Vitamin D in women of reproductive age and during pregnancy

Focus on intake, status and adiposity

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ABSTRACT

Vitamin D is attained either through synthesis in the skin by sun exposure or through diet. Vitamin D status is important for skeletal health but optimal vitamin D status may also be important in the development of other diseases such as type 2 diabetes, gestational diabetes, preeclampsia, and cancer. Circulating vitamin D is known to be decreased in obese compared to non-obese individuals. There is a lack of documented knowledge on vitamin D status and intake in Swedish women of reproductive age and during pregnancy.

The aim of this thesis was to compare vitamin D status and intake between obese and normal-weight women. In a cross-sectional study in women of reproductive age and in a longitudinal study during pregnancy, blood samples, adipose tissue biopsies, and information on dietary intake were collected. Data on lifestyle including physical activity and sun exposure were also collected.

Vitamin D status, measured as serum 25-hydroxyvitamin D [25(OH)D], was lower in obese women of reproductive age compared with normal-weight women. In contrast, circulating vitamin D-binding protein was higher in the obese women. Despite reporting a higher vitamin D intake, the obese pregnant women had lower serum 25(OH)D compared with normal-weight women in early pregnancy. A higher proportion of the obese compared with normal-weight women had 25(OH)D concentrations that might be defined as insufficient. Circulating 25(OH)D concentrations below 25 nmol/L were uncommon in both pregnant and non-pregnant women. Dietary vitamin D intake was between 7.2 and 8.8 μg/day during pregnancy and in non-pregnant obese and normal-weight women, and a major part did not reach national dietary recommendations. There were no major differences in vitamin D intake between obese and normal-weight women. Vitamin D and its metabolites were detected in adipose tissue and were localized in the lipid droplet in the adipocyte.

The present studies show that Swedish obese women of reproductive age and during pregnancy have lower circulating 25(OH)D compared with normal-weight women but few had very low concentrations. However, what effects an increased circulating 25(OH)D would have on long-term health in obese individuals is yet to be studied. The fact that obese women had higher circulating vitamin D-binding protein is interesting and should be further

examined to clarify why, and what impact that may have on the action of vitamin D. We found no evidence of a lower vitamin D intake in obese women, thus, the intake was not contributing to the lower circulating 25(OH)D. Many women do not reach the recommendations for vitamin D intake. Actions should be taken to improve dietary intake of vitamin D in women of reproductive age and during pregnancy, this might have future implications not only for women's health but for generations to come. Intervention studies are urgently needed to explore the effect of vitamin D status and intake during pregnancy and in obese subjects.

Keywords: Vitamin D, Obesity, Pregnancy, Vitamin D intake

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Sammanfattning

D-vitamin får vi antingen genom syntes i huden från solexponering eller genom kostintaget. D-vitamin är viktigt för benhälsa men kan också vara viktigt i utvecklingen av andra sjukdomar såsom typ 2 diabetes, graviditetsdiabetes havandeskapsförgiftning och olika former av cancer. Få studier har undersökt D-vitamin status och intag hos kvinnor i barnafödande ålder och under graviditeten i Sverige.

Syftet med denna avhandling var att undersöka och jämföra D-vitaminstatus och intag hos normalviktiga och obesa gravida kvinnor och hos kvinnor i barnafödande ålder. I en tvärsnittsstudie på kvinnor i barnafödande ålder och i en longitudinell studie på gravida samlades blodprover, fettvävsprover samt information om solexponering och kostintag in.

Vi fann att obesa kvinnor hade lägre D-vitaminnivåer, mätt som serum 25-hydroxyvitamin D [25(OH)D], jämfört med normalviktiga. Däremot hade de obesa icke-gravida kvinnorna högre nivåer av det protein som transporterar D-vitamin i blodet. Fler obesa i jämförelse med normalviktiga kvinnor hade serum 25(OH)D nivåer som kan anses som otillräckliga men få kvinnor hade nivåer under 25 nmol/L. Intaget av vitamin D från kosten var mellan 7.2 och 8.8 µg/dag hos gravida och icke-gravida normalviktiga och obesa kvinnor och många nådde inte de nationella rekommendationerna för D-vitaminintag. Det fanns inga större skillnader i D-vitaminintag mellan obesa och normalviktiga kvinnor. D-vitamin och dess metaboliter mättes i fettväv och återfanns i den lipiddroppe som fyller fettcellen.

