Longitudinal vitamin D status during pregnancy in Sweden

– *The GraviD cohort*

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Abstract

The aim of this thesis was to study vitamin D status among pregnant women in Sweden and if it was associated with gestational complications. A total of 2125 women were recruited at registration for antenatal care. Data were collected in early (T1, trimester 1, mean gestational week 11) and late pregnancy (T3, trimester 3, mean gestational week 34), when the women had blood drawn and answered questionnaires. In late pregnancy, women were asked to provide dietary intake data. After delivery, medical records from antenatal and obstetrics care were retrieved. Vitamin D status was measured as 25-hydroxyvitamin D (25OHD) in serum by liquid chromatography– tandem mass spectrometry.

In T1, mean 25OHD was 65 nmol/L and 10% had vitamin D deficiency (25OHD <30 nmol/L). Nearly half of the more than 300 women born in Africa and Asia were vitamin D deficient. Other risk factors associated with vitamin D deficiency were sampling in spring, lower vitamin D intake, less sun exposure and younger age. Vitamin D status increased by ~11 nmol/L during pregnancy and change in season-corrected vitamin D status was associated with origin, sun seeking behavior, clothing style, vitamin D intake and travels to southern latitudes. Vitamin D status in T1 was weakly associated with pregnancy loss, but no other outcomes. Vitamin D status in T3 was inversely associated with preeclampsia, small for gestational age and low birth weight. Change in 25OHD concentration from T1 to T3 was inversely associated with preeclampsia, small for gestational age, low birth weight and preterm delivery. A short vitamin D questionnaire was the only dietary assessment method that provided estimates of vitamin D intake that were reflected in serum 25OHD. In conclusion, vitamin D status in late but not early pregnancy, and changes in vitamin D status during pregnancy were associated with several pregnancy complications with implications for both woman and child. A short vitamin D questionnaire was a valid tool for estimation of dietary vitamin D intake.

Keywords

Vitamin D status, pregnancy, pregnancy complications, vitamin D intake, dietary assessment

Sammanfattning på svenska

D-vitaminstatus mäts som 25-hydroxyvitamin D (25OHD) i blodet och låga nivåer har förknippats med sjukdomar som cancer, hjärtkärlsjukdom och komplikationer under graviditet. Det finns mycket lite information om D-vitaminstatus hos gravida kvinnor i Sverige och huruvida 25OHD-koncentrationer är relaterade till graviditetskomplikationer. Syftet med denna avhandling var att studera gravida kvinnors D-vitaminstatus och huruvida 25OHD-koncentration i sen eller tidig graviditet var associerat till graviditetskomplikationer. Dessutom jämfördes tre olika metoder för att undersöka kostintaget av D-vitamin.

Totalt 2125 kvinnor rekryterades till studien vid inskrivning till mödrahälsovården. Blodprover och enkätdata insamlades i tidig (medelvärde för graviditetsvecka 11) och sen graviditet (medelvärde för graviditetsvecka 34). Information om graviditet insamlades från kvinnornas journaler. Koncentrationer av 25OHD i serum analyserades med liquid chromatography tandem masspektrometri av Laboratoriemedicin i region Skåne. I sen graviditet besvarade kvinnorna ett kort frågeformulär om intag av D-vitaminrika livsmedel och ombads att även besvara ett längre online-frågeformulär om sin kost. En liten andel ombads även att registrera sin kost under fyra dagar med hjälp av en matdagbok.

Resultaten av avhandlingen visar att de flesta kvinnor i studien hade god D-vitaminstatus i både sen och tidig graviditet. D-vitaminbrist var dock mycket vanligt bland kvinnor födda i Afrika och Asien, bland vilka varannan kvinna hade 25OHD-koncentrationer <30 nmol/L. Faktorer som predicerade D-vitaminbrist var härkomst, provtagning under vår, mindre solexponering, lägre D-vitaminintag och lägre ålder. Lägre D-vitaminstatus i tidig graviditet var svagt associerat till en ökad förekomst av missfall. D-vitaminbrist i sen graviditet var associerat till en ökad förekomst av preeklampsi, small for gestational age och låg födelsevikt. En ökning >30 nmol/L i 25OHD-koncentration under graviditeten var associerad med en lägre förekomst av preeklampsi, small for gestational age, låg födelsevikt och förtidsbörd. Det korta frågeformuläret om D-vitaminrika livmedel var en enkel och bra metod för att skatta D-vitaminintaget från kosten.

List of papers

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

I. Bärebring L, Schoenmakers I, Glantz A, Hulthén L, Jagner Å, Ellis J, Bärebring M, Bullarbo M, Augustin H.

Vitamin D Status during Pregnancy in a Multi-Ethnic Population-Representative Swedish Cohort

Nutrients 2016;8(10)

II. Bärebring L, Bullarbo M, Glantz A, Leu Agelii M, Jagner Å, Ellis J, Hulthén L, Schoenmakers I, Augustin H.

Preeclampsia and Blood Pressure Trajectory during Pregnancy in Relation to Vitamin D Status

PLoS One. 2016;11(3):e0152198.

III. Bärebring L, Bullarbo M, Glantz A, Hulthén L, Ellis J, Jagner Å, Schoenmakers I, Winkvist A, Augustin H.

Trajectory of vitamin D status during pregnancy in relation to neonatal birth size and fetal survival: a prospective cohort study

Submitted for publication, under revision

IV. Bärebring L, Amberntsson A, Winkvist A, Augustin H.

Validation of habitual dietary vitamin D intake using three dietary assessment tools and the biomarker 25-hydroxyvitamin D.

In manuscript

Content

Abbreviations

1,25OH2D	1,25 dihydroxyvitamin D
25OHD	25- hydroxyvitamin D
25OHD2	25- hydroxyvitamin D2
25OHD3	25- hydroxyvitamin D3
3-epi-25OHD	3- epi- 25- hydroxyvitamin D
B	Beta
BMI	Body mass index
CI	Confidence internal
DEQAS	Vitamin D External Quality Assessment Scheme
LBW	Low birth weight
LC-MS/MS	Liquid chromatography– tandem mass spectrometry
OR	Odds ratio
P	Probability
PTH	Parathyroid hormone
SD	Standard deviation
SGA	Small for gestational age
T1	First trimester of pregnancy
T3	Third trimester of pregnancy
VDQ	Vitamin D questionnaire

1. Introduction

Vitamin D plays an essential role in bone metabolism and skeletal health. In recent years, vitamin D has been attributed to other health benefits beyond bone health. It has been suggested that poor vitamin D status is associated with various illnesses, such as cardiovascular disease and cancer. Among pregnant women, poor vitamin D status is associated with gestational and neonatal complications.

The longitudinal study BUGA (Bone metabolism during pregnancy and lactation) was carried out in the Gothenburg area in 2008-2012 at our department. The BUGA study was designed to assess skeletal consequences of childbirth and lactation. The results showed that 85% of the pregnant women in the study had an insufficient vitamin D status during winter (1). The women who participated were all fair-skinned, with a generally high education level and more likely to be health literate than the general pregnant population. Therefore, vitamin D status was suspected to be better in this subgroup than in the population overall. The next step was to carry out a population based cohort study that would reflect the general pregnant population in Sweden, in terms of ethnicity, education and body mass index (BMI). This became the GraviD study; Gravidity and vitamin D.

The main aims of the GraviD study, and of this thesis, were to provide population-based data on vitamin D status of pregnant women in Sweden, and to see if vitamin D status in early or late pregnancy was associated with gestational complications.

2. Background

Vitamin D is not only a nutrient but also a prohormone that can be synthesized endogenously by humans. It plays an essential role in bone metabolism and helps maintain optimal blood levels of parathyroid hormone (PTH) and phosphate. This is achieved through the ability of vitamin D to increase intestinal calcium absorption, and to increase bone resorption and the removal of calcium from the bone. As a result of these mechanisms, blood calcium levels increase.

At the turn of the century, Rickets disease was common in industrialized countries. The disease caused malformation of the skeleton and growth retardation in children, leading to disabilities that carried through to adulthood. It was discovered that exposure to direct sunshine, ultraviolet (UV) light from a quarts lamp or cod liver oil cured the condition (2). Vitamin D was discovered in 1922 as a "calcium depositing substance" and its chemical structure was determined in the 1930's (3). Today, Rickets disease is less common but still prevalent in many parts of the world (4). In adults, clinical vitamin D deficiency is associated with muscle weakness, skeletal pain and fractures (5, 6).

Vitamin D has been attributed to health benefits beyond bone health since it was discovered that vitamin D needs a receptor in order to exert its effects, and that the vitamin D receptor is present in many of the body's tissues and organ systems (5). Poor vitamin D status has been associated with cancer, autoimmune disease, cardiovascular disease, mental disorders and lung function (7). It has also been suggested that poor vitamin D status in pregnancy is associated with gestational and neonatal complications (8).

Vitamin D metabolism

Vitamin D naturally exists in two isomers: vitamin D3 and vitamin D2. Vitamin D3 is the most common form and is present in foods such as oily fish, egg and vitamin D fortified dairy products. Vitamin D2 is present is some plant foods and is sometimes used to fortify plant-based milk substitutes (9). Both isomers are also used in dietary supplements. In addition, vitamin D3 can be formed in the body under the influence of sunlight. When solar ultraviolet B (UV-B) rays

of wavelength 290-315 nm radiate the skin, 7-dehydrocholoesterol is transformed into pre-vitamin D3, which in turn is transformed into vitamin D3 (10). If sun exposure is prolonged, vitamin D3 degrades into inactive metabolites, meaning that excessive sun exposure cannot cause toxic levels of vitamin D. Ingested vitamin D2 or D3, from diet or supplements, is incorporated into chylomicrons and transported into circulation through the lymphatic system. In the circulation, vitamin D is bound to the vitamin D binding protein, which transports the vitamin to the liver. In the liver, vitamin D is converted into 25-hydroxyvitamin D (25OHD). This is the major circulating form of the vitamin and what is usually used as a proxy for vitamin D status. However, this form is biologically inactive and is converted into the active form 1,25-dihydroxyvitamin D (1,25OH2D) in the kidneys. There is a negative feedback loop where high concentrations of 1,25OH2D increase production of inactive vitamin D metabolites (11). The active metabolite 1,25OH2D acts by binding to the vitamin D receptor. The main target of 1,25OH2D is in the intestine, where it increases production of calcium channels and calcium binding protein (11). The production of 1,25OH2D is stimulated by PTH and there is a negative feedback from 1,25OH2D to PTH.

Vitamin D status, measured as concentrations of 25OHD, is dependent on both intrinsic and external factors. External factors include those affecting the availability of UV-B radiation. Since UV-B radiation is a major determinant of vitamin D status, factors that influence the availability of UV-B are of outmost importance. These factors include season, meteorological conditions and latitude (12, 13). At northern latitudes, such as in Sweden, vitamin D synthesis is not possible all-year round (10). In addition, individual factors can restrict sun exposure, such as time spent indoors (14) and covered clothing (15, 16). In theory, sunscreen use should decrease the synthesis of vitamin D as it blocks UV radiation. However, most studies fail to see such an association (17). Darker skin pigmentation is also known to require longer sun exposure to convert the same amount of vitamin D, compared to lighter skin (18).

Vitamin D status

There is no consensus on what constitutes desirable vitamin D status, but 25OHD concentrations below 20-30 nmol/L are associated with rickets and classified as vitamin D deficiency (19, 20) (table 1). According to the American Institute of Medicine, 25OHD concentration ≥50 nmol/L is desirable for bone health (20), and this is supported by the Nordic Nutrition Recommendations (9).

A threshold of ≥75 nmol/L is advocated by many researchers to promote overall health (21-23), though the Institute of Medicine raises concerns regarding long-term effects of concentrations ≥125 nmol/L (20).

Table 1.

Cut offs commonly used to assess vitamin D status

25OHD (nmol/L)	**<30**	**30-50**	**≥50**	**≥75**	**≥125**
Definition of vitamin D status	Deficient	Insufficient	Adequate?	Adequate?	Toxic?

25OHD, 25-hydroxyvitamimn D

Measuring vitamin D status

Obtaining a measure of 25OHD in order to assess vitamin D status is associated with some difficulties. Vitamin D and its metabolites are fat-soluble molecules and inherently hydrophobic. In addition, the molecule 25OHD has structural similarities to other vitamin D metabolites and is bound to the vitamin D binding protein with high affinity (24). This makes analysing vitamin D status challenging.

Circulating concentrations of the metabolite 25OHD is most commonly used to assess vitamin D status. The metabolite is the major circulating form of vitamin D and has relatively long half-life (25). Other metabolites include the active form 1,25OH2D. In addition, less commonly measured metabolites include 3-epi-25OHD, which is an epimer of 25OHD. This metabolite cannot be distinguished from 25OHD by all assays, but contributes to approximately 4-6% of total 25OHD concentration (26, 27). As the epimer is believed to have a lower bioactivity, separating 3-epi-25OHD from 25OHD might be useful in assessing vitamin D status. Concentrations of 25OHD are stable in serum, even after prolonged periods in room temperature (28-30).

