±6.7 µg/day at 12 months postpartum. At four months postpartum, 31% (24/78) of the women were using vitamin D supplements, but at 12 months postpartum only 18% (14/78) of the women were using supplements.

### 4.1.3 Calcium intake

At four months postpartum, mean dietary calcium intake was 1110±410 mg/day and mean total calcium intake (including both diet and supplements) was 1180±420 mg/day. Thirty-three percent of the women were using supplements containing calcium at four months postpartum. Among those using calcium supplements at four months postpartum, mean intake of calcium from supplements was 230±230 mg/day. Mean dietary calcium intake at 12 months postpartum was 970±370 mg/day, and 940±340 mg/day at 18 months postpartum. Mean total intake of calcium at 12 months postpartum was 1000±370 mg/day and at 18 months 970±350 mg/day. The percentages of women using calcium supplements at 12 and 18 months postpartum were 17% and 21%, respectively.

### 4.1.4 Sun exposure

In the third trimester of pregnancy, 23% of the women preferred to stay in the sun when outdoors during summer and 77% preferred to stay in the shade or a combination of sun and shade. At four months postpartum, the number of women who preferred to stay in the sun had decreased to 12% and at 12 months postpartum the number was 14%. In the third trimester of pregnancy, 23% of the women had travelled to southern latitudes during the last six months prior to the visit. At four months postpartum, only 1% of the women had travelled to southern latitudes during the last six months prior to the visit (this travel was performed during pregnancy) and at 12 months postpartum the number was 16%.

## 4.2 Vitamin D status and its determinants during pregnancy

In the third trimester of pregnancy, mean serum concentration of 25OHD among the 95 pregnant women was 47±18 nmol/L (mean±SD), as assessed with CLIA (**Figure 7**). The median was 44 (Q1-Q3 35-64) nmol/L and the range was 10-93 nmol/L. Percentages of women having serum 25OHD <30 nmol/L, <50 nmol/L and <75 nmol in total, and during summer and winter respectively, are shown in **Table 5**.

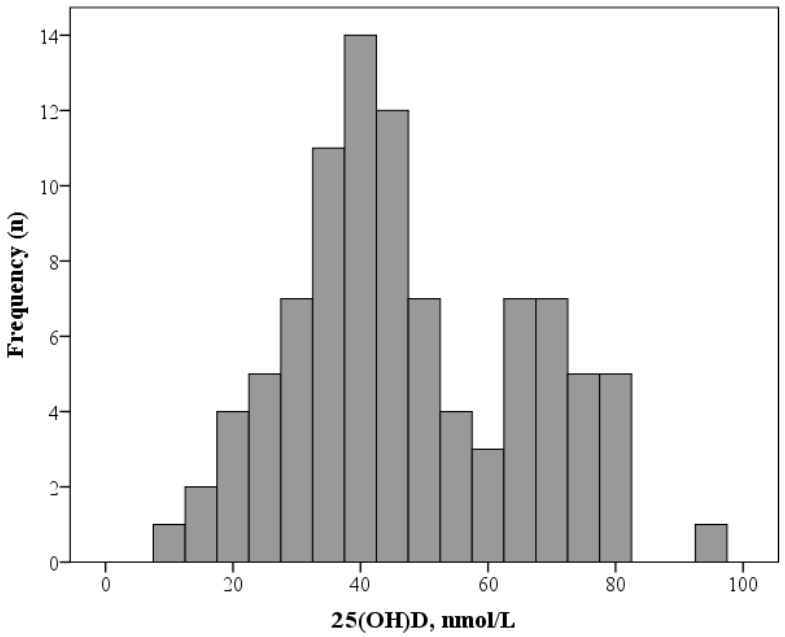


*Figure 7. Serum concentrations of 25-hydroxyvitamin D in the third trimester of pregnancy in 95 women.*

In the third trimester, serum 25OHD was highest between June-August, with highest mean in August (69 nmol/L). Lowest serum 25OHD was observed during winter and early spring with the lowest mean in April (33 nmol/L). Mean serum 25OHD was 53% higher in the summer compared to winter ($p<0.001$).

Serum 25OHD was 46% higher in women using vitamin D supplements compared to non-users ($p<0.001$), 21% higher in women who preferred to stay in the sun during the summer compared to women who preferred to stay in the shade or a mix of sun and shade ($p=0.03$), and 35% higher in women

*Table 5. Percentages of women pregnant in the third trimester having serum concentrations of 25-hydroxyvitamin D <30 nmol/L, <50 nmol/L and <75 nmol/L, respectively.*

| | Serum concentrations of 25OHD | | |
|---|---|---|---|
| | **<30 nmol/L** | **<50 nmol/L** | **<75 nmol/L** |
| All, n (%) | 16 (17) | 62 (65) | 87 (92) |
| Winter, n (%) | 27 (28) | 81 (85) | 100 (100) |
| Summer, n (%) | 2 (2) | 39 (41) | 78 (82) |

25OHD; 25-hydroxyvitamin D.

who had travelled to southern latitudes prior to the measurement compared to non-travelers (p=0.001). In addition, a positive trend was observed between use of sunscreen and serum 25OHD (p=0.07), as well as between use of sunscreen and time spent outdoors during the summer (p=0.13 for working days and p=0.031 for non-working days.

Mean serum concentration of PTH was 43.8±15.6 nmol/L. A significant inverse relation was found between serum 25OHD and serum PTH (p=0.008). Women with serum 25OHD <50 nmol/L had significantly higher serum PTH compared to women with serum 25OHD ≥50 nmol/L (p=0.005). Further, mean serum PTH was significantly higher during winter than during summer (p=0.005).

Main determinants for serum concentrations of 25OHD in the third trimester of pregnancy as analyzed with multivariable regression analyses were season, use of vitamin D supplements and travels to southern latitudes (**Table 6**). These factors could together explain 51% of the variation in serum 25OHD.

## 4.3 Changes in vitamin D status postpartum and their determinants

Mean serum 25OHD was 67±23 nmol/L at baseline and 67±19 nmol/L at 12 months postpartum among the 78 women included (**Figure 8**), as analyzed with LC-MS/MS. Mean change in serum 25OHD between baseline and 12 months postpartum was -0.2±15 nmol/L (mean±SD) and no significant change was observed in mean serum 25OHD during the first year postpartum (**Figure 9**). The range in change in serum 25OHD during the first year postpartum was -33 to + 38 nmol/L. At baseline, 1% of the women had serum 25OHD <30 nmol/L, 24% of the women had serum 25OHD <50 nmol/L and 65 had serum <75 nmol/L.

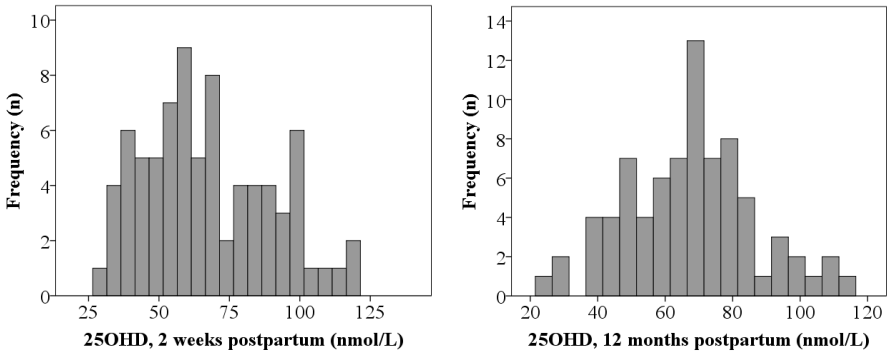


*Figure 8. Serum concentrations of 25-hydroxyvitamin D at two weeks and 12 months postpartum in the 78 women.*

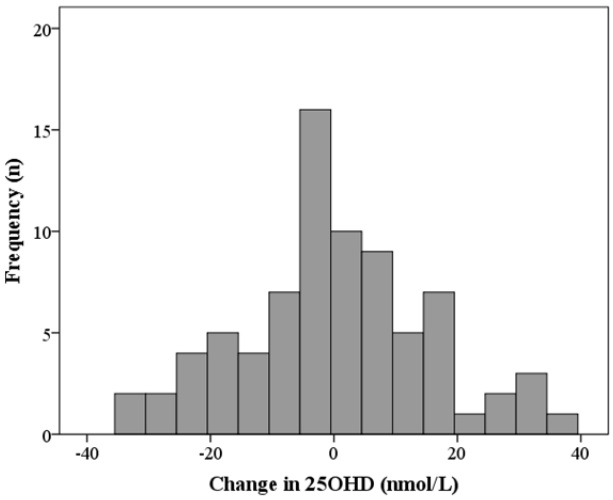


*Figure 9. Change in serum concentrations of 25-hydroxyvitamin D between baseline (2 weeks postpartum) and 12 months postpartum, as analyzed with paired sample t-test.*

Significant determinants for the change in serum concentrations of 25OHD between baseline and 12 months postpartum as analyzed with univariable regression analysis were travels to southern latitudes prior to baseline, use of estrogen contraceptives at four months postpartum, age, use of vitamin D supplements at baseline, serum 25OHD at baseline and season at baseline. No associations were found between duration of full or total lactation and change in serum 25OHD during the first year postpartum.

Paired sample t-test showed that mean serum 25OHD decreased significantly during the first year postpartum in women who had been travelling to southern latitudes prior to baseline (p=0.026), whereas no change in mean serum 25OHD was found in non-travelers. Mean serum 25OHD increased significantly during the first year postpartum in women who were using estrogen contraceptives at four months postpartum (p=0.023), whereas no change in mean serum 25OHD was found in women not using estrogen contraceptives. Mean serum 25OHD decreased significantly during the first year postpartum in women ≥33 years of age (p=0.025), whereas no significant change was observed in women <33 years of age. Mean serum 25OHD decreased significantly during the first year postpartum in women using vitamin D supplements at baseline (p=0.011), whereas no change in mean 25OHD was observed in women not using vitamin D supplements. During the same period, the percentage of women using vitamin D supplements decreased, from 37% at baseline to 18% at 12 months postpartum.