Sammantaget visar våra resultat att obesa kvinnor har ett lägre D-vitaminstatus jämfört med normalviktiga kvinnor. Att obesa hade högre nivåer av det protein som transporterar D-vitamin är intressant men behöver studeras mer för att förklara orsak och vilken betydelse detta kan ha. Vi fann inga bevis för att ett lägre D-vitaminintag hos de obesa kvinnorna kunde förklara de lägre 25(OH)D nivåerna funna hos obesa. Många kvinnor har ett D-vitaminintag under rekommendationer och det bör göras nationella insatser för att öka intaget vilket kan ha effekt inte bara för de gravida och icke-gravida kvinnornas hälsa men också hos efterföljande generationer. Interventionsstudier behövs för att undersöka effekterna av D-vitaminstatus och intag under graviditet och bland obesa.

LIST OF PAPERS

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

I. **Increased vitamin D-binding protein and decreased free 25(OH)D in obese women of reproductive age**
Therese Karlsson, Amra Osmancevic, Nina Jansson, Lena Hulthén, Agneta Holmäng, and Ingrid Larsson
Eur J Nutr 2013 E-pub ahead of print 21 April

II. **Lower vitamin D status despite higher vitamin D intake in early pregnancy in obese compared with normal-weight women**
Therese Karlsson, Louise Andersson, Aysha Hussain, Marja Bosaeus, Nina Jansson, Amra Osmancevic, Lena Hulthén, Agneta Holmäng, and Ingrid Larsson
Submitted Manuscript

III. **A new approach to measuring vitamin D in adipose tissue using time-of-flight secondary ion mass spectrometry: A pilot study**
Per Malmberg, Therese Karlsson, Henrik Svensson, Malin Lönn, Nils-Gunnar Carlsson, Ann-Sofie Sandberg, Eva Jennische, Amra Osmancevic, and Agneta Holmäng
Submitted Manuscript

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Abbreviations

ANOVA	Analysis of variance
BMI	Body mass index
BMR	Basal metabolic rate
CLIA	Chemiluminescent immunoassay
DBP	Vitamin D-binding protein
ELISA	Enzyme-linked immunosorbent assay
FM	Fat mass
FFQ	Food frequency questionnaire
GDM	Gestational diabetes mellitus
HPLC	High performance liquid chromatography
IL-6	Interleukin-6
IOM	Institute of medicine
IU	International units
LC-MS	Liquid chromatography-mass spectrometry
LGA	Large for gestational age
mRNA	Messenger ribonucleic acid
NNR	Nordic nutrition recommendations
PAL	Physical activity level
PCA	Principal component analysis
PONCH	Pregnancy obesity nutrition & child health
PTH	Parathyroid hormone
RCT	Randomized controlled trial
RIA	Radioimmunoassay
SAT	Subcutaneous adipose tissue
SD	Standard deviation
SGA	Small for gestational age
TOF-SIMS	Time-of-flight secondary ion mass spectrometry
UVB	Ultraviolet B
VAT	Visceral adipose tissue
VDR	Vitamin D receptor
WHO	World health organization
$1\alpha,25(OH)_2D$	1α,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D

1 Introduction

Rickets, a bone-deforming disease in children, was first described in the mid-17th century. The association of rickets with a lack of exposure to sunlight along with the fact that ingesting cod liver oil could cure it was suggested during the 19th century. In the beginning of the 20th century, it was fully established that this disease could be cured by either of the above two measures. Since that time, it has been discovered that it was the vitamin D in cod liver oil and the photosynthesis of vitamin D in the skin exposed to sunlight that had the antirachitic effect.[1]

Vitamin D has, throughout the 20th century, predominantly been associated with calcium homeostasis and bone health, but during recent decades an extensive interest in vitamin D status and other health outcomes has been on the rise. In observational studies, vitamin D status has been associated with non-skeletal diseases such as type 1 and 2 diabetes, cardiovascular disease, multiple sclerosis and some cancers.[2] Additionally, pregnancy complications such as gestational diabetes (GDM), pre-eclampsia, and small for gestational age (SGA) have also been associated with vitamin D status.[3] If a causal link can be proven, and subsequently a general increase in vitamin D status could reduce the prevalence of these diseases, this would have a big impact on public health. Concerns have been raised that vitamin D deficiency might be widespread in the general Swedish population, but there are few studies supporting this.