There are different methods of analysing 25OHD concentrations. The most common analyses are antibody based methods and liquid chromatography based methods, including liquid chromatography tandem mass spectrometry (LC-MS/MS) which is the closest to a gold standard (20). LC-methods can distinguish between 25OHD2 and 25OHD3, which antibody based methods cannot. Some antibody based methods underestimate 25OHD concentrations due to failure to measure 25OHD2, while some methods overestimate 25OHD concentration by detecting other vitamin D metabolites (20). The LC-MS/MS method has

higher specificity and sensitivity, and typically high reproducibility (20). Still, some LC-assays with short run-time have been reported to overestimate 25OHD concentration due to interference with 3-epi-25OHD, though the significance of this is unknown (31). Variance in results from 25OHD analyses is not only a result of the type of assay used. Results from 25OHD analysis are also known to vary between laboratories (32, 33). In order to combat the problem with large inter-laboratory variance, the Vitamin D External Quality Assurance Scheme (DEQAS) was initiated in 1989 to provide external control of accuracy (33). DEQAS now includes 1200 laboratories in 54 countries and operates by giving laboratories a number of samples with known concentrations of 25OHD to analyse and the results are thereafter reported back to DEQAS. The organization issues annual certificates to laboratories that meet the goals for performance. Another initiative to combat different results of 25OHD assays is the Vitamin D Standardization Program (34). This initiative aims to make data on vitamin D status comparable between studies by calibrating the results using a master equation. A subset of samples from e.g. a cohort study is reanalysed by the organisation, and the results are compared to the original data. This comparison is the basis for the equation to calibrate all 25OHD concentrations in the original dataset. Results from studies in Nordic populations, though not Swedish, have been standardized in 2015 and the results showed that standardization had a great impact on the reported vitamin D status (35).

Vitamin D status among pregnant women

Overall, vitamin D status among populations in North Europe tends to be higher than in South Europe, despite less sun exposure in the north. The higher vitamin D status could be attributed to higher vitamin D intake or lighter skin tone in the Nordic region (6, 36). Vitamin D status depends on several factors, such as sun exposure, vitamin D intake and life style. Contributors to vitamin D status include supplement use and sun exposure, as well as genetic factors (37, 38).

Among pregnant women in Scandinavia, 25OHD concentration is associated with season, use of vitamin D supplements, travels to southern latitudes (1), dietary vitamin D intake, gestational age (39), education level and ethnicity (40). Among non-pregnant women, use of oestrogen contraceptives or oestrogen replacement therapy is associated with higher 25OHD concentration (41, 42).

Vitamin D status among women in Sweden and Europe

There are few studies of vitamin D status among pregnant women in Sweden. The previous BUGA study indicated that vitamin D status was poor among pregnant fair-skinned women of mainly Swedish descent (1). Mean 25OHD concentration was 47 nmol/L in the third trimester of pregnancy and 65% had levels <50 nmol/L (1). Other smaller studies have found that pregnant women with immigrant backgrounds are often vitamin D deficient (43) and have a markedly lower 25OHD concentration than women of Swedish descent (44). A small Swedish study with both pregnant and non-pregnant women, found that 90% of women born in Somalia were vitamin D deficient (<25 nmol/L) (45). Obese pregnant women in Sweden have also been identified as a risk group for poor vitamin D status, and they have more often insufficient vitamin D status than normal-weight women (46). A longitudinal study of 183 pregnant women of primarily Swedish origin reported that 3-5% were vitamin D deficient during pregnancy (39).

Studies on vitamin D status among pregnant women in Sweden are scarce but more studies have been performed on non-pregnant women. One such study found that mean 25OHD concentration among women in Northern Sweden (809 women, age 25-74 years) was 71 nmol/L and 83% had adequate vitamin D status (>50 nmol/L) (47). This is in line with findings from a national study, reporting that 20% of 144 women in Sweden (age 18-80 years) had 25OHD concentrations <50 nmol/L (48). Among 61 women in the Swedish city of Uppsala (60 °N), mean 25OHD concentration was 34 nmol/L, and vitamin D status was poorer among immigrant women than women of Swedish descent (49). A study from the city of Gothenburg (58 °N) had samples taken in 1984 from 192 non-pregnant women (age 25-64 years) and reported a mean 25OHD concentration of 88 nmol/L (37). In addition, among non-pregnant women in the BUGA study, only 1% were vitamin D deficient and mean 25OHD concentration was 66 nmol/L (41). It should be noted that 25OHD was analysed using a different assay than in the paper on pregnant women (1), and the results are therefore not entirely comparable.

There are data on vitamin D status among pregnant women from other countries. Studies on pregnant populations in North Europe have reported mean 25OHD concentrations of 23-76 nmol/L (50-58). Cohort studies from Norway (50) and Denmark (52, 53) suggest that vitamin D deficiency is less common in these countries compared to the Swedish BUGA study (1) and the studies found higher mean 25OHD concentrations (figure 1). However, women from ethnic minority groups seem to be at risk of poor vitamin D status also in Norway (40). Vitamin

D status may differ between Scandinavian countries for several reasons. Firstly, recommendations on vitamin D supplement use during pregnancy are not uniform between the Scandinavian countries, and neither is the reported use. In Denmark, 65% of pregnant women reported vitamin D supplement use before supplementation was recommended (59). In a Swedish study, 27% of pregnant women used vitamin D supplements during pregnancy in the late 1990s (60). Approximately 50% of pregnant women use fish oil supplements in Iceland (61) and Norway (62), and considerably fewer in Sweden (63). This could contribute to vitamin D intake and status. Supplements accounts for roughly 75% of the total vitamin D intake among pregnant women in Norway (62). Secondly, a higher proportion of the population in Sweden originate from other countries, compared to the other Scandinavian countries (64).

***Figure 1**.Mean or median 25-hydroxyvitamin D concentrations (nmol/L) in North European pregnant women. In reference list as: 1)(50), 2)(51), 3) (52), 4) (53), 5) (54), 6) (55), 7) (56), 8) (57), 9) (58)*

Vitamin D intake

The recommended vitamin D intake in the 2012 Nordic Nutrition Recommendations is 10 µg per day for adults ≤74 years, including pregnant women (9). The recommendations are based on supplementation trials showing that 10 µg per day is needed to maintain 25OHD concentration around 50 nmol/L during winter in the majority of the population. The recommendation assumed some contribution of sun exposure during summer. For those with little or no sun exposure, an intake of 20 µg/day is recommended (9). The American Institute of Medicine recommends a vitamin D intake of 15 µg/day for pregnant women, same as for adults < 70 years of age, assuming minimal sun exposure (65). The European Food Safety Authority recommends that vitamin D intake for adults (including pregnant women) should be 15 µg per day under conditions with minimal endogenous vitamin D synthesis (66). The target 25OHD concentration of 50 nmol/L is used by the Nordic Nutrition Recommendations, Institute of Medicine and European Food Safety Authority, but the recommended dietary intake to achieve this differs slightly, because of different assumptions on endogenous vitamin D production. The UK Scientific Advisory Committee on Nutrition recommends a daily vitamin D intake of 10 µg for all adults (including pregnant women) in order to maintain 25OHD ≥25 nmol/L (67). The recommendations are based on mathematical modelling of vitamin D status. For instance, the Institute of Medicine used regression analysis to assess mean response in 25OHD concentration, following vitamin D intake in settings with minimal sun exposure (65). This method has been criticized for underestimating vitamin D requirements, by using data on group level rather than individual level (68).

In the latest nationwide Swedish dietary survey, Riksmaten, almost 1800 people between the ages 18-80 years reported their diet by using a web-based four-day food record (69). The study found that mean daily dietary vitamin D intake was 7.0 µg overall. Women had a lower intake (6.4 µg) than men (7.6 µg). In addition, young women had a lower vitamin D intake than older women. Among women age 18-30 years, the mean daily intake was 5.5 µg. The largest contributors to vitamin D intake were fish (32%), spread margarine (14%) and dairy products (12%) (69). Fortification of margarine and milk with reduced fat content is mandatory in Sweden. The current fortification program is under revision with the intention to increase vitamin D intake, due to concerns of the discrepancies between observed and recommended intake (70, 71).

Vitamin D supplementation is a major source of vitamin D and is the preferred treatment of vitamin D deficiency. In the guidelines for treatment of vitamin D

deficiency in the region of Västra Götaland, the daily dose (µg) of vitamin D needed is calculated as target level of 25OHD minus measured level of 25OHD. The target level of 25OHD is 50 nmol/L and the dose is thereby 40 µg if 25OHD concentration is 10 nmol/L and 25 µg if 25OHD is 25 nmol/L (72). Follow up is advised after 3-4 months. The target concentration of 50 nmol/L is in line with the target levels set by the Institute of Medicine and the Nordic Nutrition Recommendations (9, 65). Some researchers argue that considerably higher doses than this are needed to treat deficiency, but they also aim at a higher target 25OHD level (73). It has been suggested that vitamin D2 is less efficient than vitamin D3 in raising 25OHD concentrations (74, 75), but this is not confirmed in all studies (76, 77).

Measuring vitamin D intake

The most common methods for nutritional intake assessment are food records and food frequency questionnaires (FFQs). In the food record method, study participants keep a food diary to prospectively record all foods and beverages consumed during a stipulated amount of days. Amounts (weight or volumes) are usually indicated, as well as details about the foods consumed (fat content, brand etc.). Precision increases with the number of days food intake is recorded, but a long time period also increases the burden of participation (78). In the FFQ method, study participants approximate how often they consume different types of food or beverages. Typical amounts are sometimes indicated, usually by defining a normal portion size (78). The time-period the FFQ reflects is usually defined by asking the participants to consider the intake during e.g. the past month or year. The number of food items included in a FFQ depends on the intended use, but 130 items is a suggested maximum (78).

Dietary vitamin D intake originates from animal products and is predominantly dependent on the intake of oily fish. This can make estimating habitual dietary vitamin D intake difficult, as intake of fish may be sporadic or irregular. As with all dietary assessment, estimated vitamin D intake may be largely dependent upon methodological concerns such as general and systematic underreporting, day-to-day variation in dietary intake and seasonality in food availability (78). Vitamin D intake might be subject to extra high day-to-day variation in Sweden, as intake of oily fish such as herring and mackerel is higher than in many other European countries (79).

Previous studies indicate that vitamin D intake can be assessed by condensed FFQs with a range of 17-98 food items (80-83). Correlation between vitamin D intake from the short FFQs and food records have been r=0.5-0.6, when supplement intake was included (80, 81). Studies that have used 25OHD as a biomarker for vitamin D intake have found correlations of approximately r=0.5, in wintertime and when supplement intake was included (80-82). When supplement intake is not included in the estimate of vitamin D intake, correlation between dietary intake and vitamin D status is reduced (82). We have previously shown that dietary vitamin D intake from oily fish, milk, yoghurt/sour milk and margarine can be assessed with a FFQ targeting only these food groups in a small group of non-pregnant women (84). This short FFQ, called a vitamin D questionnaire (VDQ), was compared to a four-day food record. Overall, the VDQ underestimated vitamin D intake when compared to the food record, but produced very similar intakes on food group level. This was interpreted as promising but in need of verification in another study.

Pregnancy

Normal pregnancy lasts 40 weeks, calculated from the first day of the last menstrual period until delivery. Nowadays, pregnancy is often dated by ultrasound, as estimations based on last menstrual period are associated with some inaccuracies (85). The 40 weeks can be grouped into three parts, or trimesters. Typically, the first 12 (or sometimes 14) weeks are considered the first trimester, weeks 13-27 the second and 28-40 are the third trimester. The length of pregnancy, gestational age, is often defined as the number of completed gestational weeks plus the number of days into the following week. For instance, the third day of the 16th week of pregnancy is referred to as gestational week 15+3.

Pregnancy is achieved after successful implantation of the fertilized egg into the uterine lining. Conception usually occurs in the fallopian tube, where the initial stage of embryotic development takes place. During the ~ 3-day travel through the fallopian tube, the fertilized egg is first referred to as a morula. After an inner cell-mass can be defined, the morula is called a blastocyst. After approximately three days the fertilized egg reaches the uterus, where it develops for a few days before implanting (86). Implantation begins when the blastocyst attaches itself to the endometrium. The endometrium under goes changes, primarily under the influence of progesterone, and forms the decidua (86). The outer layer of the blastocyst consists of trophoblast cells that will develop into part of the placenta. As the blastocyst burrows into the decidua, the trophoblasts start to form the

finger like extensions, called villi, which are central structures of the future placenta. The villi will develop and interconnect with the maternal blood vessels and 17 days after fertilization, both maternal and fetal blood vessels are functional – the placental circulation is established (86). Maternal blood supply to the placenta is fully developed at the end of the third month, but the placenta continues to develop throughout pregnancy.