In women with baseline serum 25OHD <50 nmol/L, mean serum 25OHD increased significantly during the first year postpartum (p=0.002), whereas in women with baseline serum 25OHD ≥50 nmol/L, mean serum 25OHD decreased significantly during the same period of time (p=0.021). In women having their baseline measurement during summer, a non-significant decrease in mean serum 25OHD was observed (p=0.061) during the first year postpartum. However, in women having their baseline measurement during winter, a significant increase in mean serum 25OHD was observed during the same period of time (p=0.048). A significant correlation was observed between season at baseline and baseline serum 25OHD (r=0.356, p=0.0001).

In the multivariable regression analysis, use of estrogen contraceptives, use of vitamin D supplements at baseline and baseline serum 25OHD were significantly related to the changes in serum25OHD during the first year postpartum. These determinants explained 37% of the variation in the change in serum 25OHD during the first year postpartum (**Table 7**).

*Table 6. Univariable and multivariable linear regression of determinants of serum 25-hydroxyvitamin D in the third trimester of pregnancy.*

| **Variables** | **Univariable linear regression (n=95)** | | | | **Multivariable linear regression (n=95)** | | | |
|---|---|---|---|---|---|---|---|---|
| | **ß** | **SEM** | **P-value** | **$R^2$** | **ß** | **SEM** | **P-value** | **Adjusted $R^2$** |
| Season[a] | 19.40 | 3.19 | 0.000 | 0.29 | 16.25 | 2.76 | 0.000 | 0.51 |
| Use of vitamin D supplements[b] | 17.34 | 3.31 | 0.000 | 0.23 | 14.06 | 2.76 | 0.000 | |
| Travels to southern latitudes[c] | 15.57 | 4.61 | 0.001 | 0.11 | 10.17 | 3.55 | 0.005 | |
| Sun preference[d] | 9.50 | 4.34 | 0.031 | 0.005 | 4.26 | 3.23 | 0.190 | |

[a]1=winter (November-April), 2= summer (May-October)

[b]1=no, 2=yes

[c]Travels to latitude 35º North or below, during the last six months; 1=no, 2=yes

[d]1= shade or sun and shade, 2=sun

*Table 7. Univariable and multivariable linear regression of lactation and other determinants of changes in serum 25-hydroxyvitamin D between two weeks and 12 months postpartum.*

| **Variables** | **Univariable linear regression (n=78)** | | | | **Multivariable linear regression (n=78)** | | | |
|---|---|---|---|---|---|---|---|---|
| | **ß** | **SEM** | **P-value** | **$R^2$** | **ß** | **SEM** | **P-value** | **Adjusted $R^2$** |
| Total lactation (months) | -0.61 | 0.50 | 0.272 | 0.019 | 0.10 | 0.50 | 0.846 | 0.373 |
| Season[a] | -9.36 | 3.35 | 0.007 | 0.093 | -8.87 | 3.06 | 0.005 | |
| Use of estrogen contraceptives[b] | 25.79 | 7.36 | 0.001 | 0.139 | 18.30 | 7.21 | 0.013 | |
| Use of vitamin D supplements[c] | -10.63 | 3.41 | 0.003 | 0.113 | -9.44 | 3.06 | 0.003 | |
| Travels to southern latitudes[d] | -10.75 | 4.87 | 0.030 | 0.060 | -6.27 | 4.20 | 0.140 | |
| Age (years) | -1.33 | 0.49 | 0.008 | 0.088 | -0.80 | 0.47 | 0.090 | |

[a]1=winter (November-April), 2= summer (May-October)

[b]At four months postpartum; 1=no, 2=yes

[c]At baseline; 1=no, 2=yes

[d] Travels to latitude 35º North or below, during the last six months prior to baseline; 1=no, 2=yes

# 4.4 Changes in bone parameters postpartum and its determinants

## 4.4.1 Changes in bone parameters postpartum

Changes in bone parameters during the first 18 months postpartum were evaluated for postpartum women and for controls. Results for the whole group of postpartum women and controls respectively are shown in **Table 12** and **13** (Appendix).

Number of women participating within each lactation category at the different study visits are shown in **Figure 10**.

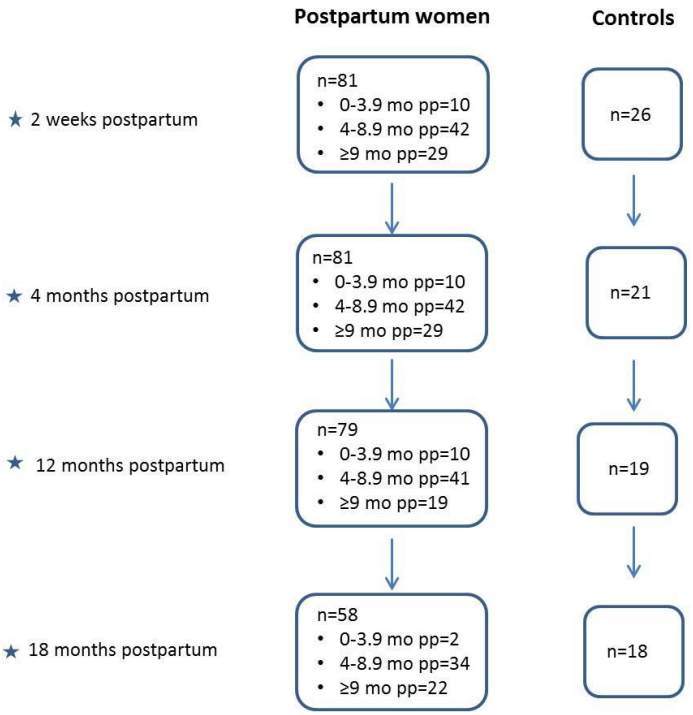


*Figure 10. Number of women participating at the different study visits depending on duration of total lactation (0-3.9 months, 4-8.9 months or ≥9 months). Mo pp; months postpartum.*

Results from the DXA-measurements showed that during the first *four months postpartum*, aBMD decreased significantly in the range -0.73±0.21% to -3.98±0.76% (mean±SE) only in women lactating 4-8.9 months and ≥9 months at the following skeletal sites: lumbar spine, femoral neck, femoral shaft, femoral trochanter, total femur and whole body **(Figure 11**). No changes were observed in women lactating less than four months or in controls during the first four months postpartum.

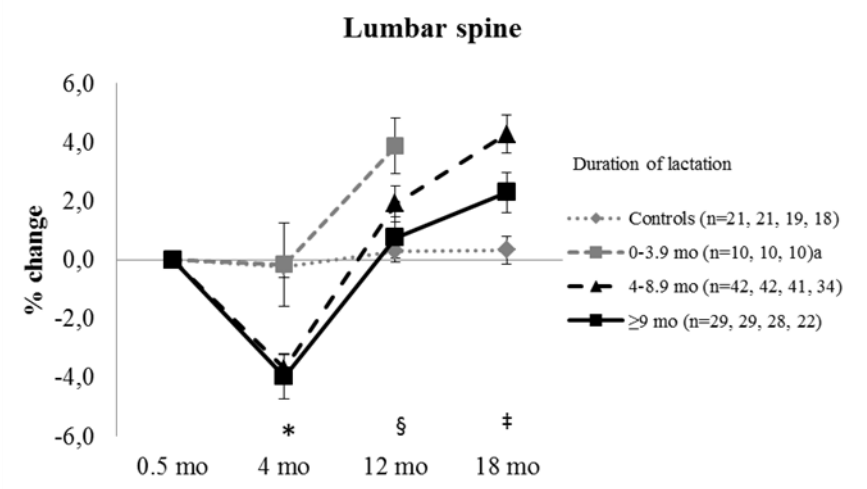


*Figure 11. Mean percentage change from baseline ± SE in lumbar spine areal bone mineral density, as assessed with dual-energy X-ray absorptiometry, in relation to duration of total lactation. Mo; months postpartum. *Significant change compared to baseline for lactation groups 4-8.9 months and ≥9 months, § Significant change compared to baseline for lactation group 4-8.9 months, ‡ Significant change compared to baseline for lactation groups 4-8.9 months and ≥9 months. a; Because of a small number of women at 18 months postpartum in lactation group 0-3.9 months, this measurement was excluded from all analyses.*

At *12 months postpartum*, femoral neck, femoral shaft and total femur were still significantly lower compared to baseline (range -1.05±0.44% to -4.00±0.69%) in women lactating 4-8.9 months and ≥9 months. In addition, for women lactating 4-8.9 months, aBMD at lumbar spine was significantly higher compared to baseline (1.91±0.61%). No decreases in aBMD were found in women lactating less than four months or in controls during this period. Instead, radius ultradistal, lumbar spine and femoral shaft was higher compared to baseline in women with short duration of lactation.

At *18 months postpartum*, aBMD at the lumbar spine was significantly higher than baseline in women lactating 4-8.9 and ≥9 months, as well as the femoral trochanter and radius ultradistal in women lactating 4-8.9 months. No significant decreases compared to baseline were found in any lactation group or in controls at 18 months postpartum.

Results from the HR-pQCT measurements showed that during the first *four months postpartum*, cortical vBMD and cortical area at the ultradistal tibia decreased significantly in the range -0.26±0.08% to -1.54±0.33% only in women lactating 4-8.9 months and ≥9 months (**Figure 12**). In women lactating 4-8.9 months, significant decreases compared to baseline were also evident for cortical thickness (-2.46±1.63%). No changes were observed among women with short duration of lactation. In controls, significant changes compared to baseline were found only in trabecular vBMD and trabecular bone volume fraction (-1.03±0.35% and -1.12±0.37%, respectively) during this period.