What to expect when expecting

Physiological adaptations of the female body are manifested very early during pregnancy. Blood volume starts to expand in gestational week 6-7 and reaches its peak of 130-150% in gestational week 32 (86, 87). However, not all components of blood expand equally. For instance, red cell mass increases to a lesser extent, which leads to a reduction in haemoglobin and risk of anaemia. Other physiological changes are increased heart size, increasing cardiac output and increased oxygen consumption. During normal pregnancy, blood pressure typically decreases in the first trimester as vascular resistance is reduced. In the second trimester, blood pressure increases until mid-pregnancy to return to pre-pregnancy levels in the third trimester (88). Levels of hormones such as oxytocin, peptide hormones and steroid hormones increase. Maternal metabolism also adapts to pregnancy. Calcium absorption is increased and renin secretion is higher, which stimulates aldosterone-driven absorption of sodium and the secretion of potassium. As pregnancy progresses, insulin resistance develops and glucose tolerance is reduced which leads to increments in circulating glucose. The plasma concentration of free fatty acids and triglycerides is also increased (86). Nutrients are transported across the placenta through several different mechanisms. Lipids are usually transported through simple diffusion, while amino acids require active transport. Glucose is the main energy substrate for the growing foetus and is transported via facilitated diffusion (86, 89). Disrupted placental nutrient transport can result in impaired fetal growth and development (89).

Complications of pregnancy

Pregnancy loss

The most common complication of pregnancy is pregnancy loss, often referred to as spontaneous abortion or miscarriage. This occurs in approximately 15% of

established pregnancies and up to 50% of preclinical pregnancies (90). In some cases, pregnancy loss does not present for a prolonged period after fetal or embryonic demise. This is called missed abortion or missed miscarriage. Pregnancy loss after gestational week 22+0 is referred to as intrauterine fetal death. After gestational week 8, approximately 95% of live embryos will continue to a live birth if there are no signs of threatening pregnancy loss, such as cramping or bleeding. The rate of pregnancy loss among live foetuses after gestational week 14-16 is 1% (90). Thus, the rate of pregnancy loss is dependent on gestational duration. Maternal risk factors for pregnancy loss include older age, tobacco use, alcohol consumption, psychological distress and a history of pregnancy loss but embryonic defects and defective implantation are the most common causes (90, 91).

Hypertensive disorders of pregnancy

Hypertensive disorders of pregnancy include pre-existing hypertension, new onset hypertension during pregnancy and preeclampsia. The general definition of hypertension as systolic/diastolic blood pressure at or above 140/90 mmHg applies also in pregnancy. Pre-existing, or chronic, hypertension is generally defined as at least two measures at or above 140/90 mmHg before gestational week 20. Preeclampsia is defined as at least two measures at or above 140/90 mmHg after gestational week 20, in addition to the presence of significant amounts of proteinuria (defined as ≥300 mg per day) (92). Preeclampsia can be divided into several subgroups: severe or moderate preeclampsia, and early or late onset preeclampsia. Severe preeclampsia is generally defined by the symptom severity, with higher blood pressure (160/110 mmHg) or signs of liver, renal, coagulation or neurological involvement (92). Early onset preeclampsia typically presents before gestational week 34. Early onset and severe preeclampsia poses greater risks to both woman and foetus. Pregnancy-induced hypertension is defined as at least two measures at or above 140/90 mmHg after gestational week 20. Among women who will consequently develop preeclampsia or pregnancy-induced hypertension, early pregnancy blood pressure is higher (93, 94) and the initial decrease in blood pressure is less pronounced (95).

It is not fully understood what causes hypertension and preeclampsia during pregnancy. Some risk factors are common for both conditions while others differ. Pregnancy-induced hypertension and preeclampsia are possibly not the same condition with differing symptom severity, but rather two separate conditions with different clinical characteristics (96). However, 10-50% of women who

presents with pregnancy-induced hypertension will subsequently develop preeclampsia (96). A large Swedish study showed that risk factors for preeclampsia but not for pregnancy-induced hypertension were type 1 diabetes, gestational diabetes and twin birth (97). A common risk factor for both disorders was maternal overweight, while non-Nordic origin, smoking and summer birth were protective factors for both disorders. This study concluded that the risk factors were sufficiently similar to suspect joint aetiology (97). In addition, previous preeclampsia constitutes a risk factor for recurrence in a subsequent pregnancy. This risk is further exacerbated by higher maternal age, higher pre-pregnancy BMI, inter-pregnancy weight gain and longer inter-pregnancy interval. Possibly, non-Nordic origin may be an additional risk factor for early preeclampsia recurrence (98). However, smoking and partner change are associated with a smaller probability of preeclampsia recurrence (98). Studies have shown that also paternal factors could contribute to preeclampsia development (99), but these probably play a limited role (100).

Fetal growth restriction

Fetal growth is not linear during pregnancy but fetal mass grows exponentially during initial gestation (101) (90). Low birth weight (LBW) can be a consequence of preterm delivery. In approximately one third of LBW cases, birth weight is low also when considering the gestational age at delivery (87). This is referred to as small for gestational age (SGA). Normal fetal growth is dependent on placental function and placental dysfunction can cause of abnormal growth. Consequently, conditions that are associated with placental dysfunction, such as hypertension and preeclampsia, are risk factors for impaired fetal growth (86). In addition, fetal infections and congenital abnormalities can also cause restricted growth. Maternal risk factors for impaired fetal growth include low pre-pregnancy BMI, low gestational weight gain, pre-existing disease and use of tobacco, drugs or alcohol. Multifetal pregnancy is also a risk factor. Excessive fetal growth is associated with maternal diabetes and obesity (102). A Swedish study also found that income and occupation affects the risk of having a child that is SGA (103).

Preterm delivery

Birth weight is largely dependent on gestational age at delivery. Normal gestational duration is 40±2 weeks. Delivery is considered preterm if it occurs before

gestational week 37+0. Delivery before gestational week 34 is often referred to as early preterm delivery. Preterm delivery is a major cause of perinatal mortality and morbidity, and is a major cause of cerebral palsy in Sweden (104). Preterm delivery can be a consequence of either spontaneous onset labour or induced labour. Risk factors for spontaneous preterm delivery are previous preterm delivery, multiple gestations (e.g. twin or triplet pregnancy), low socioeconomic status and African American ethnicity (in America) (105, 106). Major causes of induced preterm labour are intrauterine growth restriction and preeclampsia (86). Scandinavian studies also show that inter-pregnancy weight gain (107), maternal grief (108) and unhealthy diet (109) are risk factors for preterm delivery.

Swedish antenatal care

In Sweden, antenatal care is free of charge for all pregnant women. Approximately 8-10 visits to the antenatal care are typical during an uncomplicated pregnancy. Routine visits are conducted according to a "basic program" as seen in table 2. Typically, the pregnant women only meet an obstetrician if there is a gestational complication. Most visits and tests are therefore performed by midwives. Midwives also typically also deliver the baby. A routine ultrasound in gestational week 18-20 is offered to all pregnant women to accurately date the pregnancy, find multifetal pregnancies and to screen for fetal complications. However, many women are also offered an ultrasound before gestational week 14, as a part of screening for chromosomal abnormalities.

Table 2.
Example of basic program for antenatal care in the study region during data collection

GW	Weight	Iron status	Blood pressure	Glucose	SF	Fetal sounds
4-12	X	X	X	X		
25	X	X	X	X	X	X
29			X	X	X	X
32	X	X	X	X	X	X
35			X		X	X
37	X	X	X	X	X	X
40			X		X	X
40+			X		X	X

GW, gestational week; SF, symphysis fundus measure

The characteristics of the pregnant Swedish population at entry to antenatal care in 2014 are shown in table 3. The average age of the women was 30 years. Mean BMI was 24.8, and 25% of the women were overweight and 13% were obese. Almost 27% were born outside of Sweden and most immigrant women were born in Asia. Before pregnancy, 13% smoked while less than 6% did so in early pregnancy (110).

In 2014, preeclampsia was diagnosed in 2.8% of pregnancies in Sweden. The total rate of preterm birth was 5.8% and 2.5% of infants were born SGA, while 4.5% were born with LBW (110).

Table 3.

Characteristics of pregnant women in Sweden at entry to antenatal care during 2014, according to the Swedish Medical Birth Register (110)

	Sweden N=113 963*
Age, mean	30.3
Age, mean nulliparous	28.6
BMI, mean (kg/m^2)	24.8
Nulliparity (%)	43.1
University level education	52.3
Country of birth	
Sweden (%)	73.3
Europe, other (%)	9.4
Asia (%)	10.6
Africa (%)	5.2
America (%)	1.6
Tobacco use	
Smoking before pregnancy (%)	13.6
Smoking in early pregnancy (%)	5.5
Snuff use in early pregnancy (%)	1.3

**Number of deliveries in 2014, where pregnancy exceeded gestational week 22*

There is evidence to suggest that gestational complications differ depending on the ethnicity of the pregnant woman. Some of these differences are due to the different characteristics of women depending of their origin. Previous research

show that smoking and chronic hypertension is less common among pregnant women born outside the Nordic region who also are shorter, lighter and older (111). However, even after adjusting for these differences, women who are born outside the Nordic region have a higher incidence of gestational diabetes but a lower incidence of pregnancy-induced hypertension (111). Also, women born outside Sweden have higher rates of perinatal death and stillbirth (112, 113). Research also shows that women born outside Sweden utilize antenatal care to a lesser extent than women born in Sweden. Women born outside Sweden have fewer visits and register for antenatal care later in pregnancy (114).

Vitamin D status and gestational complications

Due to the plasma volume expansion in pregnancy, the plasma concentration of substances and nutrients is generally reduced. It is not entirely known how 25OHD concentrations are affected by pregnancy. Still, it would be reasonable to assume that also concentrations of vitamin D would be lower during pregnancy due to the plasma volume expansion. On the other hand, 25OHD is more closely related to the endocrine system than are other nutrients and might therefore be affected by hormonal changes due to pregnancy. This theory is supported by the fact that women who consume oestrogen, either in the form of hormonal replacement therapy or contraceptives, have higher 25OHD concentrations than non-users (41, 42).

Maternal vitamin D status has been associated with several complications of pregnancy. Generally, lower 25OHD has been associated with increased risk of complications such as miscarriage (115), preeclampsia (116), preterm delivery (117), gestational diabetes (117), impaired fetal growth (118), small for gestational age (117), delivery by caesarian section (119) and birth asphyxia (120).

Vitamin D status and preeclampsia

Preeclampsia is likely caused, at least in part, by suboptimal placental development. Some believe that this causes inflammatory processes, oxidative stress and a systemic condition that leads to the symptoms. Others believe that the reason for preeclampsia is that the mothers immunological adaptations to pregnancy are suboptimal (121). Vitamin D is suggested to play a role in the development of preeclampsia; either for placentation in early pregnancy or for the subsequent systemic condition. Vitamin D could potentially modify placentation and placen-

tal development by regulating the transcription of genes relating to implantation, placental invasion or artery formation (122-124). Further, vitamin D could modify the systemic response to a suboptimal placental development by modulating the inflammatory response or immune function (125, 126). Finally, vitamin D may protect the endothelial cells from negative effects of hypertension and oxidative stress (127).

Previous observational studies have found disparate results regarding the association between 25OHD concentration and preeclampsia. Typically, cohort studies do not see such an association (128-130) but some studies are likely underpowered to study this outcome. One previous cohort study, by Wei and colleagues, studied 25OHD concentration among Canadian women both early and late in the second trimester. The study found an association between 25OHD and preeclampsia in late but not in early second trimester (131). Most nested case-control studies have seen that poor vitamin D status is a risk factor for preeclampsia (132-135).

Vitamin D status, fetal growth and pregnancy duration

Previous studies of the association between maternal vitamin D status and infant birth size have not yielded homogenous results. Associations between poor vitamin D status and higher risk of infant SGA are quite consistent across studies (136-139) but there are exceptions where no such associations are found (140). Findings by Bodnar and colleagues indicate that 25OHD concentrations between 60-80 nmol/L are associated with the lowest risk of SGA while both higher and lower vitamin D status is associated with an increased risk (141). Fewer studies have investigated maternal vitamin D status in relation to infant LBW. An association between poor maternal vitamin D status and LBW is seen in some (139, 142) but not all studies (140). There might be a genetic component to the association between poor vitamin D status and lower birth weight, and some genetic variants might modify the effect associated with 25OHD concentration (143). Since the duration of pregnancy at birth is a major determinant of infant birth size, preterm birth is associated with LBW. Some studies have associated poor maternal vitamin D status with preterm birth (142, 144) while other have not (136, 140).