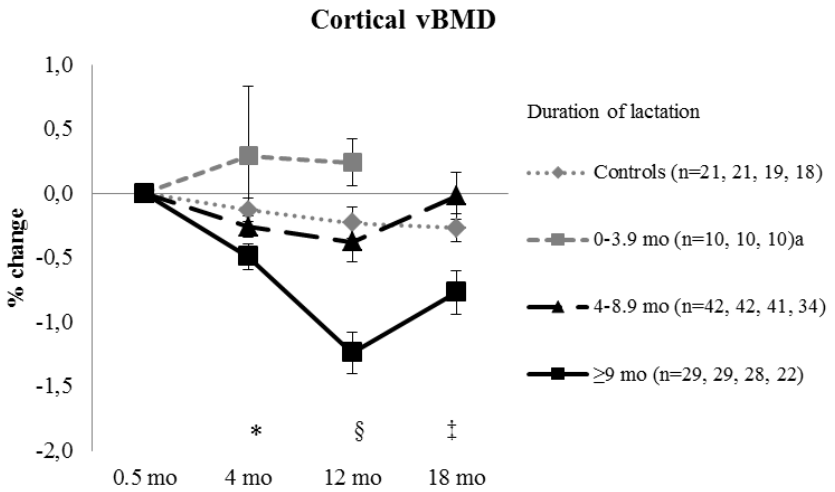


*Figure 12. Mean percentage change from baseline ± SE in cortical volumetric bone mineral density at the ultradistal tibia, as assessed with high-resolution peripheral quantitative computed tomography, in relation to duration of total lactation. Mo; months postpartum. *Significant change compared to baseline for lactation groups 4-8.9 months and ≥9 months, § Significant change compared to baseline for lactation groups 4-8.9 months and ≥9 months, ‡ Significant change compared to baseline for lactation group ≥9 months. a; Because of a small number of women at 18 months postpartum in lactation group 0-3.9 months, this measurement was excluded from all analyses.*

At *12 months postpartum*, cortical vBMD and trabecular thickness were significantly lower compared to baseline in women lactating 4-8.9 and ≥9 months (range 0.38±0.15% to -2.56±1.21%). In addition, in women lactating ≥9 months, significant decreases compared to baseline were evident for cortical thickness, cortical area, trabecular vBMD and trabecular bone volume fraction (range -2.48±0.41% to -2.60±0.70%) (**Figure 13**). No changes were found in women with short duration of lactation or in controls during this period.

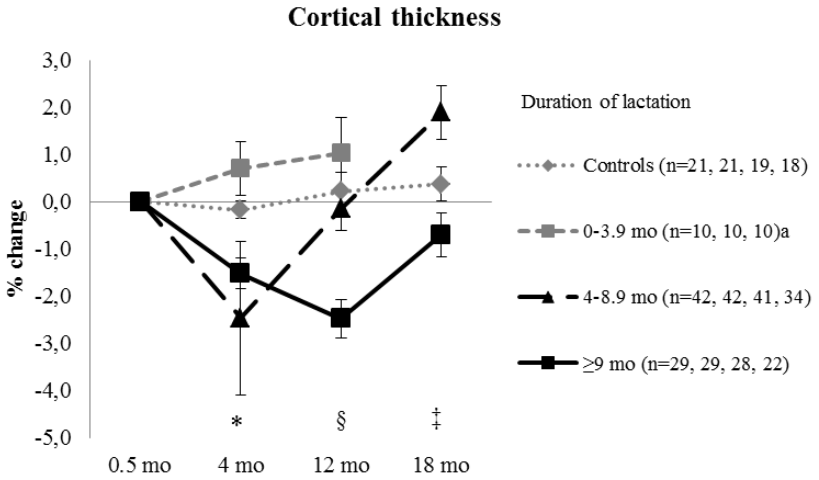


*Figure 13. Mean percentage change from baseline ± SE in cortical thickness at the ultradistal tibia, as assessed with high-resolution peripheral quantitative computed tomography, in relation to duration of total lactation. Mo; months postpartum. *Significant change compared to baseline for lactation group 4-8.9 months, § Significant change compared to baseline for lactation group ≥9 months, ‡ Significant change compared to baseline for lactation group 4-8.9 months. a; Because of a small number of women at 18 months postpartum in lactation group 0-3.9 months, this measurement was excluded from all analyses.*

At *18 months postpartum*, cortical vBMD was still significantly lower compared to baseline (-0.77±0.17%), but only for women with longest duration of lactation (≥9 months). Trabecular thickness was still significantly lower compared to baseline for women lactating both 4-8.9 and ≥9 months (-2.25±1.25% and -3.21±1.41%, respectively) (**Figure 14**). In women lactating 4-8.9 months, cortical thickness and cortical area were instead significantly higher compared to baseline (+1.90±0.57% and +1.99±0.57%, respectively).

In controls, significant changes compared to baseline were found only in trabecular vBMD and trabecular bone volume fraction (-2.38±0.81% and -2.45±0.83%, respectively) during this period.

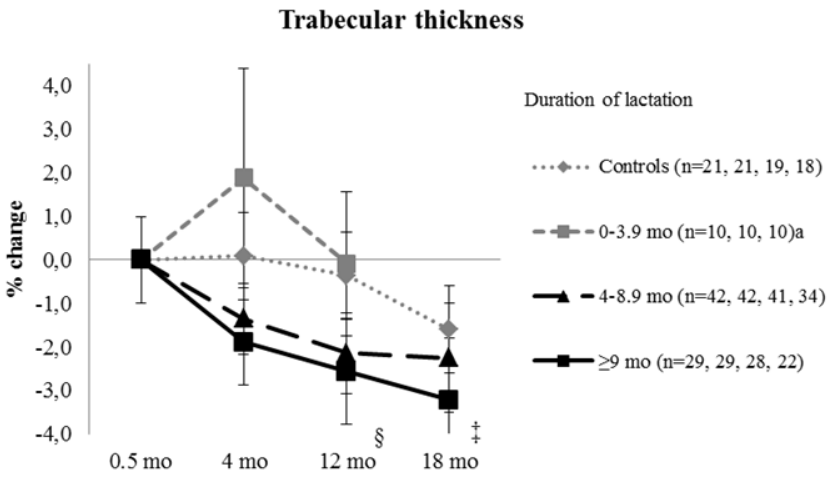


*Figure 14. Mean percentage change from baseline ± SE in trabecular thickness at the ultradistal tibia, as assessed with high-resolution peripheral quantitative computed tomography, in relation to duration of total lactation. Mo; months postpartum. § Significant change compared to baseline for lactation groups 4-8.9 months and ≥9 months, ‡ Significant change compared to baseline for lactation groups 4-8.9 months and ≥9 months. a; Because of a small number of women at 18 months postpartum in lactation group 0-3.9 months, this measurement was excluded from all analyses.*

### 4.4.2 Determinants of changes in bone parameters postpartum

As shown above, significant changes postpartum were observed in lumbar spine aBMD and cortical vBMD, cortical thickness and trabecular thickness at the ultradistal tibia. Thus, determinants for these changes were subsequently analyzed. Specifically, calcium intake and 25OHD concentrations were evaluated as possible determinants for the postpartum changes in bone parameters. Further, baseline body weight, postpartum body weight change, height, age, parity, lactation, PAL, use of estrogen

contraceptives and baseline bone value were analyzed as possible determinants for the changes in bone parameters postpartum.

## Lumbar spine

During the first *four months postpartum*, multivariable linear regression showed that baseline serum 25OHD (ß=-0.073±0.018, p=0.000), lactation at four months postpartum (no/yes) (ß=-1.997±0.835, p=0.021) and baseline aBMD (ß=-8.498±3.499, p=0.018) were associated with the changes in lumbar spine during the first four months postpartum (**Table 8**). At *12 months postpartum*, duration of full lactation (ß=-0.558±0.167, p=0.001) and baseline aBMD (ß=-17.452±3.386, p=0.000) were still associated with the changes in lumbar spine compared to baseline, whereas at *18 months postpartum*, only baseline aBMD (ß=-15.066±3.800, p=0.000) was associated with the changes in lumbar spine compared to baseline.

*Table 8. Significant determinants from the univarible regression analysis for percentage change compared to baseline, in lumbar spine aBMD postpartum.*

| **Determi-nants** | **Univariable linear regression** | | | **Multivariable linear regression** | | |
|---|---|---|---|---|---|---|
| | **4 mo pp** | **12 mo pp** | **18 mo pp** | **4 mo pp** | **12 mo pp** | **18 mo pp** |
| **N** | 81 | 79 | 58 | 81 | 79 | 58 |
| Lactation[a] | -1.944 ±0.942* | -0.587 ±0.192** | -0.138 ±0.185 | -1.997 ±0.835* | -0.558 ±0.167** | -0.185 ±0.176 |
| 25OHD (nmol/L)[b] | -0.071 ±0.017*** | -0.033 ±0.020 | -0.021 ±0.021 | -0.073 ±0.018*** | | |
| PAL[c] | -5.638 ±2.627* | -2.658 ±2.724 | -0.288 ±3.008 | -3.287 ±2.381 | | |
| Estrogen contracep-tives[d] | +4.554 ±1.999* | +2.032 ±2.069 | -0.748 ±3.678 | +2.568 ±2.042 | | |
| Baseline lumbar spine aBMD ($g/cm^2$) | -9.239 ±3.836* | -17.201 ±3.585*** | -14.266 ±3.786*** | -8.498 ±3.499* | -17.452 ±3.386*** | -15.066 ±3.800*** |

Baseline; two weeks postpartum
aBMD; areal bone mineral density
*p<0.05, **p<0.01, ***p<0.001
[a]Lactation variables used were at four months postpartum if the women were fully lactating (0=no, 1=yes), at 12 months postpartum duration of full lactation (months) and at 18 months postpartum duration of total lactation (months)
[b]At baseline
[c]PAL; physical activity level, at four months postpartum
[d]Use of estrogen contraceptives at four months postpartum; 0=no, 1=yes

## Cortical vBMD

During the first *four months postpartum*, multivariable linear regression showed that use of estrogen contraceptives at four months postpartum (ß=+0.596±0.287, p=0.041) and baseline body weight (ß=+0.020±0.007, p=0.006) were associated with the changes in cortical vBMD at the ultradistal tibia compared to baseline (**Table 9**). At *12 months postpartum*, baseline body weight (ß=+0.035±0.012, p=0.005) was still associated with the changes in cortical vBMD compared to baseline, and so was duration of full lactation (ß=-0.127±0.050, p=0.014). Also at *18 months postpartum*, baseline body weight (ß=+0.035±0.14, p=0.016) was related to the changes in cortical vBMD during the first 18 months postpartum. At 18 months postpartum, also calcium intake at four months postpartum (ß=+0.708±0.298, p=0.021) was related to the changes in cortical vBMD during the first 18 months postpartum.

*Table 9. Significant determinants from the univarible regression analysis for percentage change compared to baseline, in cortical vBMD at the ultradistal tibia postpartum.*

| **Determinants** | **Univariable linear regression** | | | **Multivariable linear regression** | | |
|---|---|---|---|---|---|---|
| | **4 mo pp** | **12 mo pp** | **18 mo pp** | **4 mo pp** | **12 mo pp** | **18 mo pp** |
| **N** | 81 | 79 | 58 | 81 | 79 | 58 |
| Body weight (kg)[a] | +0.021 ±0.006** | +0.032 ±0.012** | +0.038 ±0.013** | +0.020 ±0.007** | +0.035 ±0.012** | +0.035 ±0.014* |
| Lactation[b] | -0.233 ±0.142 | -0.151 ±0.049** | -0.177 ±0.053* | -0.168 ±0.136 | -0.127 ±0.050* | -0.080 ±0.050 |
| Ca intake (g/day)[c] | +0.154 ±0.154 | +0.371 ±0.281 | +0.887 ±0.307** | | | +0.708 ±0.298* |
| Estrogen contraceptives[d] | +0.752 ±0.285* | +1.124 ±0.510* | -0.467 ±1.024 | +0.596 ±0.287* | +0.677 ±0.520 | |

Baseline; two weeks postpartum
vBMD; volumetric bone mineral density
*p<0.05, **p<0.01, ***p<0.001
[a]At baseline
[b]Lactation variables used were at four months postpartum if the women were fully lactating (0=no, 1=yes), at 12 months postpartum duration of full lactation (months) and at 18 months postpartum duration of total lactation (months)
[c]Total calcium intake (from diet and supplements) at four months postpartum
[d]Use of estrogen contraceptives at four months postpartum; 0=no, 1=yes

## Cortical thickness

No significant associations were found between any of the investigated variables and changes in cortical thickness at the ultradistal tibia during the first *four months postpartum*. At *12 months postpartum*, duration of full lactation (ß=-0.387±0.133, p=0.005) and baseline body weight (ß=+0.120±0.034, p=0.001) were related to the changes in cortical thickness compared to baseline **(Table 10**). At *18 months postpartum*, baseline body weight (ß=+0.132±0.043, p=0.001) was still related to the changes in cortical thickness during the first 18 months postpartum. At 18 months postpartum, also calcium intake at four months postpartum (ß=+1.990±0.915, p=0.034) was related to the changes in cortical thickness during the first 18 months postpartum.

*Table 10. Significant determinants from the univarible regression analysis for percentage change compared to baseline, in cortical thickness at the ultradistal tibia postpartum.*

| **Determinants** | **Univariable linear regression** | | | **Multivariable linear regression** | | |
|---|---|---|---|---|---|---|
| | **4 mo pp** | **12 mo pp** | **18 mo pp** | **4 mo pp** | **12 mo pp** | **18 mo pp** |
| **N** | 81 | 79 | 58 | 81 | 79 | 58 |
| Body weight (kg)[a] | +0.085 0.090 | +0.104 ±0.033** | +0.123 ±0.041** | +0.074 ±0.103 | +0.120 ±0.034** | +0.132 ±0.043** |
| Lactation[b] | -2.132 ±1.910 | -0.400 ±0.141** | -0.409 ±0.164* | -2.175 ±1.925 | -0.387 ±0.133** | -0.279 ±0.154 |
| Ca intake (g/day)[c] | +0.861 ±2.072 | +1.141 ±0.786 | +2.634 ±0.968** | | | +1.990 ±0.915* |

Baseline; two weeks postpartum
*p<0.05, **p<0.01, ***p<0.001
[a]At baseline
[b]Lactation variables used were at four months postpartum if the women were fully lactating (0=no, 1=yes), at 12 months postpartum duration of full lactation (months) and at 18 months postpartum duration of total lactation (months)
[c]Total calcium intake (from diet and supplements) at four months postpartum

## Trabecular thickness

During the first *four months postpartum*, no significant change in trabecular thickness compared to baseline was observed. A relation between baseline trabecular thickness (ß=-8.882±3.566, p=0.015) and changes in trabecular thickness was found during this period (**Table 11**). At *12 months postpartum*, duration of full lactation (ß=-0.579±0.286, p=0.046) and baseline trabecular thickness (ß=-10.289±3.712, p=0.007) were related to the changes in trabecular thickness compared to baseline. At *18 months postpartum*, only

baseline body weight (ß=+0.271±0.107, p=0.014) was related to the changes in trabecular thickness during the first 18 months postpartum.

*Table 11. Significant determinants from the univarible regression analysis for percentage change compared to baseline, in trabecular thickness at the ultradistal tibia postpartum.*

| **Determinants** | **Univariable linear regression** | | | **Multivariable linear regression** | | |
|---|---|---|---|---|---|---|
| | **4 mo pp** | **12 mo pp** | **18 mo pp** | **4 mo pp** | **12 mo pp** | **18 mo pp** |
| **N** | 81 | 79 | 58 | 81 | 79 | 58 |
| Body weight (kg)[a] | +0.115 ±0.065 | +0.123 ±0.070 | +0.281 ±0.092** | +0.056 ±0.075 | +0.073 ±0.075 | +0.271 ±0.107* |
| Lactation[b] | -1.542 ±1.390 | -0.594 ±0.297* | -0.280 ±0.390 | -1.403 ±1.341 | -0.579 ±0.286* | -0.199 ±0.363 |
| Baseline trabecular thickness (mm) | -9.888 ±3.777** | -11.353 ±3.563** | -11.321 ±4.833* | -8.882 ±3.566* | -10.289 ±3.712** | -7.787 ±4.865 |

Baseline; two weeks postpartum
*p<0.05, **p<0.01, ***p<0.001
[a]At baseline
[b]Lactation variables used was at four months postpartum if the women were fully lactating (0=no, 1=yes), at 12 months postpartum duration of full lactation (months) and at 18 months postpartum duration of total lactation (months)

# 5 DISCUSSION

## 5.1 Study population

The study population may not be fully representative for pregnant and postpartum women in the general population in Sweden in that it was rather homogenous and with socio-demographic characteristics that may be expected from a group of self-selected study participants. Eighty percent of the postpartum women and 71% of the controls had studied for at least three years at the university level, which is higher than the corresponding number of 37% among women in the same age group in the general population (145). The mean self-reported pre-pregnancy body weight among postpartum women of 64.1 kg and mean baseline body weight among controls of 63.4 kg were both lower than the corresponding number in the general population (67.0 kg) (146). None of the postpartum women in the study population were smoking at two weeks postpartum, compared to 4.6% of the mothers of infants in the age 0-4 weeks in the general population (147). In sum, the women in this study were leaner and more highly educated than women in the general population, which has to be kept in mind when interpreting the results. Possibly, they were also more health conscious, since none of the women in the study smoked and also since the women had actively chosen to participate in a longitudinal study on health. Due to the relatively small study population, sub-groups became very small, e.g., women with short duration of lactation at 18 months postpartum and women using estrogen contraceptives, and so any conclusions drawn regarding these groups need to be handled with care.

Further, a higher number of the women in this study were breastfeeding during the first six months postpartum compared to women in the general population. At two weeks postpartum, 99% of the study women were breastfeeding to at least some extent, and at four months postpartum, 87% of the women were breastfeeding to some extent. Corresponding numbers in the general population are 96% at one week postpartum and 75% at four months postpartum (147). Median (Q1-Q3) duration of breastfeeding to some extent in our study was 8.1 (6.8-10.4) months. However, the number of women who were breastfeeding to some extent at nine months postpartum was the same as in the general population (36%), and at 12 months postpartum almost the same (17% in this study versus 18% in the general population) (147). This implicates that a higher number of the women in this study may initiate breastfeeding compared to national numbers, but the number of women with

extended lactation appear to be similar to the national population. Previous studies have found that women with higher education breastfeed for longer periods than do women with lower education (148). Also, the women in this study were older than women in childbirth in the general population (32.9 years vs 30.8 years) and earlier studies have found that maternal age is positively associated with breastfeeding duration (149). In sum, the higher number of women who were breastfeeding during the first six months postpartum in this study compared to the general population might in part be explained by their higher age and their high education. Still, more than every third woman in the same age group in the general population has studied for at least three years at the university (145), making the results comparable for a considerable percentage of women in Sweden.

Mean dietary vitamin D intake in our study during pregnancy was 6.1±3.1 µg/day and at 12 months postpartum 6.0±3.1 µg/day. This is comparable with national data of 6.2 µg/day among non-pregnant and non-lactating women in the same age group (74). Still, it's lower than the recommended daily intake by NNR of 10 µg/day and the average requirement in NNR of 7.5 µg/day (1). The recommended intake of IOM, 15 µg/day, is even higher (25). However, when adding vitamin D supplements to the dietary intake, mean total intake of vitamin D in our study was 9.3±4.9 µg/day in the third trimester of pregnancy and 7.4±6.7 µg/day at 12 months postpartum. In the third trimester of pregnancy, 56% of the women were using supplements containing vitamin D, while only 18% were using supplements containing vitamin D at 12 months postpartum. Still, these values are higher than comparable national data, however among non-pregnant and non-lactating women. The national Swedish survey Riksmaten reported that 27% of the women were using supplements, and of these, 29% were using supplements containing multivitamins, vitamin D or calcium and vitamin D (74).