Vitamin D supplementation trials during pregnancy

Intervention trials with vitamin D during pregnancy show disparate results with regards to gestational and neonatal outcomes (145). A few American randomized controlled trials have studied the effect of vitamin D supplementation on pregnancy outcomes, but they lack a true placebo control group. One of these trials randomized 502 pregnant women to three doses of vitamin D (10, 50 or 100 µg daily) from gestational week 12-16 to delivery, and saw no differences in mode of delivery, gestational duration or birth weight (146). A second study with similar design was performed in 192 women with Arab background and low baseline vitamin D status. This study found no effects on birth size or gestational duration (147). A third American trial randomized 440 women to receive either 10 or 110 µg vitamin D per day during pregnancy, and reported no between group differences in birth weight, delivery mode or rate of preterm delivery (148). A placebo controlled trial from England randomized 1134 women with 25OHD concentration >25 nmol/L to either 25 µg vitamin D daily or placebo, from gestational week 14. There were no overall differences in infant anthropometry, though offspring bone mineral content was higher in the intervention group among those who delivered in winter (149). It is possible that studies would have an effect on obstetric outcomes if vitamin D supplementation was compared to true placebo, in women with vitamin D deficiency. It has been suggested that vitamin D supplementation should be targeted toward individuals with deficiency, since supplementation of women overall seems ineffective in order to reduce gestational complications (150).

A meta-analysis of 13 supplementation trials that gave women either vitamin D and placebo or vitamin D in combination with other nutrients such as calcium, concluded that vitamin D supplementation resulted in higher birth weight and length, but did not affect the rate of gestational complications (145). A 2016 Cochrane review concluded that there is some indication that vitamin D supplementation might reduce the risk of preeclampsia and increase length and head circumference at birth, but that confirmation in other trials is needed (151). Supplementation trials have differed in many ways, which might explain the disparate results. Such explanations can be differences in study design, study populations, vitamin D doses and statistical power (152).

Summary

Vitamin D status in pregnancy has been studied in many countries but not within a population-based setting in Sweden. As the Swedish population in many as-

pects differs from the populations in the neighboring countries, an investigation of the vitamin D status among pregnant women is warranted. Previous studies indicate that vitamin D status among pregnant women in Sweden is poor, but the studies are small. Previous research also relates poor maternal vitamin D status to complications of pregnancy that affect both mother and child. It is not known if these associations exist also in Sweden, where the antenatal care is utilized to a large extent, and the seasonal variation in vitamin D status is pronounced. It is also not known if associations between vitamin D status and complications differ in early and late pregnancy.

3. Aims

The overall aim of this thesis was to study vitamin D status, measured as 25OHD, and its associations to gestational complications in a population-based cohort of pregnant women in Sweden.

The specific aims of this thesis were to study:

1. Vitamin D status among pregnant women in Sweden and its determinants, and if the determinants differed between subgroups

2. If poor vitamin D status was a risk factor for preeclampsia and gestational blood pressure development.

3. If poor vitamin D status during pregnancy was a risk factor for neonatal SGA, LBW, preterm delivery or pregnancy loss.

4. Agreement between three methods of estimating dietary vitamin D intake, and the biomarker 25OHD.

4. Subjects and methods

Recruitment

Women were recruited to participate in the GraviD study when registering for antenatal care in parts of the region of Västra Götaland in Sweden. In total, 43 antenatal care clinics within the primary care participated in the study. The study area was located at latitudes 57-58° North, in southwest Sweden. Recruitment was performed during two time-periods; fall 2013 (September 2nd-November 8th) and spring 2014 (February 24th-June 13th). All women registering for antenatal care at the clinics included in the study were eligible for inclusion, as long as the pregnancy had not exceeded 16 gestational weeks. Ultra sounds to date the pregnancy is generally not performed until gestational week 18-20, so the inclusion criterion for gestational age was based on last menstrual period. During the six months of study recruitment, approximately 6600 women registered for antenatal care, though all were probably not eligible for inclusion. The midwives were asked to provide some data (age, country of origin and education) on the women who declined participation.

In order to promote participation in all ethnic groups, study information and informed consent were translated into eight languages besides Swedish: English, Polish, Arabic, French, Persian, Somali, Sorani and Turkish. Also, questionnaires were translated into English. In line with standard practice of care, interpreters were consulted when needed.

Data collection

All data collection was performed at two routine visits to the antenatal care (figure 2). Women who were included in the study were sampled for blood at gestational week <17 and >31, when they also answered a questionnaire regarding vitamin D exposure, education and origin. Education was classified as having attended school at primary level, secondary level and university level. Country of origin was defined by country of birth, and was classified according to continent. Europe was divided into North Europe (Nordic countries, the UK, Latvia and Lithuania) and Continental Europe (South, Central and East Europe). South

and North America were considered as one category. No woman was born in Oceania. The women also provided information on their skin colour, using the Fitzpatrick scale (153), based on how their skin reacts to the first hour of sun in the spring (scale from 1-6). They were also asked about their eye colour (blue, green, hazel or dark brown).

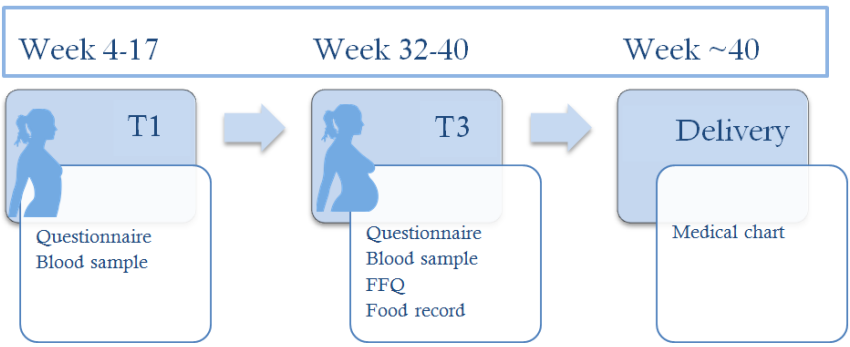

Figure 2. *The design of the GraviD study.*
T1, first trimester; T3, third trimester

Assessment of vitamin D exposure

At both study visits, participants were asked if they had travelled abroad in the past six months. The travel was considered relevant for vitamin D exposure if the latitude of the destination was <35°N. No criterion on the time spent on the destination was added to the definition. Clothing style was assessed by asking how often (often, seldom, never) the women exposed more skin than face and hands to the sun, in warm weather. Sun-seeking behaviour was defined by whether the women preferred sun or shade in sunny weather (prefer sun, shade or both). Season was defined as either November-April or May-October or as winter (December-February), spring (March-May), summer (June-August) or autumn (September-November).

In both T1 and T3, the women were asked if they used a food supplement containing vitamins, and if so, the brand, dose and duration of use. Nutritional content of the supplements was retrieved from manufacturers as soon as possible and classified by vitamin D content. All current use of supplements containing vitamin D was defined as vitamin D supplement use, and there were no criteria on dose or duration of use.

Assessment of vitamin D intake from diet was performed using a short VDQ on both occasions. The T1 questionnaire included questions on intake of oily fish and milk, while the T3 questionnaire also included questions on margarine and yoghurt/sour-milk. These forms could be filled out with support from midwives or interpreters, if needed. After the T3 visit, women were asked to fill out an online FFQ, called MealQ (154, 155) and an assessment tool for physical activity, ActiveQ (156). These questionnaires were only available in Swedish, and women were encouraged to use help from a relative or friend if needed. The FFQ has been validated but not among pregnant women. Therefore, a subgroup of 420 women was also asked to fill out a four-day food record. These women were study participants recruited at five antenatal care clinics, chosen to be representative of the cohort. In the food records, weights were measured if the women had scales, or estimated using provided illustrations of different portion sizes. A dietitian calculated the dietary intake using computer software Dietist XP (version 3.2), based on the Swedish National Food Agency's database (version 2013-10-04).

Medial records and definition of outcomes

After delivery, medical records from antenatal and obstetrics care were collected. Data were retrieved regarding parity, employment, BMI, weight, tobacco use, pre-existing medical conditions and complications during pregnancy or labour. Pregnancy loss was defined as miscarriage before gestational week 22 and intrauterine fetal death as pregnancy loss from gestational week 22+0. Preeclampsia was defined as ≥2 measures of high blood pressure (≥140/90 mmHg) and significant proteinuria (+1 on dipstick), after gestational week 20 in previously normotensive women. Pregnancy-induced hypertension was defined as ≥2 measures of high blood pressure (≥140/90 mmHg), after gestational week 20 in previously normotensive women. SGA was defined as weight and/or length at birth, below 2 SD of the population mean, specific for gender and gestational age. LBW was defined as weight at birth <2500 grams. Preterm delivery was defined as delivery before the completion of gestational week 37. Gestational age at delivery was defined by routine ultrasound. Measures of blood pressure and weight were performed according to standard practice of care and collected from medical records. According to standard practice, blood pressure is measured after 10 minutes of rest. Usually, weight is measured in clothes but should not include shoes or outerwear. Gestational weight gain was calculated as the difference between weight at registration for antenatal care and weight in gestational week 37 (±2 weeks). Excessive gestational weight gain was defined ac-

cording to the Institute of Medicines recommendations based on pre-pregnancy BMI (157).

Laboratory analysis

Blood sampling was first performed at registration for antenatal care in gestational week <17 and again at gestational week >31. Blood was drawn in serum gel tubes, and centrifuged for 10 minutes after 0.5-2 hours of sampling. Clinics that did not have access to a centrifuge were provided with one for the duration of the study. The centrifuged blood samples were kept in cardboard boxes and refrigerated until transport to the Sahlgrenska University Hospital where they were refrigerated until study personnel collected them. Serum was extracted and aliquoted by study personnel. For more than half of the samples (56%), serum was extracted within 12 hours of sampling, in 59% within 24 hours and in 95% within 36 hours. A few samples (1.7%) that had just exceeded 48 hours at extraction were included. The aliquots were frozen and stored at -70° C until analysis. Sets of samples were sent for analysis when both samples from each woman were collected. Serum samples were sent to the central laboratory at the University hospital in Malmö, Sweden for 25OHD analysis by LC-MS/MS (API 4000). The method separates 25OHD2 and 25OHD3 but not 3-epi-25OHD. The lower limit of detection is 6 nmol/L for both 25OHD2 and 25OHD3. The upper limit is 450 nmol/L for 25OHD3 and 225 nmol/L for 25OHD2. At 40 nmol/L, the coefficient of variation is 6% and at 120 nmol/L 4% for 25OHD3 and 5% for 25OHD2 (158). In total, eight groups of samples were sent to the laboratory between spring 2014 and spring 2016.

The last shipment (analysed in mid-2016) contained samples from the women who miscarried before gestational week 22, as these had not been prioritized initially. At this time, the LC-MS/MS instrument had been moved from the laboratory in Malmö to Lund. Eight samples that had previously been analysed were re-tested to study the reproducibility among low, midrange and high 25OHD concentrations. The results of these analyses are shown in table 4. There overall coefficient of variation was 4%.

Table 4.

Results from eight serum samples analysed twice for total 25-hydroxyvitamin D (25OHD).

	25OHD 2014 (nmol/L)	**25OHD 2016 (nmol/L)**	**Mean**	**SD**	**CV**
Sample 1	21	19	20.0	1.41	7%
Sample 2	17	20	18.5	2.12	11%
Sample 3	36	33	34.5	2.12	6%
Sample 4	40	39	39.5	0.71	2%
Sample 5	62	60	61.0	1.41	2%
Sample 6	76	78	77.0	1.41	2%
Sample 7	100	98	99.0	1.41	1%
Sample 8	104	99	101.5	3.54	3%
Mean	**57**	**56**	**56**	**1.77**	**4%**

25OHD, 25-hydroxyvitamin D; CV, coefficient of variation

Statistical analysis

Power calculation for the study was based on pregnancy-induced hypertension, and showed that 2000 study participants would yield 85% power to detect a doubled incidence of pregnancy-induced hypertension among women with 25OHD concentration <25 nmol/L compared women with concentrations ≥25 nmol/L.

Paper I

Determinants of continuous numeric outcomes (change in vitamin D status) were assessed using multivariable linear regression analysis. Determinants of dichotomous outcomes (vitamin D deficiency) were assessed using logistic regression analysis. Subgroup analysis of women in risk groups for vitamin D deficiency (women born in Africa and Asia) was performed. Potential confounding was identified using an ad hoc approach. Students' T-test was used to study differences between mean 25OHD at T1 and T3, during different seasons of the year. Chi square tests were performed to test differences in proportions.