Mean dietary calcium intake at four months postpartum in our study was 1110±410 mg/day, at 12 months postpartum 970±370 mg/day and at 18 months postpartum 940±340 mg/day, which can be compared to national data among non-pregnant and non-lactating women in the same age group of 849±306 mg/day (74). It can also be compared to the recommended daily intake by NNR of 800 mg/day among adults and of 900 mg/day among pregnant and lactation women (1). At four months postpartum, 33% of the women were using supplements containing calcium, at 12 months postpartum, 17% of the women were using supplements containing calcium and at 18 months, 21% of the women. As specified above, Riksmaten reported that 27% of the women were using supplements, and of these, 28% were using supplements containing multivitamins, calcium or calcium and

vitamin D (74). In sum, the calcium intake was higher among the postpartum women at four months postpartum compared to the national data of non-pregnant and non-lactating women. This is probably explained by the fact that at four months postpartum a majority of the women were still fully breastfeeding. The calcium intake decreased during the postpartum period and at 18 months postpartum, it approached the national data of calcium intake among non-pregnant and non-lactating women (74).

Finally, all women participating in this study were fair-skinned. Dark-skinned women are generally found to have lower 25OHD concentrations (57), and so mean serum 25OHD in the general population of pregnant women at northern latitudes may be even lower than the mean in the study group. Despite the high education level and the normal body weight, a majority of the women were vitamin D insufficient during pregnancy.

Thus, the descriptive results from this study may not represent those of pregnant and postpartum women in the general population in Sweden. However, there is no reason to believe that the associations between exposures and outcomes found in the studies presented here should differ between our study population and the general population of adult women who have reached peak bone mass and who have a calcium intake close to the recommendations. Though, there are some subgroups where the results may differ. Peak bone mass is reached in the late teenage years/early adult years (102). In women who become pregnant during the teenage years, previous studies have found that markers of calcium and bone metabolism during both pregnancy and lactation may differ from women who become pregnant as adults (150). This is why women below the age of 25 were not included in this study. We believe, however, that the results regarding the postpartum bone changes are representative for adult pregnant women who have reached peak bone mass.

We also believe that the results are representative for all pregnant/postpartum women with a medium or high calcium intake, i.e., a calcium intake that is rather close to existing guidelines. In women with a very low calcium intake the results may differ. A previous study among pregnant women in The Gambia with a low daily calcium intake of approximately 350 mg found that supplementation with 1500 mg calcium/day during pregnancy were actually associated with lower BMC, BA and BMD at the hip throughout the subsequent 12 month lactation, compared to women with a lower calcium intake (151). One hypothesis is that the supplementation altered the mother's ability to adapt to a low calcium intake (151). However, in women with a medium or high calcium intake we believe that our findings are

representative. Studies of vitamin D intake during pregnancy and lactation and its relation to 25OHD concentrations are very few. Therefore, for the time being, we see no reason why our findings regarding the association between vitamin D intake and 25OHD concentrations during pregnancy and lactation should differ between our study population and the general population.

# 5.2 Methodology

## 5.2.1 25-hydroxyvitamin D measurements

Vitamin D status is regarded to be most accurately estimated by measuring the circulating 25OHD (152). Concentrations of 25OHD can by analyzed by different methods. In *Paper I*, 25OHD concentrations were analyzed with CLIA, since CLIA was at that time the standard method used for 25OHD analyses by the Sahlgrenska University Hospital in Gothenburg. The CLIA, and other immunoassays, are considered to give the lowest 25OHD concentrations among the methods (51). In *Papers II* and *IV*, 25OHD concentrations were analyzed with LC-MS/MS. At that point, the Sahlgrenska University Hospital no longer used the CLIA as the standard method for 25OHD analyses. The reason for choosing LC-MS/MS was partly that this was the same analysis method used by the Swedish national survey Riksmaten, which makes the numbers comparable, and because it is considered to have high validity. The MS, and different kinds of HPLC-methods, have been found to give the highest 25OHD concentrations (51, 53). In a study by Snellman et al., highest validity was observed for HPLC-APCI-MS, while lowest validity was observed for CLIA (51). The greatest inter-seasonal difference was also observed for HPLC-APCI-MS, which may be interpreted as it being a more accurate and reliable method than both CLIA and RIA (51). There is currently no golden standard for measuring 25OHD concentrations, but lately LC-MS/MS has been considered a candidate, since it can differentiate between and accurately quantitate both $25OHD_3$ and $25OHD_2$, and it potentially offer improved specificity (53, 54, 152). According to the results in the study by Snellman et al., 43% of the individuals were classified as vitamin D deficient by the CLIA assay if using the IOM cut-off (<50 nmol/L), while only 8% were classified as vitamin D deficient by the HPLC-APCI-MS assay (51). Similar results were found by Black et al. (52) Black et al. also observed that while the chemilumnescence assay gave lower 25OHD concentrations compared to a LC-MS/MS assay at a certified laboratory, a LC-MS/MS assay at a non-certified laboratory gave higher 25OHD concentrations compared to the certified laboratory (52). It is

important to keep this in mind when comparing results from different studies or when comparing results to the NNR and IOM guidelines.

This may also be a reason why we found a lower mean serum 25OHD and a higher proportion of women who were vitamin D deficient (<50 nmol/L) among the women when pregnant in *Paper I*, than during the postpartum period in *Papers II* and *IV*. Another explanation for our results might be that women actually have lower 25OHD concentrations in the third trimester of pregnancy than during the postpartum period, possibly due to the fact that 25OHD passes through the placenta to the fetus. The cord blood concentrations of 25OHD have been found to be approximately 75% of the maternal 25OHD concentrations (36). Also, plasma volume expansion during pregnancy occurs with a peak between gestational weeks 28 and 34, which may give a lower 25OHD concentration (153). However, most studies have observed that 25OHD concentrations do not decrease during pregnancy (36). Yet, the aim of the BUGA-study was not to compare 25OHD concentrations during pregnancy and postpartum. Instead, we wanted to investigate 25OHD concentrations and its determinants during pregnancy, as well as changes in 25OHD concentrations and its determinants during the postpartum period.

### 5.2.2 Bone measurements

This study is the first study to measure the compartmental bone changes postpartum, using the HR-pQCT methodology. The DXA is the golden standard for measuring aBMD and it has many advantages, since it measures many skeletal sites of the body and provides measurements of BA, BMC and aBMD. It also gives information about body composition, such as fat mass and fat free mass. However, DXA is unable to differentiate between cortical and trabecular bone, which is possible with HR-pQCT. The new HR-pQCT method further gives an estimate of the bone with higher resolution than does the DXA, and gives information about microstructural changes such as trabecular thickness and number and trabecular bone volume fraction, and dimensional changes such as cortical thickness and area. Thus, it might be possible to detect small bone changes with the HR-pQCT which is not detectable with the DXA (112).

The compartmental bone changes during the postpartum period that can be captured using HR-pQCT has not been studied before and such information will increase our knowledge about and understanding for postpartum bone changes. A previous study has shown that among postpartum women, cortical and trabecular vBMD and cortical thickness are major determinants for fracture risk (112). Our results highlight that by only using DXA and not HR-

pQCT, information may be missed, i.e., that the postpartum bone losses are more long-lasting in cortical than trabecular bone and that some postpartum bone changes are still evident at 1.5 years postpartum in women with long duration of lactation. Further, our results indicate that the determinants for the postpartum bone changes in cortical and trabecular bone may partly differ, which may also be missed with the DXA methodology.

Bone measurements were performed at two weeks postpartum, to give a baseline value, and at four, 12 and 18 months postpartum. Previous studies have shown that largest decreases in bone minerals are found during the first months postpartum (41, 118, 119, 122, 124, 125), when the women are still breastfeeding to a large extent. In addition, we wanted to capture a time point when the majority of the women were still fully breastfeeding. At four months postpartum, 52% of the women in the general population were exclusively breastfeeding while at six months postpartum the number has decreased to only 15% (147). This is why a bone measurement at four months postpartum was performed. By 12 months postpartum, the vast majority of the women in Sweden have stopped breastfeeding (147), why a measurement at 12 months postpartum was included. Further, the measurement at 12 months postpartum was performed during the same or adjacent month as the baseline measurement, which covers seasonal aspects such as vitamin D concentrations. Previous studies have observed that bone minerals appear to recover three to six months after weaning (92, 118, 123, 129), which is why a measurement at 18 months postpartum was performed.

### 5.2.3 Measurements of vitamin D and bone determinants

Individual, habitual dietary vitamin D intake may be difficult to measure due to its presence in relatively few foods. Information of vitamin D and calcium intake was collected from four-day food diaries and from a short food frequency questionnaire at all study visits. Data collection of dietary intake is prone to miscalculations. For example, study participants may under- or over-report their total energy intake or certain foods, may change their eating behavior during the days of data collection or they may simply forget to report some food intake. This has to be kept in mind when interpreting our results. Still, the reported vitamin D and calcium intake were in line with the intake reported from Riksmaten (74). However, a somewhat higher intake is to be expected from our study, since the women in our study were either pregnant or lactating during most of the study visits. Even so, previous studies have indicated that vitamin D intake does not seem to be affected by underreporting of energy intake (154).

We investigated several different determinants of sun exposure, including season, time spent outdoors during summer, winter, weekends and weekdays, preference of sun and shade, sunscreen use, sunbed use, travels to southern latitudes and skin type. There are no validated questions regarding sun exposure, but we chose to use questions that had been used in previous studies in Sweden by Burgaz et al. (62)

Detailed information about breastfeeding habits was collected at each study visit postpartum, including number of lactation sessions per day, number and amount of formula feedings per day, date of introduction of solid foods and amount of solid foods given. Women were also asked to record the last date of breastfeeding. We did not analyze breast milk output or vitamin D content in milk, and so we cannot say anything about the amount of breast milk produced or the vitamin D content in breast milk in this population of postpartum women. What we do have information about is the duration of full breastfeeding and the duration of some breastfeeding, which are also the breastfeeding parameters that we have used in the analyses. We choose to focus on the duration of breastfeeding, since we considered that it was the breastfeeding estimate with the largest impact of the postpartum bone changes and also since we considered it to be a reliable breastfeeding estimate.