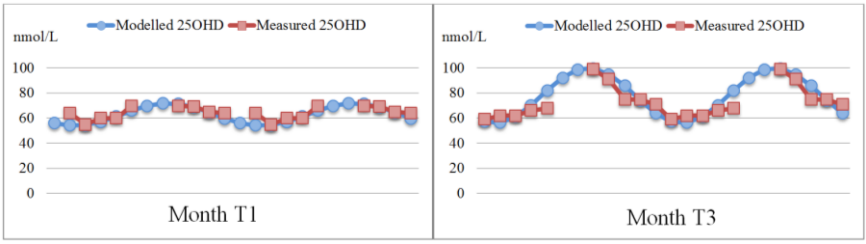

Figure 3. *The cosine function and the data it is based on, for season-correction of vitamin D status measured as 25-hydroxyvitamin D (25OHD) concentration in the first (T1) and third (T3) trimester of pregnancy*

In order to remove the seasonal variation in vitamin D status, a customized cosine function was created based on the data (figure 3). This was performed on T1 and T3 samples from the whole cohort and the subgroup born in Africa and Asia. These functions were used to predict season-corrected 25OHD concentrations.

Paper II

Determinants of continuous numeric outcomes (baseline blood pressure) were assessed using multivariable linear regression analysis. Determinants of dichotomous outcomes (preeclampsia and pregnancy-induced hypertension) were assessed using logistic regression analysis. Determinants of repeated measures continuous numeric outcomes (blood pressure throughout pregnancy) were assessed using mixed models analysis. Vitamin D status in T1 and T3, and change in vitamin D status from T1 to T3 were the independent variables in the analyses. Regression models included other relevant risk factors and potential confounding was identified using an ad hoc approach.

Paper III

Determinants of dichotomous outcomes (SGA, LBW, preterm delivery and pregnancy loss) were assessed using multivariable logistic regression analysis. Vitamin D status, modeled as both continuous and categorical variables, was the independent variable. Potential confounding was identified using directed acyclic graphs (159).

Paper IV

Wilcoxon signed ranks test for related samples was used to compare vitamin D intakes between methods. Linear regression and correlation was used to compare vitamin D intakes between methods and with 25OHD concentration. The triads method was used to calculate validity coefficients for the short VDQ, food record and 25OHD concentration (80). In addition, subgroup analysis among wintertime samples was performed.

Ethical considerations

This study has ethical approval from the Regional Ethics Committee in Gothenburg (Dnr 897-11, T439-13). All procedures were conducted in line with the Declaration of Helsinki. Study information and consent forms were available in nine languages in order to promote participation of women from many ethnic groups, and to ensure that information was understood. In line with standard practice of care, interpreters were present at the visit if needed. The participants were informed that they could withdraw from the study at any time, without giving cause. Written and informed consent was provided by all included women to ensure voluntary participation. Out of the 2134 women who agreed to participate, nine failed to provide complete consent forms and were therefore not included. All their data and biological samples were discarded.

Risks of participation were considered limited to discomfort at blood sampling. Sampling in early pregnancy was performed at routine blood sampling when an additional 15 ml blood was drawn for study purposes. In late pregnancy, an additional 15 ml blood was drawn at a visit when blood sampling is not routinely performed. Thus, discomfort at sampling was only attributed to participation in the study at one occasion. After analysis of vitamin D status, the results were entered into the women's medical records and could be shared at a post-partum visit to the antenatal care. Women with vitamin D deficiency were also notified via mail and encouraged to consult their physician.

5. Results

Study participants

In total, 2125 women were included to participate in the study (figure 4). Three participants were excluded from all analyses, due to gestational duration >17 weeks at inclusion. Therefore, 2122 participants were included in these papers. There were 44 women who were excluded due to terminated pregnancy or were lost to follow up. T1 samples were available from 2078 women, but six were discarded due to diversions from the study protocol. Thus, T1 25OHD was analysed for 2072 women. In T3, 1836 of the women were sampled for 25OHD but four samples were discarded due to diversions from the study protocol. After delivery, 2000 medical records could be retrieved from the obstetrics care.

Women who miscarried between T1 and gestational week 22 were included only in paper III. Women with multifetal pregnancy (twins or triplets) were included in paper I and II but not in paper III.

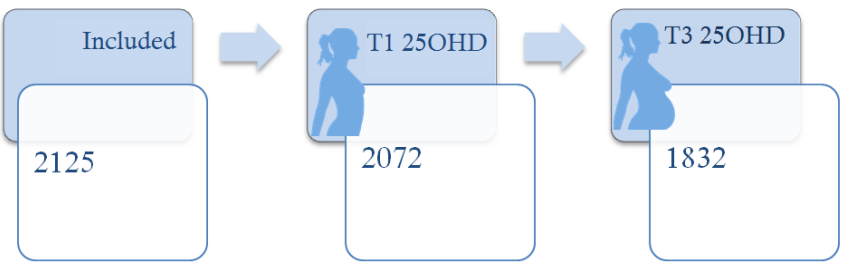

Figure 4. *Flow chart of the GraviD study.*
T1, first trimester; T3, third trimester; 25OHD, 25-hydroxyvitamin D

The T1 blood sample was drawn in mean gestational week 11 and the T3 sample in mean gestational week 34. The women who were included in the study were 31 years old on average, had a BMI of 24.5 and a mean gestational weight gain of 13.5 kg (table 5). Almost half of the women expected their first child and 35% were either overweight or obese at inclusion. In total, 13% reported smoking before pregnancy and 4% still smoked in T1. More than half of the women had a university level education and 75% were employed. Almost half of the women

used vitamin D supplementation in either T1 or T3. Most women had not travelled to a southern latitude (<35°N) prior to T1 (26% had travelled) or T3 (17% had travelled). In total, 26% of the women were born outside of Sweden. Most women who were not born in Sweden were born in Asia (10%), mainly West Asia, followed by Continental Europe (7%), Africa (6%), America (2%) and North Europe (1%). In total, the participating women were born in 91 different countries. The most common countries of birth besides Sweden were Somalia, followed by Iraq, Poland, Bosnia and Iran. The study sample is comparable to the general Swedish pregnant population in terms of mean BMI and ethnicity, while mean age and the proportion with higher education and tobacco use are similar to the population (110).

Table 5.
Characteristics of the GraviD study participants

	Mean (SD)	
Age (years)	31.3	(4.9)
Height (cm)	166.8	(6.3)
Weight T1 (kg)	68.1	(12.6)
BMI T1 (kg/m^2)	24.5	(4.2)
Gestational age T1 (weeks)	10.8	(2.0)
Gestational age T3 (weeks)	33.4	(1.9)
Gestational weight gain[1] (kg)	13.5	(5.1)
	N	**(%)**
Born in Sweden	1479	(74)
Overweight T1 (BMI 25–29.9)	489	(25)
Obese T1 (BMI ≥30)	203	(10)
Vitamin D supplement use T1	868	(43)
Vitamin D supplement use T3	842	(42)
Travel <35°North before T1[2]	516	(26)
Travel <35°North before T3[2]	347	(17)
Tobacco use at T1 (any)	89	(4)
University education level T1	1190	(60)
Employment T1	1501	(75)
Nulliparity T1	836	(42)
Preeclampsia	80	(4.1)
Pregnancy-induced hypertension	160	(8.0)
Small for gestational age	99	(5.0)
Low birth weight (<2500 gr)	70	(3.5)
Preterm delivery (<37 weeks)	92	(4.2)

[1]From gestational week <12 until gestational week ≥35; [2]Travelled <35° North in the past six months
T1, first trimester; T3, third trimester

Non-participation

During the study's six-month recruitment period, approximately 6600 women registered for antenatal care in the study area. In total, 2509 women were approached to participate in the study and 383 women (15%) declined participation. They did not have to give a reason as to why. Women who declined participation had a mean age of 30 years and 45% had a university level education. In total, 69% were born in Sweden and 70% in North Europe, while 10% were from Continental Europe, 8% from Africa, 11% from Asia and 1% from America.

Paper I. Longitudinal vitamin D status and its determinants

Of the 2122 included women, 1985 serum samples were analyzed for 25OHD in T1 and 1832 in T3. The mean (SD) 25OHD concentration was 65 (25) nmol/L in T1 and 75 (34) nmol/L in T3 and the mean change was an increase of 10 (30) nmol/L.

Vitamin D deficiency in early pregnancy

In the whole cohort, 10% were vitamin D deficient with 25OHD concentrations <30 nmol/L in T1. Among women born in Africa and Asia, 51% and 46% were vitamin D deficient.

The significant determinants of vitamin D deficiency in T1 were non-North European origin, sampling in spring, never exposing skin when sunny, no vitamin D supplementation, lower dietary vitamin D intake and younger age. Among women born in Africa and Asia, the determinants of vitamin D deficiency in T1 were no vitamin D supplementation, never exposing skin when sunny and younger age (table 6).

Change in vitamin D status during pregnancy

In T1, 6% of the women had 25OHD ≥100 nmol/L while 24% had concentrations ≥100 nmol/L in T3 ($p<0.001$). Mean (SD) season-corrected 25OHD concentration was 63 (24) nmol/L at T1 and 75 (31) nmol/L at T3.

The significant determinants of season-corrected change in 25OHD concentration during pregnancy were country of origin, sun-seeking behaviour, clothing style, dietary vitamin D intake, vitamin D supplementation and having travelled <35°N. In the subgroup born in Africa and Asia, the only significant determinant of change in 25OHD concentration was vitamin D supplementation (table 7).

Table 6.

Determinants of vitamin D deficiency in the first trimester of pregnancy

	ALL WOMEN		WOMEN BORN IN AFRICA AND ASIA	
	OR	**95% CI**	**OR**	**95% CI**
Country of origin				
North Europe (ref)				
America	5.13*	1.01-26.15		
Continental Europe	4.55***	2.16-9.57		
Asia	22.09***	11.51-42.42		
Africa	9.74***	4.09-23.18		
Season T1				
Sept-Nov (ref)				
Mar-May	2.17**	1.35-3.49	1.22	0.62-2.41
Dec-Feb	1.91	0.29-12.38	10.01	0.63-160.02
Jun-Aug	0.59	0.13-2.63	0.22	0.03-1.72
Sun-seeking behaviour				
Prefer sun (ref)				
Prefer both sun and shade	0.91	0.48-1.74	0.78	0.33-1.89
Prefer shade	0.64	0.23-1.82	0.47	0.14-1.64
Clothing when sunny				
Often expose skin (ref)				
Seldom expose skin	1.68	0.88-3.21	1.44	0.56-3.72
Never expose skin	6.04***	2.80-13.02	6.52***	2.56-16.60
Vitamin D supplement T1				
No (ref)				
Yes	0.09***	0.05-0.17	0.04***	0.02-0.12
Travel <35°North before T1				
No (ref)				
Yes	0.70	0.40-1.22	0.97	0.47-1.98
Tobacco use T1				
No (ref)				
Yes	2.18	0.88-5.39	4.71	0.42-52.74
Vitamin D dietary intake T1	0.82**	0.71-0.95	0.84	0.70-1.01
BMI T1	1.02	0.97-1.07	1.01	0.93-1.09
Age	0.88***	0.83-0.93	0.89**	0.82-0.96

p<0.005, ** p<0.001, *<0.001*

T1, first trimester

Table 7.

Determinants of change in vitamin D status during pregnancy

	ALL WOMEN Adjusted R2= 0.186		**WOMEN BORN IN AFRICA AND ASIA** Adjusted R2=0.115	
	B	**P**	**B**	**P**
Country of origin				
North Europe (ref)				
America	-4.61	0.227		
Continental Europe	-4.75	0.018		
Africa	-10.95	<0.001		
Asia	-16.99	<0.001		
Sun-seeking behaviour				
Prefer sun (ref)				
Prefer both sun and shade	-3.18	0.022	-2.56	0.437
Prefer shade	-1.13	0.718	-4.60	0.348
Clothing when sunny				
Often expose skin (ref)				
Seldom expose skin	-4.69	0.003	-5.85	0.085
Never expose skin	-7.54	0.007	-5.37	0.105
Vitamin D supplement T3				
No (ref)				
Yes	16.68	<0.001	13.67	<0.001
Travel <35°North before T3				
No (ref)				
Yes	3.57	0.006	-0.92	0.780
Vitamin D dietary intake T3	0.99	0.002	1.07	0.119
BMI T1 (kg/m2)	-0.09	0.489	0.30	0.301
Age T1 (years)	0.22	0.082	0.27	0.271
Gestational weight gain (kg)	-0.15	0.126	0.06	0.805

T1, first trimester; T3, third trimester

One quarter of the women had an increment ≥30 nmol/L from T1 to T3. Significant determinants of a ≥30 nmol/L increase in 25OHD from T1 to T3 were season at T3, vitamin D supplement use, country of origin and clothing style. Compared to women who were sampled during autumn, women who were sampled in T3 during summer were more likely to have a ≥30 nmol/L increase (OR=5.8, p<0.001). Women sampled in spring (OR=0.7, p<0.001) or winter (OR=0.6, p<0.001) were less likely to have a ≥30 nmol/L increase, compared to those sampled during autumn in T3. Women who used vitamin D supplementation in T3 were more likely to have a ≥30 nmol/L increase than non-users (OR=2.7, p<0.001). Lastly, women born in Africa (OR=0.3, p=0.004) and Asia (OR=0.14, p<0.001) were less likely to have a ≥30 nmol/L increase, compared to women born in North Europe.

Paper II. Vitamin D status and hypertension

In total, 4% (n=80) of the women in the study developed preeclampsia and 8% (n=160) pregnancy-induced hypertension. Data on vitamin D status in early pregnancy were available for all cases of preeclampsia and for all but two cases of pregnancy-induced hypertension. Data on vitamin D status in late pregnancy were available for 70 of the preeclampsia cases and 151 of the pregnancy-induced hypertension cases.

Preeclampsia and pregnancy-induced hypertension

Preeclampsia was inversely associated with vitamin D status in T3 (OR 0.99, p=0.043) but not in T1. In addition, change in vitamin D status was inversely associated with preeclampsia (OR 0.99, p=0.021). Women with an increase in 25OHD ≥30 nmol/L had lower odds of preeclampsia, compared to the other women. Other predictors of preeclampsia were multifetal gestation, obesity, nulliparity, diastolic blood pressure in T1 and preexisting medical condition (table 8).

Vitamin D status was not associated with pregnancy-induced hypertension in the fully adjusted model. The predictors of pregnancy-induced hypertension were height, diastolic blood pressure in T1 and a trend toward significance for excessive gestational weight gain (appendix II).

Blood pressure

Systolic blood pressure in T1 was associated with T1 25OHD concentration, obesity, parity, height and age. Diastolic blood pressure in T1 was associated with T1 25OHD concentration, obesity, parity and preexisting medical condition (table 9). In mixed models analysis, T1 25OHD was positively associated with systolic blood pressure trajectory, while the trajectory of 25OHD was associated with diastolic blood pressure trajectory (table 10).

Table 8.

Determinants of preeclampsia

	OR	95% CI	P
Δ25OHD ≥30 (nmol/L)	0.221	0.08-0.58	0.002
Multifetal gestation	11.332	2.34-54.82	0.003
Obesity T1	3.391	1.73-6.66	<0.001
Nulliparity	4.188	2.28-7.70	<0.001
Diastolic blood pressure T1	1.094	1.06-1.13	<0.001
Preexisting medical condition T1	3.435	1.03-11.45	0.045
Excessive gestational weight gain	1.677	0.95-2.96	0.074
Assisted reproduction	1.271	0.64-2.52	0.491
Age ≥40 years T1	1.126	0.24-5.28	0.880

25OHD, 25-hydroxyvitamin D; T1, first trimester

Table 9.

Determinants of systolic and diastolic blood pressure in early pregnancy

	Systolic blood pressure[1]		Diastolic blood pressure[1]	
	B	P	β	P
25OHD (nmol/L) T1	0.03	0.022	0.02	0.016
Obesity T1	4.91	<0.001	4.26	<0.001
Nulliparity	2.40	<0.001	1.22	0.001
Preexisting medical condition	1.36	0.147	2.33	0.001
Height (cm) T1	0.12	0.004	0.00	0.901
Assisted reproduction	1.00	0.401	-0.22	0.802
Age ≥40 T1	2.57	0.043	1.20	0.200

25OHD, 25-hydroxyvitamin D; T1, first trimester

[1]Adjusted for baseline tobacco use, multifetal pregnancy, North European birth country, baseline employment status, gestational age at sampling, month of conception

Table 10.

Mixed models analysis of the determinants of systolic and diastolic blood pressure trajectory during pregnancy

	Systolic blood pressure		**Diastolic blood pressure**	
Adjusted[1]	**Coefficient**	**P**	**Coefficient**	**P**
25OHD trajectory (nmol/L)	0.007	0.223	0.009	0.047
BMI ≥30 T1	-2.794	<0.001	-1.569	0.004
Nulliparity	3.067	<0.001	2.202	<0.001
Preexisting medical condition T1	1.619	0.027	1.836	0.001
Age ≥40 years T1	1.992	0.043	0.864	0.226
Assisted reproduction	0.247	0.791	-0.441	0.514
Height (cm) T1	-0.088	0.009	-0.134	<0.001
Weight trajectory (kg)	0.282	<0.001	0.205	<0.001

25OHD, 25-hydroxyvitamin D; T1, first trimester

[1]*Adjusted for baseline tobacco use, multifetal pregnancy, Northern European birth country, baseline employment status, gestational age at sampling and month of conception*

Paper III. Vitamin D status in relation to fetal growth and survival

In total, 4.5% infants were born SGA, 2.8% with LBW and 3.8% were delivered preterm. In total, 4.7% of the women experienced pregnancy loss and of these, nine cases were intrauterine fetal death.

First trimester vitamin D status

The 25OHD concentration in T1 was only significantly associated with pregnancy loss (OR 0.99, p=0.046). T1 25OHD concentration was not associated with SGA, LBW or preterm delivery.

Third trimester vitamin D status

The 25OHD concentration in T3 was associated with SGA and LBW, and there was a trend for preterm delivery (figure 5). Women with 25OHD concentration ≥100 nmol/L in T3 had lower odds of SGA (OR=0.32, p=0.03) and LBW (OR=0.22, p=0.046), compared to women with vitamin D deficiency (<30 nmol/L).

Change in vitamin D status from the first to the third trimester

Change in vitamin D status during pregnancy was related to SGA, LBW and preterm delivery. Compared to women with a large increase in 25OHD concentration (≥30 nmol/L) from T1 to T3, those with a decrease in 25OHD concentration had higher odds of SGA, LBW and a trend for preterm delivery. A small increase (<30 nmol/L) in 25OHD concentration from T1 to T3 was associated with higher odds of SGA, preterm delivery and a trend for LBW (figure 6), compared to a large increase.

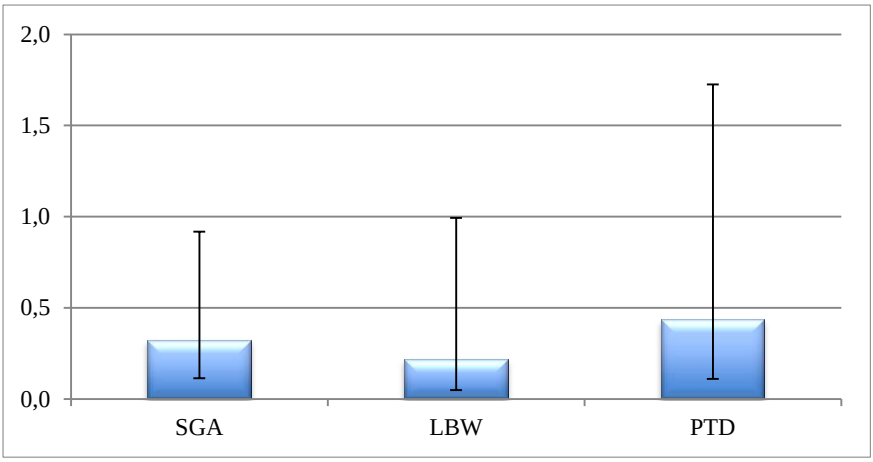

Figure 5. *Odds ratios (95% CI) of small for gestational age (SGA), low birth weight (LBW) or preterm delivery (PTD) among women with third trimester 25-hydroxyvitamin D concentration≥100 nmol/L, compared to <30 nmol/L. Adjusted for education, origin, season of conception and BMI at T1.*

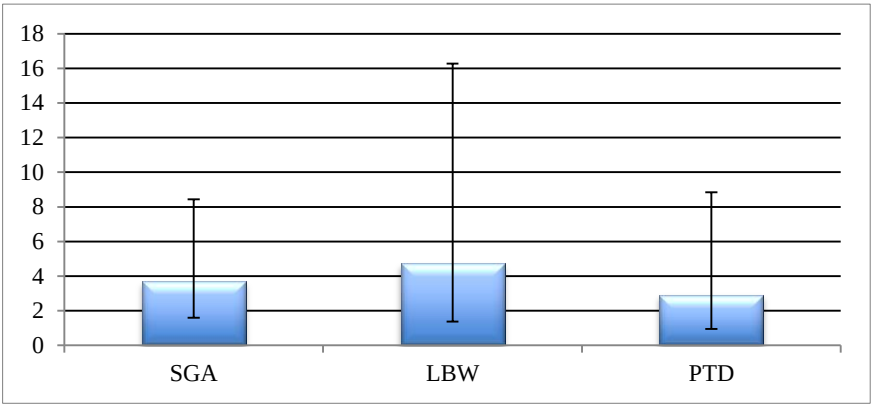

Figure 6. *Odds ratios (95% CI) of small for gestational age (SGA), low birth weight (LBW) or preterm delivery (PTD) among women with a decrease in 25-hydroxyvitamin D concentration from early to late pregnancy, compared to women with an increase ≥30 nmol/L. Adjusted for education, origin, season of conception, BMI at T1 and 25OHD at T1.*

Paper IV. Assessment of vitamin D intake

A total of 1800 women answered the short VDQ in T3, while 1160 answered the FFQ and 85 women provided a complete four-day food record. In total, 64 women provided data on dietary vitamin D intake using all three methods.

According to the food records, the largest contributor to dietary vitamin D intake was oily fish, followed by margarine, milk, lean fish and yoghurt/sour-milk. The four foods included in the short VDQ (oily fish, milk, yoghurt/sour-milk and margarine) contributed to 56% of the total amount of vitamin D obtained from the food record.

Median (IQR) dietary vitamin D intake was 5.0 (3.4-9.0) µg/day assessed by food record, 5.2 (3.8-7.0) µg/day assessed by FFQ and 3.8 (2.7-5.3) µg/day assessed by the short VDQ (figure 7). The median vitamin D intake from the food record differed significantly from the short VDQ but not from the FFQ. Energy intake in eight FFQs was considered implausible (<500 or >5000 kcal/day). When they were excluded, median dietary vitamin D intake was 5.3 µg/day. Median dietary vitamin D intake per 1000 kcal was 2.7 µg assessed by food record and 3.0 by FFQ. The short VDQ did not provide an estimate of energy intake, and vitamin D density could therefore not be calculated.

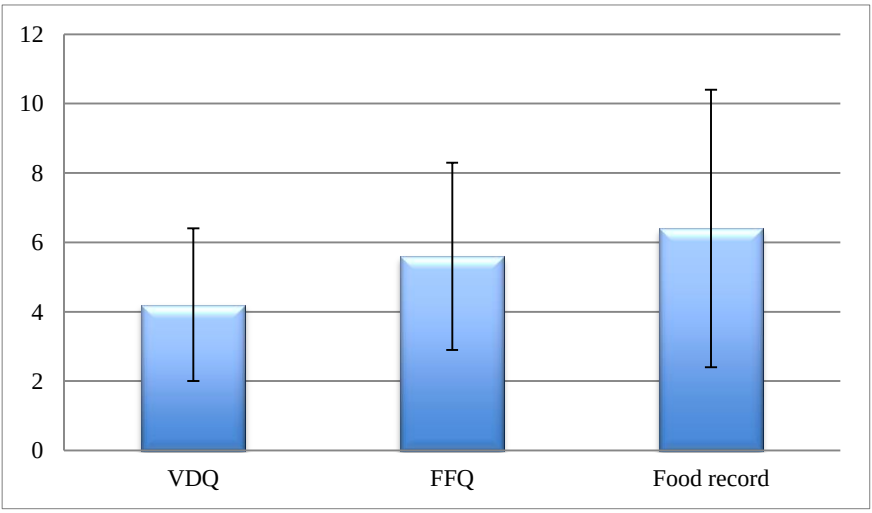

Figure 7. *Mean (SD) daily dietary vitamin D intake (µg) assessed by the vitamin D questionnaire (VDQ), food frequency questionnaire (FFQ) and four-day food record.*

Among the women who completed all three methods, vitamin D intake from the food record correlated significantly with the vitamin D intake from the FFQ and the short VDQ (appendix IV). Vitamin D status, measured as concentration of 25OHD, correlated significantly only with the intakes estimated by the short VDQ (appendix IV). This result did not change when limiting the correlation to those who participated during winter. In addition, results from regression analysis showed that dietary vitamin D intake assessed by the short VDQ but not FFQ or food record, was significantly associated with 25OHD concentration. The validation coefficient was higher for the short VDQ than for the four-day food record and the FFQ (appendix IV).

6. Discussion

The main findings of this thesis are that vitamin D status of pregnant women in Sweden is overall adequate, and is associated with pregnancy complications. The initial concern that many would have poor vitamin D status was unsupported. However, vitamin D deficiency was common among women born in Africa and Asia. In this subgroup of the pregnant Swedish population, approximately 50% were vitamin D deficient. The results also show an inverse association between vitamin D status in late pregnancy and preeclampsia, SGA and LBW. These associations were not seen for early pregnancy vitamin D status. A novel finding is that changes in 25OHD during pregnancy are related to preeclampsia, SGA, LBW and preterm delivery. An increase in vitamin D status ≥30 nmol/L from early to late pregnancy seems protective. Lastly, the method chosen for assessment of vitamin D intake has an impact on the intake estimate. The short VDQ tool performed better than a longer FFQ or a food record in assessing dietary vitamin D intake, when compared to vitamin D status.