In *Paper III*, women were categorized according to duration of total lactation. Women in lactation category 0-3.9 months captured those women who were both fully and totally lactating for a short period, as well as the one woman who was not breastfeeding at all. Lactation category 4-8.9 months postpartum captured those women whose median duration of full and total lactation was similar the group medians, while lactation category ≥9 months captured the women with extended duration of both full and total lactation. These women also had a long duration of both full and total lactation compared to the national means. In the national Swedish population, 36% of the women were lactating to some extent at nine months postpartum (147).

# 5.3 Main findings

## 5.3.1 Vitamin D status during pregnancy and postpartum and its determinants

### Pregnancy

The main finding in *Paper I* is that mean serum 25OHD among the included women in the third trimester of pregnancy was 47(±18) nmol/L. Sixty-five percent of the women had serum 25OHD under 50 nmol/L. Further, main determinants for serum 25OHD in the third trimester of pregnancy were use of vitamin D supplements and estimates of sun exposure including season and travels to southern latitudes. No previous study of determinants of 25OHD concentrations among pregnant women has included measurements of vitamin D intake from diet and supplements separately, and only one Belgian study has included different estimates of sun exposure (32).

The finding that serum 25OHD varies with season and is highest during the summer months has been shown in previous studies among pregnant women (13, 35, 72, 81). We found that during the summer months, mean serum 25OHD was 58 nmol/L, i.e., above the IOM (25) and NNR (1) guidelines of 50 nmol/L as cutoff for vitamin D insufficiency. During the winter months, however, mean serum 25OHD was only 38 nmol/L, i.e. below the recommendations by the IOM and the NNR. Studies among pregnant fair-skinned women in Sweden (155), Denmark (156) and Belgium (32, 34) have reported higher mean 25OHD concentrations than in this study. Aside from differences in methodology, as discussed above, the differing results may be explained by differences in season and trimester for blood sampling. For example, in the Swedish study by Sääf et al., blood samples were collected only during autumn after the summer season with high sun exposure, the sample size was smaller than ours and constituted of 20 women of Swedish ethnicity and 20 women of Somalian ethnicity and only a few possible determinants for 25OHD concentrations were investigated (155).

Women who had been travelling to southern latitudes during the last six months prior to the study visit also had significantly higher serum 25OHD. This is in accordance with the results from a Belgian study among pregnant women (32) and a Swedish study among elderly women (62), and could probably be explained by the fact that at northern latitudes, cutaneous production of vitamin D is not possible during the winter months (57). A travel to latitudes where cutaneous production of vitamin D is possible all year round could therefore increase serum 25OHD, since sunlight exposure is

the primary source of vitamin D (1, 157). We also found that women who preferred to stay in the sun had higher serum 25OHD than women who preferred to stay in the shade or a combination of shade and sun. In line with our results, a Belgian study has reported that preference for sun was associated with higher 25OHD concentrations among pregnant women (32). Our finding on an association between sun preference and higher 25OHD concentrations was however no longer significant in the multivariable regression model.

In the third trimester, a significant relationship was found between total intake of vitamin D (from both diet and supplements) and serum 25OHD, which supports similar findings from previous studies among pregnant women (7, 35, 72). This relationship is probably explained by the strong association between use of vitamin D supplements and serum 25OHD, which has also been reported from previous studies among pregnant women (32, 72, 73).

An inverse relationship was observed between serum 25OHD and serum PTH. Serum PTH was also significantly higher at serum 25OHD below 50 nmol/L compared to above 50 nmol/L. Some previous studies among non-pregnant adults have found that above 25OHD concentrations of 50 nmol/L, no further increase is seen in serum PTH (158). Other studies among non-pregnant adults have however found that serum PTH continues to increase until 25OHD concentrations reach levels of 75-100 nmol/L (18).Weaker associations between PTH concentrations and 25OHD concentrations than in our study have also been found in previous studies among pregnant women (21, 23, 159, 160). Some previous studies have suggested that PTH may be suppressed during pregnancy and instead PTHrP has been observed to increase during pregnancy (21, 22).

## Postpartum

The main findings in *Paper II* are that mean serum 25OHD did not change between two weeks and 12 months postpartum, but the variation in change of serum 25OHD was large. Further, no relation was found between duration of lactation and changes in serum 25OHD during the first year postpartum. Instead, the main determinants for the variation in changes in serum 25OHD during the first year postpartum were season, use of vitamin D supplements and use of estrogen contraceptives. This study was the first study to examine change in serum 25OHD postpartum and its determinants among women in Sweden.

It has been suggested that maternal serum 25OHD may decrease postpartum, due to the fact that vitamin D is transferred from mother to child through breast milk (42). This might in turn lead to an increased maternal need of vitamin D during lactation (42). The amount of vitamin D transferred is small, between 0.1-3.4 ug/L (37), but in women who are breastfeeding for a long period the amount of vitamin D transferred may theoretically reach substantial amounts. However, even though the median duration of total lactation among the women in our study was quite long, over eight months, no relationship was found between changes in serum 25OHD and lactation during the first year postpartum. Hence, our study does not support the theory of an increased maternal need of vitamin D during lactation due to breast milk production. This is in line with the results observed by Specker et al. among American women (49) and Möller et al. among Danish women (118) where no changes in 25OHD concentrations postpartum were observed. However, Narchi et al. did observe a decrease in serum 25OHD during the first six months postpartum among lactating women living in the United Arab Emirates (42). One explanation for the differing results might be that the study population in the study by Narchi et al. differed from ours, since the majority of the women in the study by Narchi et al. had their heads and arms covered and were exclusively breastfeeding for a longer period than were the women in our study (42). At six months postpartum, 85% of the women were exclusively breastfeeding in the study by Narchi et al., whereas only 37% of the women in our study were fully breastfeeding at six months postpartum.

A positive relationship was found between use of estrogen contraceptives and changes in serum 25OHD during the first year postpartum. In women using estrogen contraceptives at four months postpartum, mean serum 25OHD increased from 52 nmol/L at baseline to 76 nmol/L at 12 months postpartum. In women not using estrogen contraceptives, mean serum 25OHD was almost the same at baseline and at 12 months postpartum (68 vs 66 nmol/L). However, since only 5% of the women were using estrogen contraceptives at four months postpartum, the results need to be carefully handled and interpreted. Still, the results support previous findings by Harris et al., where a decrease in serum 25OHD with over 20 nmol/L was observed in women who ceased using estrogen contraceptives (161). During lactation, estrogen levels are low and amenorrhea often occurs (22). This is caused by the child's suckling during breastfeeding and the high prolactin levels during the breastfeeding period, which both in turn lead to low estrogen levels (22). The suggested theory behind the observed finding between use of estrogen contraceptives and higher 25OHD concentrations is that estrogen may increase the vitamin D binding protein (161-163), which in turn would

decrease the free concentrations of all vitamin D metabolites and result in an overall increase in the circulating 25OHD (162).

In line with the results in *Paper I*, sun exposure was a major determinant for the changes in serum 25OHD postpartum. In women giving birth during the winter season, a significant increase in serum 25OHD was observed during the first year postpartum, while no change in serum 25OHD was found during the first year postpartum in women giving birth during the summer season. Also in line with the results in *Paper I*, travels to southern latitudes prior to the baseline visit was found to be a determinant for the changes in serum 25OHD during the first year postpartum in the univariable analyses. Women who had travelled to southern latitudes during the last six months prior to the baseline visit had a significant decrease in serum 25OHD during the first year postpartum. However, in *Paper* II the association between changes in serum 25OHD postpartum and travels to southern latitudes was no longer significant in the multivariable regression analysis. Travels to southern latitudes have previously been associated to serum 25OHD during pregnancy (32) and in non-pregnant and non-lactating women (62), but not until now in lactating women.

Like in *Paper I*, supplement use was found to be a major determinant for the changes in 25OHD postpartum. Women who were using vitamin D supplements at baseline had a mean baseline serum 25OHD of 75 nmol/L, while women who were not using vitamin D supplements at baseline only had a mean serum baseline 25OHD of 63 nmol/L. During the first year postpartum, mean serum 25OHD decreased from 75 nmol/L to 68 nmol/L among the women who were using vitamin D supplements at baseline. However, the percentage of women using vitamin D supplements decreased from 37% at baseline to 18% at 12 months postpartum, which might explain the finding. Use of vitamin D supplements have previously been associated with 25OHD concentrations (59, 62, 71) and our study contributes with the finding that use of vitamin D supplements is also associated with changes in serum 25OH postpartum.

## Conclusions for *Paper I* and *II*

In conclusion, during the winter a majority of the fair-skinned women pregnant in the third trimester had serum 25OHD below 50 nmol/L. More than every fourth woman was vitamin D deficient during the winter. The main determinants for serum 25OHD in third trimester of pregnancy were season, travels to southern latitudes and use of vitamin D supplements. Therefore, also fair-skinned women at northern latitudes may be at risk of vitamin D insufficiency, especially during winter. Higher vitamin D intake

among pregnant women living at northern latitudes may therefore be needed during the winter to avoid vitamin D insufficiency. The results from *Paper I* and *Paper II* confirm each other. *Paper I* shows that women who had travelled to southern latitudes had higher serum 25OHD than non-travelers, while *Paper II* shows that women who had traveled to southern latitudes but then did not travel again for a year were decreasing in serum 25OHD. Further, *Paper I* shows that women using vitamin D supplements had higher serum 25OHD than non-users, while *Paper II* shows that women who were using vitamin D supplements but then discontinued supplement use decreased in serum 25OHD. Hence, estimates of sun exposure and use of vitamin D supplements were found to be major determinants both for serum 25OHD during pregnancy and for changes in serum 25OHD postpartum.

Our findings regarding 25OHD concentrations during pregnancy and postpartum and its determinants could be used for national and international guidelines. Today, the findings from *Paper I* constitute an expansion of the underlying principles used by the NNR in their guidelines regarding vitamin D intake during pregnancy. The findings from *Paper II* also support the latest NNR guidelines that there is no need for an extra vitamin D intake during lactation (1).