Study participants

Participation rate

No reliable data exist on the number of women who did not receive information about participation the study. During the study's recruitment period, approximately 6600 women were registered for antenatal care in the study area. As 2509 women were approached, most women do not seem to have received an invitation to the study. Midwives provided information that 15% of the approached women declined to participate. If all 6600 women who registered at the time of recruitment had been eligible for inclusion, 38% would have been invited to participate and 32% would have agreed. However, it is unlikely that all women fulfilled the inclusion criterion of gestational duration <17 gestational weeks at inclusion. Therefore, participation rate among eligible women is probably closer to 40%. Among women who declined participation, age at registration was slightly younger, education level lower and more were born outside Sweden.

This does not seem to have had a major impact of the characteristics of the final study sample, as it seems to represent the Swedish population.

Population-based cohort?

The aim of the GraviD study was to recruit a cohort of pregnant women that reflected the general pregnant Swedish population. This was successful, with minor diversions between the sample and the population with regards to education level and age. Women in the GraviD cohort are approximately one year older (31 years) than the national average (30 years) for women who register for antenatal care (110). In the GraviD cohort, 60% had a university level education, compared to 52% in the pregnant population. These small differences can be a reflection of that the GraviD study's main recruitment was performed in the city of Gothenburg, the second largest city in Sweden. Thus, the study cohort is likely a reflection of an urban population and might therefore be population-based within this setting. It is however important to keep in mind that education level is known to affect health. In 2016, The Public Health Agency of Sweden reported that lower education is associated with poorer overall health and that the citizens with low education are almost invariably the ones with the poorest health (160). Pre-pregnancy use of tobacco in the study (13%) was comparable to national statistics (13.6%). Tobacco use in early pregnancy in the study (4.5%) was more common in Sweden at large (5.5%) (110). The proportion of women born outside Sweden and mean BMI are almost identical to the national average for pregnant women (110). Most who were born outside Sweden were born in Asia followed by other parts of Europe and Africa, and this is also representative (110). Due to slight differences in education and tobacco use, it is possible that the women in the GraviD cohort are healthier than the average pregnant woman in Sweden. Again, it is possible that the women in the study reflect the pregnant population within the study area also in these aspects.

Incidence of pregnancy complications

The large sample size of over 2000 women is a strength of this study, although most pregnancy complications are uncommon and power may be inadequate to provide robust risk estimates. Preeclampsia and SGA seems more common in the GraviD cohort, while there is a lower rate of preterm delivery and LBW compared to national statistics (161). When defining SGA only by birthweight the study rate (2.1%) is comparable to the national incidence (2.5%). The higher

preeclampsia rate in the study could be explained by under-diagnosis by obstetrics care. In the GraviD cohort, 4% met the criteria for preeclampsia but only 2.8% were registered preeclampsia cases, which is identical to the national statistics. The rates of preterm delivery and LBW in the GraviD cohort are approximately 1% lower than the national statistics. This could be a consequence of higher education and less tobacco use during pregnancy among women in the GraviD cohort, compared to national data. In addition, slight variations in incidence due to chance are likely.

Main findings in relation to other research

Paper I

The mean 25OHD concentration in early pregnancy in the GraviD cohort was comparable to findings from Norway (50) and Denmark (51, 52), all using LC-MS/MS assays. However, the pregnant population in Sweden is not identical to those of other Nordic countries. For instance, use of fish oil supplementation is more common in Norway and that could contribute to vitamin D status (62, 63). Also, the fraction of the population with an immigrant background is larger in Sweden than in Norway or Denmark (64). This could suggest that the Swedish national average vitamin D status is somewhat lower than in other Nordic countries, though this is not supported by our findings. Both first and third trimester 25OHD concentrations in our study are higher than what was presented in a smaller Swedish study from 2016 (39). That study was conducted in the northern city of Umeå (latitude 64°N), and it is possible that vitamin D status lowers with increasing latitude even within the country. The LC-MS/MS assay also had a higher coefficient of variation than the assay used in our study, which could imply that it is less reliable (39). This is hypothetical since a high coefficient of variation does not necessarily affect the mean value. In addition, the previous BUGA study found a lower vitamin D status among women in the same study area, though used a different method for 25OHD analysis (1). Ideally, data on vitamin D status among pregnant women in the Nordic region should be harmonized (e.g. by the Vitamin D Standardization Program) in order to enable national comparisons and the possibility of pooling data.

Few studies have assessed vitamin D status longitudinally during pregnancy, and even fewer cohort studies with power to study the determinants of 25OHD concentration and changes in 25OHD during pregnancy. We found that there is a

significant increase of approximately 10 nmol/L in 25OHD concentration from mean gestational week 11 to 34. A Canadian cohort study has previously analyzed 25OHD concentrations twice during pregnancy, and the findings suggested a slight increment in vitamin D status during pregnancy of approximately 1 nmol/L in 10 weeks (131). In contrast, data from the Southampton's Women's Survey indicate a slight decrease in 25OHD concentration from gestational week 11 to 34 (55). These data should perhaps be interpreted with caution since early pregnancy samples were analyzed five years after the late pregnancy samples, using a different 25OHD assay. Smaller studies have also assessed pregnancy-related changes in vitamin D status with different results. The Swedish study from the city of Umeå included 184 women and found that 25OHD concentration increased with ~10 nmol/L from gestational week 12 to 35 (39). A study from Ireland found that 25OHD concentration decreased throughout pregnancy, though recruitment of the 30 women in the study took place in summer and decreasing 25OHD concentrations might be expected (162). Interestingly, the same study found that concentrations of vitamin D binding protein increased with pregnancy duration, which confirms previous findings (163). The assay used to assess 25OHD concentration could perhaps explain some of the disparate findings, as the GraviD study and the Umeå study used LC-MS methods while the studies that did not find an increase used antibody based methods (55, 162). In addition, changes in vitamin D status during pregnancy are likely dependent on external factors such as season and lifestyle that can vary between studies.

Determining changes in vitamin D status during pregnancy is associated with an inherent problem with seasonality, as a pregnancy typically lasts nine months and therefore covers almost all four seasons. Consequently, vitamin D status is expected to change regardless of any pregnancy-related influence on 25OHD concentration. In order to combat this dilemma, season-correction of vitamin D status has been utilized to make samples taken at different gestational ages, and therefore seasons, comparable. This was performed on the data from the Southampton's Women's Survey and season-correction yielded a higher correlation between early and late pregnancy samples (55). That same study also found that determinants of season-corrected changes in 25OHD concentration were gestational weight gain, physical activity and vitamin D supplementation. In the GraviD study, the determinants of vitamin D deficiency and of change in season-corrected 25OHD concentrations were similar, highlighting the role of sun exposure and vitamin D intake for vitamin D status. Sun exposure might be a smaller contributor to vitamin D status among women born in Africa and Asia.

Approximately one quarter of the women had an increase of at least 30 nmol/L during pregnancy and this large increase was associated with season, vitamin D supplement use, origin and clothing style. The GraviD study sampled women in early pregnancy during either autumn or spring, and in late pregnancy approximately six months later. Therefore, sampling in early pregnancy was performed when vitamin D status was at its highest or lowest and the consecutive sample was taken when the season had changed. This means there is a methodological explanation for the large seasonal variation in 25OHD concentration in our study. Interestingly, few women had a large decrease in vitamin D status during pregnancy, despite being in early pregnancy during autumn. This could indicate that hormonal changes associated with pregnancy has an impact on 25OHD concentrations and that the pregnancy-related increments previously observed in vitamin D binding protein (162) and 1,25OH2D (163) are also reflected in 25OHD. The observed increment in 25OHD could also be the result of an accumulative effect of the low dose vitamin D supplementation used in this cohort.

Paper II

We found that late pregnancy 25OHD concentration, but not early, was inversely associated with preeclampsia. Earlier studies that have studied vitamin D status in relation to preeclampsia show disparate results. A potential explanation for this is that the populations in different studies differ both in terms of vitamin D status but also in rates of pregnancy complications. Other probable explanations are differences in study design, statistical testing and power. However, our results suggest that the gestational duration at 25OHD sampling is also a factor to take into consideration.

Few previous cohort studies have sampled women as early in pregnancy as we have, but many have sampled very early in the second trimester. A prospective cohort study carried out in a socially disadvantaged part of USA suggested that vitamin D status in mean gestational week 14 was inversely associated with preeclampsia (164). This is contradicted by cohort studies from New Zealand and China that sampled women early in the second trimester and found no association with preeclampsia (128, 130). Further, an American study found no association between preeclampsia and vitamin D status in mean gestational week 28, but a positive association with pregnancy-induced hypertension (129). A Canadian study is, to our knowledge, the only other prospective cohort study to assess vitamin D status at two time-points in relation to preeclampsia, and found that lower vitamin D status in gestational week 24-26 was associated with

preeclampsia (131), but there was no association with vitamin D status in week 12-18. They also found an indication, though not significant, that women with normal pregnancy have a small increase in 25OHD concentration that was not seen in those who developed preeclampsia (131). This supports our findings that associations between vitamin D status and preeclampsia are not constant during the course of pregnancy, and that increment in 25OHD concentration is associated with a decreased probability. Overall, most prospective cohort studies do not find an association between preeclampsia and vitamin D status, and most have sampled in the second trimester of pregnancy.

Findings from nested case-control studies usually indicate associations between 25OHD concentration and preeclampsia (132, 134) or severe preeclampsia (133, 135) but not in all studies (136). Nested case-control studies usually have a high proportion of cases (i.e. women who have developed preeclampsia) and therefore have better statistical power to detect differences. However, biased differences between the selected controls and the cases cannot be ruled out.

Previous research has also found that women with preeclampsia have a lower response in vitamin D status to seasonal changes, which also support our findings regarding the protective associations of increments in 25OHD (165). In addition, preeclampsia incidence has been associated with season (128, 166).

Paper III

We found that late pregnancy 25OHD concentration, but not early, was inversely associated with SGA and LBW, and that change in vitamin D status was associated with SGA, LBW and preterm delivery.

Previous findings relating vitamin D status to infant size or gestational age at delivery are conflicting. Data from New Zealand and Spain show no associations between vitamin D status in gestational week 14-15 with SGA or preterm birth (128, 140). This is supported by a Chinese study that found no association between second trimester 25OHD and SGA or LBW, but a positive relation to preterm delivery (130). American studies show an increased risk of SGA among women with lower vitamin D status in the second trimester (137, 167). Findings also show that associations between vitamin D status and birth weight are modified by factors that can differ between populations. The association between vitamin D status and birth weight might be more pronounced in women with certain genetic variants (143). In addition, adequate vitamin D status has been

shown to reduce the probability of SGA in Caucasian but not African American women, and in non-obese but not in obese women (167). This could mean that the disparate results from different studies are due to differences in the study populations.

In the GraviD study, we also found an association between early pregnancy 25OHD concentration and pregnancy loss. This association was quite weak, and was not seen when vitamin D status was expressed as a categorical variable. Previous studies have found disparate results regarding the association between vitamin D status and miscarriage. A Danish cohort study found that early pregnancy 25OHD concentration was lower among those who would subsequently miscarry in the first trimester, but not in the second (115). An Australian cohort study found no association between miscarriage and vitamin D status in gestational week 10-14 (136). A possible reason for the association in our study, is that women who would subsequently lose their pregnancy registered earlier for antenatal care. An inclination to register for antenatal care early could reflect lifestyle or health choices that could decrease the risk of pregnancy loss. It is also possible that women who were not born in Sweden (and thus had higher risk of low vitamin D status) utilized antenatal care to a lesser extent and therefore were less likely to report pregnancy loss. This theory is supported by previous research from Sweden that found foreign-born women to have a lower utilization of planned antenatal care and they also registered for care later in pregnancy (114). Since our results were adjusted for gestational age at registration for antenatal care, this is probably not the entire explanation.

Paper IV

Previous research supports our finding that a short FFQ, such as the VDQ, is a valid tool to assess dietary vitamin D intake (80-83). However, previous studies have used considerably longer FFQs to assess vitamin D intake, compared to the short VDQ used in the GraviD study. In addition, previous studies all seem to have included supplementary vitamin D intake in the estimated total intake of vitamin D, and have only sampled during wintertime (80-83). These differences in methodology might explain the lower correlation coefficient for the short VDQ compared to 25OHD concentration in our study.

There are many reasons why vitamin D intake is not always reflected in vitamin D status. Content of 25OHD in animal foods has been suggested to contribute to vitamin D status but not to estimated vitamin D intake, as 25OHD is not meas-

ured in food or included in nutrient databases (168). The absorption of ingested vitamin D might also be impacted of the fat content of the meal (169) which could interfere with the association between ingested vitamin D and vitamin D status. In addition, farmed fish may have lower vitamin D content than wild-caught fish, which might not be reflected in all dietary databases (170). Further, the cooking method might considerably decrease the vitamin D content in various foods (18, 171). Our findings suggest that also the dietary assessment method used is pivotal in determining if vitamin D intake is reflected in vitamin D status. The short VDQ tool was the best method to estimate dietary vitamin D intake, when using vitamin D status as a reference. It is uncertain if the VDQ would perform as well in a population where dairy products do not contribute substantially to vitamin D intake.

Mechanisms for vitamin D in pregnancy

There are several hypotheses for the physiological role of vitamin D in pregnancy, and how it relates to gestational complications.

There are some potential windows of opportunity for vitamin D status to affect the development of gestational complications. There is early pregnancy, when trophoblast invasion and placental development occurs and late pregnancy when the increasing fetal growth demands more nutrients and oxygen (102). Results from in vitro studies suggest that vitamin D (25OHD and 1,25OH2D) can facilitate trophoblast invasion and thereby placental development (172). Vitamin D is also believed to regulate inflammation in the placenta, which could contribute to normal placental development (173). Previous studies indicate that metabolites of vitamin D act by upregulating the transcription of transport proteins that carry amino acids across the placenta (174, 175) and 25OHD concentration is correlated with expression of amino acid placental transport proteins (175). Transport and supply of amino acids is important for fetal growth and development (176).

Besides placental function, vitamin D status has also been linked to endothelial function in middle-aged men and women (177) and in normotensive women (178). This could indicate a systemic effect of vitamin D status on e.g. blood pressure. In vitro studies indicate that vitamin D might have the ability to reduce the negative effects of preeclampsia (179). These results could point to a protective effect of 25OHD on the progression of gestational complications, and attenuate the effects of faulty implantation and placental development. This would

support our findings that late pregnancy (rather than early) vitamin D status is associated with pregnancy complications.

Whether vitamin D status during pregnancy has an actual effect on risk of complications is difficult to determine from an observational study such as the GraviD study. It is unclear if the beneficial properties of increasing vitamin D status observed in this study are due to other factors associated with vitamin D status. These potential unknown confounders could be associated with metabolism, endocrinology or aspects associated with healthy pregnancy. It is possible that the associations seen between vitamin D status and pregnancy complications are the results of confounding variables that were not adequately measured in this study.

A possible explanation for the associations observed in this thesis, is that vitamin D status could be a reflection of overall nutritional status or intake. This could be due to supplementation or dietary intake. Supplementary vitamin D intake is associated with a lower incidence of preeclampsia (180), but also other nutrients could contribute. For instance, calcium and selenium supplementation seems to decrease the risk of developing preeclampsia (181, 182), while supplementation with vitamin C and E does not seem to prevent development of gestational complications (183). In the GraviD study, use of supplements containing vitamin D was associated with both higher vitamin D status and larger increase in 25OHD during pregnancy. Multivitamins and minerals were the most common supplements. Therefore, in increasing vitamin D status by supplementation, other nutrients were often also consumed (e.g. iron, zinc, folate). However, supplement use did not confound the associations between 25OHD concentration and complications. This indicates that supplement use was not the reason for the associations observed. Dietary intake may also be associated with gestational complications such as preeclampsia (184, 185) and preterm delivery (186). Since dietary intake is a minor source of vitamin D, vitamin D status is probably not a major reflection of dietary intake. However, we did not adjust for dietary vitamin D intake in any analysis and confounding can therefore not be ruled out. Vitamin D status also changes with season, and season is associated with several gestational complications and outcomes (187). One could speculate that change in 25OHD concentration is a function of season, and that season could be the real culprit in the associations with preeclampsia, SGA, LBW and preterm delivery. However, season was included in the regression models and was not a major confounder. The results from the GraviD cohort show that vitamin D supplementation and summer season increases 25OHD concentration, and that vitamin D status is

associated with gestational outcomes. However, these associations do not seem to be caused by vitamin D exposure, but rather vitamin D status per se.

Finally, a major determinant of vitamin D status was ethnicity. It is possible that normal pregnancy differs between women of different ethnicities. For instance, African American women have higher rates of preterm delivery but not higher rates of neonatal morbidity (90), which might indicate that normal gestational duration may be shorter in this group. This could mean that women in Sweden who are born in Africa have a shorter normal gestational duration and that the association between vitamin D deficiency and preterm delivery is a reflection of ethnic differences in the progression of normal pregnancy.

Methodological considerations

Analysis of 25-hydroxyvitamin D

The data on vitamin D status in this cohort have not been standardized according to the Vitamin D Standardization Programme. However, a 2015 standardization of Nordic data on vitamin D status showed that mean standardized 25OHD concentrations in non-pregnant populations were in line with our findings on early pregnancy vitamin D status (35). In the GraviD study, only 25OHD was measured. It would have been interesting to see if increasing concentrations of the vitamin D binding protein could explain the increasing vitamin D status during pregnancy. In addition, the routine LC-MS/MS method used in this study can separate 25OHD3 from 25OHD2 but not 3-epi-25OHD from 25OHD (158). The significance of this is unclear, but it is an unlikely source of major measurement error, as 3-epi-25OHD is found in relatively small amounts in pregnant women (26). This molecule also seems to act in the same manner as 25OHD, albeit with a lower bioactivity, which might warrant its inclusion in total 25OHD concentration.

The LC-MS/MS method used to analyze 25OHD is a strength of this study, and the method estimated vitamin D status with high reproducibility. In addition, two 25OHD measures were available for most women. The GraviD study is to our knowledge the largest cohort study to measure 25OHD concentrations longitudinally during pregnancy. The results from the study suggest that gestational duration at blood sampling matters, for both vitamin D status and for associations

with gestational complications. Therefore, gestational week at sampling should be considered in future research.

Assessment of vitamin D exposure

FFQ data on vitamin D intake was only available from about half the cohort, and food records from only 4%. This could mean that there was a selection bias, whereby only the most motivated and possibly health-aware women answered the FFQ and food record. However, subgroup analyses among women who provided data from all three methods did not substantially change the effect estimates, though statistical power was lost. This suggests that the potential selection bias was probably not a major source of error. Also, comparisons to 25OHD enabled an objective reference method. Even though 25OHD does not only reflect dietary intake of vitamin D, the other contributors to vitamin D status (such as supplementation and sun exposure) should not differ between methods and 25OHD is therefore most likely a useful reference method.

Comparing three methods for assessment of dietary vitamin D intake is a strength, as the three methods have inherent different sources of bias and are therefore suitable for comparisons. For instance, food records are not associated with the recall bias of a questionnaire method (78). In addition, using 25OHD as a biomarker of vitamin D intake was successful as it correlated with vitamin D intake from the short VDQ even though only four dietary sources were included. The study also had enough participants to allow for subgroup analyses among those sampled in winter, though the low response rate for the food record probably reduced the statistical power for this method. The short VDQ does not provide dietary intake beyond vitamin D, and might therefore be suitable as a complement to a method that provides more comprehensive dietary data, such as a FFQ or food record. Even though the short VDQ does not reflect the total dietary intake of vitamin D, it is probably a useful tool in identifying individuals with low and high vitamin D intakes.

Measures of sun exposure in this study were season, sun-seeking behaviour, clothing style and travels to southern latitudes. Travels to southern latitudes were defined as <35°N but exact locations were mostly unspecified. The 35th latitude on the northern hemisphere crosses the southern parts of the Mediterranean Sea – a popular destination for the women in the GraviD study. As the exact location was not specified, some of these travels were possibly incorrectly classified as <35°N. This may have led to errors in the classification of the variable. Howev-

er, this is probably not a major source of error, since UV-B exposure does not abruptly disappear above the 35th latitude. All trip durations were considered but all were at least three days long. Sun-seeking behaviour and covered clothing style were estimated with rather crude questions, but were still reflected in vitamin D status.

The importance of skin pigmentation for vitamin D status is well known. Skin pigmentation was estimated using the Fitzpatrick scale (1-6), based on how easily the skin tans and burns. However, the data seemed to be centred on the middle of the scale, regardless of where the women were born. Therefore, a question on eye colour was added to the T3 questionnaire. Eye colour has previously been used as a proxy of skin pigmentation (188) but this data was highly correlated with origin and was therefore not included in the same regression models. Consequently, no estimate of skin pigmentation was used. Having a measure of skin pigmentation might have provided additional information, especially in the large group of women born in Sweden.

Definition of outcomes

In this study, definitions of preeclampsia, pregnancy-induced hypertension, preterm delivery and LBW were based on well-established and commonly used criteria (86, 87, 90, 92). However, the definition of SGA as either length or weight below two SD of the population mean is perhaps a bit more uncommon. This definition of SGA is regarded as applicable, especially in combination with head circumference (189), which we did not include in the definition. In choosing to define SGA by length and/or weight, the number of cases doubled and the statistical power was therefore increased. In reviewing the medical records, most neonates who were SGA by length, were close to being SGA also by weight. This was regarded as further support for the definition used in paper III.

Statistical analysis

All regression models were adjusted for other variables, which decreases the risk of confounding bias. As a rule of thumb, the number of variables included in linear regression models should not exceed one per 10 observations (i.e. maximum of 10 variables if the outcome includes 100 observations). Likewise, the number of variables included in logistic regression models should not exceed 1 per 10 cases (i.e. maximum 10 variables if the outcome has 100 cases). Some

logistic regression models had more variables included, in particular the preeclampsia models. However, the models were run with fewer variables and this did not change the results for vitamin D status. Fewer variables were included in the models for SGA, LBW and preterm delivery. Overall, very little confounding was detected even when adjusting for major determinants of vitamin D status such as season and ethnicity. This suggests that the associations between vitamin D status and gestational outcomes were not caused by other factors such as sun exposure, ethnicity or supplementation.

All results presented in these papers are based on the same data. Multiple testing is a concern, and p-values should be interpreted with caution. Further, power calculations were only based on pregnancy-induced hypertension. The low rate of complications is a limitation that reduces statistical power. Insufficient power is probably not a major limitation in the study since significant associations were found for 25OHD in late pregnancy, despite that there were fewer T3 samples than T1 samples. It is possible that there is an association between early pregnancy vitamin D status and gestational complications, but that the effect was too small to detect in the GraviD study.

7. Conclusions

In paper I, the results show that vitamin D status was generally adequate among pregnant women in Sweden, but deficiency was common among women born in Africa and Asia. These women might benefit from extra support from the health care system in order to increase their vitamin D status. Overall, vitamin D status increased from early to late pregnancy, by approximately 10 nmol/L. Determinants of vitamin D status and of changes in vitamin D during pregnancy were sun exposure and vitamin D intake.

Results from papers II and III show that vitamin D status in late pregnancy, but not early, was related to several complications for both mother and child. Vitamin D status in early pregnancy was inversely related to miscarriage. The results from papers II and III also show that an increase of at least 30 nmol/L in 25OHD concentration from early to late pregnancy is inversely related to preeclampsia, small for gestational age, preterm delivery and low birth weight. However, causality cannot be established from this observational study. Therefore, the results need to be confirmed in a randomized controlled trial of vitamin D supplementation in pregnancy.

The results from paper IV highlight the impact of the chosen method for assessment of vitamin D intake. A short FFQ with only four food items was a valid method to assess vitamin D intake from dietary sources.

8. Future Perspective

The GraviD study showed that vitamin D status in late pregnancy and changes in vitamin D status during pregnancy are associated with several gestational complications. However, we also found that vitamin D status was generally adequate among pregnant women in Sweden. This finding suggests that a nationwide supplementation program might not be the way to go, in order to prevent complications during pregnancy. Nevertheless, some complications were more common among women with vitamin D deficiency. Therefore, a nationwide screening for vitamin D deficiency during pregnancy might be a strategy to prevent these complications. It is unclear however, if vitamin D supplementation can reduce these risks. The largest trials conducted so far do not support this hypothesis. The previous trials have for ethical reasons either been unable to use a true placebo or unable to include women with vitamin D deficiency. Therefore, well-designed supplementation trials that include vitamin D deficient women should be carried out to verify the causality before vitamin D supplementation is recommended to all pregnant women. It would be motivated to carry out such a study in a Swedish setting, as many women belong to ethnic groups with high risk of vitamin D deficiency.

Regardless of whether vitamin D status has any impact on pregnancy outcomes, pregnancy could be a good time to identify deficient women. For some women, pregnancy might be the first real contact with health care. Therefore, identification and treatment of women with poor vitamin D status during pregnancy could be beneficial for overall bone health throughout the life course. Also, the largest intervention trial conducted so far found beneficial effects on infant bone health in subgroups, which could further motivate treatment of vitamin D deficient pregnant women. This could mean that improving vitamin D status among pregnant women could be beneficial, not only for her but also for the future health of her child.

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