### 5.3.2 Bone changes postpartum and its determinants

#### Bone changes postpartum

The main findings in *Paper III* are that among women lactating four months or longer, cortical vBMD, cortical thickness and trabecular thickness at the ultradistal tibia decreased significantly during the first year postpartum. Still at 18 months postpartum both cortical vBMD and trabecular thickness at the ultradistal tibia were significantly lower compared to baseline among women lactating nine months or longer. This study is the first to describe the compartmental changes in microstructural and dimensional bone parameters and vBMD during different durations of lactation, using HR-pQCT in postpartum women.

Several previous studies have observed decreases in aBMD postpartum (41, 118, 119, 122, 124, 125). Some of these studies have found that the changes in aBMD are at first most pronounced in the lumbar spine, but that the decreases in aBMD are more long-lasting in the femoral neck (118, 124, 128). These results are supported by the findings from our study. We can also support previous results that the bone decreases are both more pronounced and more long-lasting in women with longer duration of lactation compared

to women with shorter duration of lactation or non-lactating mothers (41, 118, 120, 122, 124, 126, 127). At 18 months postpartum, decreases in aBMD were no longer observed at any skeletal site, in any lactation category. Instead, at 18 months postpartum, lumbar spine aBMD was significantly higher compared to baseline in all lactation categories.

The finding from previous studies, that the decreases in aBMD are at first most pronounced at the trabecular-rich lumbar spine, but more long-lasting at the cortical-rich femoral neck, has led to the suggestion that lactation influences cortical and trabecular skeletal sites differently (118, 124, 128). These suggestions are partly confirmed by our study. The data from the HR-pQCT showed that cortical vBMD, cortical thickness and cortical area at the ultradistal tibia decreased significantly during the first 12 months postpartum, only in women lactating four months or longer. Thus, it seems that lactation influences both cortical bone quality and dimensional bone parameters in women lactating four months or longer, at least temporarily. No decreases were found in women lactating less than four months or in controls in cortical bone. At 18 months postpartum, cortical vBMD was still significantly lower than baseline, but only in women with the longest duration of lactation (≥9 months). Hence, the influence of lactation seems to be more long-lasting on cortical bone quality than on cortical dimensional and microstructural bone parameters.

We also found decreases in trabecular bone postpartum. Trabecular vBMD at the ultradistal tibia was significantly lower than baseline at 12 months postpartum, but only in women with the longest duration of lactation (≥9 months). At both 12 and 18 months postpartum, trabecular thickness at the ultradistal tibia was significantly lower than baseline in women lactating four months or longer. Decreases in trabecular bone volume fraction compared to baseline were also observed at four months postpartum for women lactating 4-8.9 months, and at 12 months postpartum for women lactating ≥9 months. However, decreases in trabecular vBMD and trabecular bone volume fraction postpartum were also evident for controls, which makes it harder to interpret the results for trabecular bone. No changes in trabecular bone were found in women with short lactation. Hence, we found that lactation mainly influenced cortical vBMD, cortical thickness and cortical area, but also trabecular thickness, in women lactating four months or longer, during the first 18 months postpartum.

Since this study is the first to investigate the compartmental changes in bone microstructural and dimensional parameters and vBMD postpartum, the clinical implications are unknown. It might be that the decreases in cortical

vBMD and trabecular thickness persist longer than 1.5 years postpartum in women with extended lactation and may increase fracture risk in later life. Melton et al. observed that among postmenopausal women cortical and trabecular vBMD, cortical thickness and cortical area were major determinants for fracture risk (112). However, a study among Gambian women with subsequent periods of long lactations found no relation between lactation and persistent decreases in aBMD, which may indicate that a full recovery takes place also after extended lactation (164).

## Determinants of bone changes postpartum

The main findings in *Paper IV* are that lactation and body weight were the main determinants of both cortical and trabecular bone changes during the first 18 months postpartum. Further, calcium intake and serum 25OHD appear to be differently associated with changes in cortical and trabecular bone postpartum. This is the first study to investigate the determinants, including calcium intake and serum 25OHD, of the compartmental changes in microstructural and dimensional bone parameters and vBMD in postpartum women. In addition, it is the first study to evaluate serum 25OHD as a determinant also for the changes in aBMD in postpartum women.

Previous studies have indicated that high serum 25OHD may protect against bone decreases postpartum (135). The hypothesis is that at higher serum concentrations of 25OHD, the calcium uptake from the intestine increases and less calcium is reabsorbed from the skeleton to the breast milk production (135). The calcium demand for the breast milk production would then instead to a larger extent be supplied by the increased intestinal calcium uptake (135). This hypothesis is however not supported by the findings from our study since we found that higher serum 25OHD were associated with larger decreases in the trabecular-rich lumbar spine aBMD during the first four months postpartum. This is surprising and reasons for this are unclear. Our finding was not influenced by season, differences in proportions of women breastfeeding during summer versus during winter or differences in baseline serum 25OHD depending on breastfeeding duration. However, the results may still be confounded by other, unknown variables. Neither did Krebs et al. or Möller et al. find any relation between dietary intake of vitamin D and aBMD at lumbar spine or mid-radius postpartum (92) or between dietary intake of vitamin D and changes in lumbar spine, total hip or whole-body during the first 18 months postpartum (118). No association in any direction was observed between serum 25OHD and changes in microstructural and dimensional bone parameters or vBMD postpartum.

Our results indicate that a high total calcium intake at four months postpartum (including both dietary calcium intake and calcium intake from supplements) may protect against decreases in cortical, but not trabecular, bone during the first 18 months postpartum. Most previous studies have found no association between calcium intake and changes in aBMD postpartum (41, 119, 122). Krebs et al., however, did observe a positive relationship between calcium intake and lumbar spine aBMD postpartum (92). Also most calcium intervention studies have found no (165, 166) or only a transient (129) association between calcium supplementation and radial and lumbar spine aBMD postpartum. However, none of these previous studies investigated the influence of calcium intake on microstructural and dimensional bone parameters or vBMD postpartum. We found that a higher total calcium intake at four months postpartum was positively associated with the changes in cortical vBMD and cortical thickness at the ultradistal tibia during the first 18 months postpartum, but not with trabecular thickness or the trabecular-rich lumbar spine.

The positive associations found between use of estrogen contraceptives and changes in lumbar spine aBMD (in the univariable regression analyses) and cortical vBMD (in the multivariable regression analyses) at the ultradistal tibia during the first four months postpartum might be explained by differences in durations of lactation. The few women using estrogen contraceptives at four months postpartum (5%), were all women with short duration of full lactation (not more than one month). Many previous studies have shown that women with shorter duration of lactation have smaller decreases in aBMD postpartum (41, 118, 122, 124). Hence, the observed positive relation between use of estrogen contraceptives and bone decreases postpartum may be a proxy for a short duration of lactation. Still, due to the small sample size, these results should be interpreted with caution.

The major determinants for changes in bone variables postpartum observed in our study were lactation and body weight at baseline. Associations were found between a lower baseline body weight and larger decreases in cortical bone variables, and to some extent also trabecular thickness, at the ultradistal tibia postpartum. One explanation to this finding might be that a lower body weight leads to a lower mechanical load on the skeleton (137, 167). It may also lead to a lower body fat content, which in turn may reduce the peripheral estrogen production (137, 167). Both the lower mechanical load and the lower estrogen production may influence the bone decreases postpartum (137, 167). In addition, previous studies have also found additional factors that were negatively associated with the bone losses or aBMD postpartum. These include maternal age (128, 137) and height (41), parity (92), breast

milk output (41), duration of amenorrhea (128, 165) and PTHrP concentrations (168).

## Conclusions for *Paper III* and *IV*

In conclusion, cortical vBMD, cortical thickness and trabecular thickness at the ultradistal tibia decreased significantly during the first year postpartum in women lactating four months or longer. At 18 months postpartum, cortical vBMD and trabecular thickness at the ultradistal tibia were still lower than baseline in women lactating nine months or longer. Lactation and body weight were the main determinants of the postpartum bone changes in this population of fair-skinned women. In addition, calcium intake and serum 25OHD appears to have different influences on cortical and trabecular bone, which is a novel finding. Further studies with a follow-up period longer than 18 months are needed to evaluate whether women with long duration of lactation fully recover at all skeletal sites after weaning, or if the bone changes postpartum could potentially lead to an increased fracture risk in later life.

# 6 OVERALL CONCLUSIONS

For our sample of fair-skinned women living in Sweden, mean 25OHD concentration during pregnancy was lower than the NNR and IOM guidelines and a majority of the pregnant women were vitamin D insufficient (<50 nmol/L). More than every fourth woman was vitamin D deficient (<30 nmol/L) during winter. Higher vitamin D intake during winter may therefore be needed also for fair-skinned pregnant women living at northern latitudes to avoid vitamin D insufficiency and deficiency. During the first year postpartum, no change in mean 25OHD concentration was found and no association between changes in 25OHD concentrations and duration of lactation was observed. Estimates of sun exposure, i.e., season and travels to southern latitudes, and use of vitamin D supplements, were found to be major determinants both for 25OHD concentrations during pregnancy and for changes in 25OHD concentrations postpartum.

Postpartum, cortical vBMD, cortical thickness and trabecular thickness at the ultradistal tibia decreased significantly during the first year postpartum in women lactating four months or longer. At 18 months postpartum, cortical vBMD and trabecular thickness at the ultradistal tibia were still significantly lower than at baseline in women lactating nine months or longer. The major determinants for the bone changes postpartum were lactation and body weight. Calcium intake and 25OHD concentrations appear to have different influences on the cortical and trabecular bone changes postpartum, which is a novel finding. A negative association was observed between 25OHD concentrations and decreases in trabecular-rich bone, while calcium intake may protect against the decreases in cortical-rich bone postpartum. Further studies with a follow-up period longer than 18 months are needed to evaluate whether women with long duration of lactation fully recover at all skeletal sites after weaning, or if the bone changes postpartum could potentially lead to an increased fracture risk in later life.

# 7 FUTURE PERSPECTIVES

Low 25OHD concentrations have been associated both with sub-optimal bone health, as well as many chronic diseases. Serum concentrations of 25OHD during pregnancy and lactation and its determinants are however not thoroughly studied, especially not among women at northern latitudes, where cutaneous production of vitamin D is not possible all year round. Postpartum, decreases in bone mineral are known to occur, but the importance of vitamin D is only rarely studied.

For future studies, it would be of interest to study 25OHD concentrations from prepregnacy, through pregnancy and throughout lactation, using the same analysis method, to investigate how 25OHD concentrations may change during a whole reproductive cycle. It would also be of interest to study other populations of women living at northern latitudes, such as dark-skinned women or women wearing covering clothing, or a more heterogeneous population. Serum concentrations of 25OHD may not be the same in other populations and other determinants may be observed. Further, it would be of importance to investigate whether maternal 25OHD concentrations during pregnancy affect the child's bone health - both cortical and trabecular bone health - later in life. Since recent studies have implicated the importance of maternal 25OHD concentrations on health aspects concerning the child during childhood, it would be of interest to further investigate these issues.

With a larger study population, we might have found more or other determinants both for vitamin D status/changes in vitamin D status and for bone changes. We found that use of estrogen contraceptives was positively related to both changes in 25OHD postpartum and bone changes postpartum. However, only a few women were using estrogen contraceptives. It would be of interest to study whether the relationship also persisted in a larger study population.

For future studies, it would be of importance to study bone status both prepregnancy and postpartum. This would give information about prepregnancy bone values, which would make it possible to evaluate whether or not the women return to their prepregnancy values after weaning. It would also be of interest to study whether decreases in BMD during pregnancy are mostly located to trabecular or cortical bone. It may be that the decreases in BMD during pregnancy are mostly located to the trabecular bone, followed by a postpartum decrease in cortical bone. This would be interesting to evaluate. Further, a longer follow-up period would be needed to evaluate

whether also women with extended lactation reach their baseline or prepregnancy bone values, or if the postpartum bone changes may increase fracture risk in later life for these women. It would also be interesting to study the effect of multiple pregnancies/lactations on bone changes. Studies that investigate if similar bone changes/determinants of bone changes are observed in other populations of postpartum women would also increase our knowledge about bone changes postpartum.

The study population was rather homogenous. It is difficult to say if the results would have been different if the study population had not been composed of mostly highly educated fair-skinned women, although we have no reason to believe that the associations found would differ among other groups of adult women who have reached peak bone mass and who have a calcium intake close to the recommendations. The subgroup of women with short duration of lactation or formula-feeding women was also quite small. The number of women with long duration of lactation, however, was the same as in the general population, which is why our opinion is that the results regarding women with long duration of lactation are representative. We believe that our results have contributed to an increased understanding of the etiology behind postpartum bone changes and that the observed relationships remain strong also in a broader population of pregnant and postpartum women. To further confirm these findings, future studies including a more heterogeneous study population or other minority groups will be needed.

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# APPENDIX

*Table 12. Areal bone mineral density (aBMD) in postpartum women and controls at baseline[a] and four, 12 and 18 months thereafter, as assessed with dual-energy X-ray absorptiometry*

| aBMD (g/cm$^2$) | Postpartum women (n=81) | | | | Controls (n=21) | | | |
|---|---|---|---|---|---|---|---|---|
| | **Baseline** | **4 months** | **12 months** | **18 months** | **Baseline** | **4 months** | **12 months** | **18 months** |
| Body weight (kg) | 70.2 (64.2-76.3) | 66.7 (60.4-72.2) | 65.3 (58.2-70.3) | 65.2 (59.1-71.4) | 64.2 (57.2-69.7) | 63.3 (57.1-68.3) | 65.0 (56.0-70.5) | 64.0 (57.5-67.7) |
| Ultra-distal radius[b] | 0.334 (0.306-0.372) | 0.332 (0.303-0.370) | 0.330 (0.302-0.362) | 0.339** (0.313-0.379) | 0.348 (0.317-0.368) | 0.345 (0.307-0.377) | 0.343 (0.315-0.358) | 0.344 (0.314-0.367) |
| Lumbar spine[§§§b] | 1.167 (1.080-1.252) | 1.127*** (1.053-1.223) | 1.186*** (1.123-1.274) | 1.211*** (1.135-1.316) | 1.209 (1.105-1.300) | 1.206 (1.103-1.303) | 1.205 (1.089-1.296) | 1.201 (1.089-1.302) |
| Femoral neck[§§§b,c] | 0.993 (0.920-1.072) | 0.957*** (0.871-1.030) | 0.967*** (0.877-1.042) | 0.982 (0.905-1.073) | 1.009 (0.928-1.101) | 1.006 (0.918-1.102) | 0.997 (0.912-1.102) | 0.990 (0.907-1.088) |
| Femoral shaft[§§§c] | 1.204 (1.091-1.309) | 1.172*** (1.062-1.259) | 1.190** (1.080-1.303) | 1.219 (1.123-1.347) | 1.202 (1.149-1.260) | 1.207 (1.135-1.259) | 1.197 (1.137-1.262) | 1.197 (1.146-1.270) |
| Femoral trochanter[§§§] | 0.783 (0.710-0.866) | 0.767*** (0.703-0.837) | 0.781 (0.710-0.860) | 0.807*** (0.742-0.890) | 0.775 (0.696-0.863) | 0.770 (0.692-0.853) | 0.760 (0.692-0.847) | 0.753 (0.689-0.841) |
| Total femur[§§§b,c] | 1.012 (0.913-1.090) | 0.986*** (0.891-1.067) | 1.002* (0.919-1.100) | 1.027** (0.955-1.126) | 1.011 (0.935-1.039) | 1.012 (0.931-1.057) | 1.003 (0.921-1.043) | 1.001 (0.929-1.047) |
| Wholebody | 1.180 (1.131-1.232) | 1.173*** (1.120-1.214) | 1.175 (1.121-1.220) | 1.187 (1.129-1.237) | 1.195 (1.151-1.246) | 1.192 (1.137-1.249) | 1.197 (1.142-1.249) | 1.198 (1.126-1.267) |

Values are presented as geometrical means (Q1-Q3)
Mixed procedure repeated measure ANOVA with least square means showed significant differences in change over time in areal bone mineral density (aBMD) between postpartum women and controls, §§§=p<0.001, and significant change in aBMD compared to baseline, *=p<0.05, **=p<0.01, ***=p<0.05
[a]Two weeks after delivery, [b]Adjusted for body weight, [c]Adjusted for age

*Table 13. Ultradistal tibia bone variables in postpartum women and controls at baseline[a] and four, 12 and 18 months thereafter, as assessed with high-resolution peripheral quantitative computed tomography*

| Ultradistal tibia bone variables | Postpartum women (n=81) | | | | Controls (n=21) | | | |
|---|---|---|---|---|---|---|---|---|
| | **Baseline** | **4 months** | **12 months** | **18 months** | **Baseline** | **4 months** | **12 months** | **18 months** |
| Body weight (kg) | 70.2 (64.2-76.3) | 66.7 (60.4-72.2) | 65.3 (58.2-70.3) | 65.2 (59.1-71.4) | 64.2 (57.2-69.7) | 63.3 (57.1-68.3) | 65.0 (56.0-70.5) | 64.0 (57.5-67.7) |
| Cortical vBMD ($mg/cm^3$) | 892.5 (867.8-918.7) | 890.0*** (863.5-916.9) | 886.2*** (861.8-914.2) | 893.4** (864.4-916.9) | 906.3 (893.4-942.0) | 905.2 (880.1-937.3) | 903.1 (870.4-939.2) | 905.8 (877.4-942.0) |
| Cortical thickness (mm)[§] | 1.134 (0.977-1.300) | 1.115* (0.960-1.290) | 1.127** (0.990-1.300) | 1.165** (1.030-1.305) | 1.158 (1.000-1.489) | 1.156 (0.975-1.361) | 1.150 (0.960-1.380) | 1.163 (0.975-1.390) |
| Cortical area ($mm^2$)[§§] | 117.6 (106.6-132.0) | 116.6*** (105.7-129.8) | 117.1** (106.3-131.5) | 120.7* (109.9-136.3) | 118.2 (106.1-133.4) | 118.2 (105.0-133.5) | 116.9 (103.8-134.6) | 117.7 (104.8-135.1) |
| Trabecular vBMD ($mg/cm^3$)[§] | 156.9 (133.0-177.5) | 156.2 (131.9-178.2) | 155.3** (131.2-175.4) | 155.2 (130.7-173.5) | 164.4 (151.3-172.3) | 162.7* (146.6-171.7) | 159.2 (146.5-172.1) | 156.7* (141.2-168.3) |
| Trabecular thickness (mm) | 0.070 (0.060-0.079) | 0.069 (0.061-0.078) | 0.069** (0.060-0.077) | 0.068** (0.061-0.078) | 0.074 (0.066-0.084) | 0.074 (0.066-0.083) | 0.073 (0.065-0.081) | 0.072 (0.066-0.080) |
| Trabecular number($mm^{-1}$) | 1.860 (1.696-2.016) | 1.877 (1.711-2.061) | 1.881 (1.719-2.071) | 1.901** (1.730-2.102) | 1.849 (1.680-2.020) | 1.826 (1.639-2.044) | 1.819 (1.680-2.010) | 1.810 (1.751-1.990) |
| Trabecular bone volume fraction[§] | 0.131 (0.111-0.148) | 0.130* (0.110-0.148) | 0.129** (0.109-0.146) | 0.129 (0.109-0.144) | 0.137 (0.126-0.144) | 0.135* (0.122-0.143) | 0.133 (0.122-0.143) | 0.130* (0.117-0.140) |

Values are presented as geometric means (Q1-Q3)
Mixed procedure repeated measure ANOVA with least square means showed significant differences in change over time in volumetric bone mineral density (vBMD) and microstructure between postpartum women and controls, [§]=p<0.05, [§§]=p<0.01, and significant change in vBMD and microstructure compared to baseline, *=p<0.05, **=p<0.01, ***=p<0.001
vBMD; volumetric bone mineral density
[a]Two weeks after delivery