Obesity is prevalent all over the world and is associated with increased morbidity and mortality. Obesity is common in women of reproductive age and hence also during pregnancy. During pregnancy and consequently in women of childbearing age, nutritional status is of particular importance. Nutritional status during these times affects not only women's health but also has the potential to affect the health of generations to come. Maternal obesity during pregnancy increases the risks for complications for both the woman and her child, and some of the complications associated with lower vitamin D status also coincide with risks due to maternal obesity. If an optimal vitamin D status improves health during pregnancy and could easily be achieved in obese women, this has the potential to have large public health effects.

2 Background

2.1 Obesity

Obesity is defined by the World Health Organization (WHO) as having a body mass index (BMI) ≥30 kg/m^2. BMI is calculated as body weight in kilograms divided by height in meters squared (kg/m^2). Obesity is the result of an accumulation of excess fat over a period of time stemming from positive energy balance, i.e. that energy intake exceeds energy expenditure. Worldwide, the prevalence of obesity has increased since the 1980s and is one of the most important public health problems. There is an increased risk for morbidity such as type 2 diabetes, cardiovascular disease, musculoskeletal disease, and some cancers in obesity.[4] WHO has classified overweight and obesity, primarily based on the association between BMI and mortality (Table 1).[5] The prevalence of obesity in women aged 20-49 in Sweden 2010-2011 was between 7.0 and 11.7%.[6]

Table 1. BMI classifications of obesity in adults

Classification	BMI (kg/m^2)
Underweight	<18.5
Normal weight	18.5 – 24.9
Overweight	25.0 – 29.9
Obesity class I	30.0 – 34.9
Obesity class II	35.0 – 39.9
Obesity class III	≥40.0

2.2 Obesity in pregnancy

Obesity is not uncommon during pregnancy. In 2010, 25 and 13% of women registering for antenatal care in Sweden were overweight or obese, respectively.[7] Maternal obesity during pregnancy has been linked with an increased risk for GDM,[8] pre-eclampsia,[9] caesarean section,[10] large for gestational age (LGA),[11] and preterm delivery.[12, 13]

2.3 Vitamin D

2.3.1 Photosynthesis

When exposed to solar ultraviolet B (UVB) radiation (wave length 290-315 nm), cholecalciferol (vitamin D_3) can be synthesized in human skin. The cholesterol precursor 7-dehydrocholesterol located in the plasma membrane in skin absorbs the penetrating UVB photons and pre-vitamin D_3 is formed.[14] Previtamin D_3 is readily transformed to vitamin D_3 by thermally induced isomerization and then released into the circulation bound to the vitamin D-binding protein (DBP) (also named Gc-protein or Gc-globulin).[15] Humans are thought to be protected from vitamin D toxicity from UVB radiation since UVB exposure also converts previtamin D_3 to the inert molecules lumisterol and tachysterol,[16] and vitamin D_3 to 5,6-*trans*-vitamin D_3, suprasterol I and suprasterol II.[17] The process of synthesis of vitamin D_3 in the skin is illustrated in Figure 1.

The level of synthesis will be affected by any factors altering the amount of UVB radiation entering the skin. Factors such as cloudiness, ozone, latitude, time of day, and time of year will all affect the amount of radiation available to the skin.[18, 19] Studies performed at latitudes similar to those in Sweden (latitude 55 to 69 degrees N) show that during late autumn to early spring there is little or no synthesis of vitamin D in the skin, due to the quality and quantity of solar radiation.[18, 20, 21] Synthesis in the skin will decrease with increased skin pigmentation,[22] age,[23] use of sunscreen,[24] and clothing.[25] The sun exposure behavior of the individual will subsequently also affect the amount synthesised in the skin.[19] Additionally, use of sunbeds initiates synthesis of vitamin D in the skin.[26]

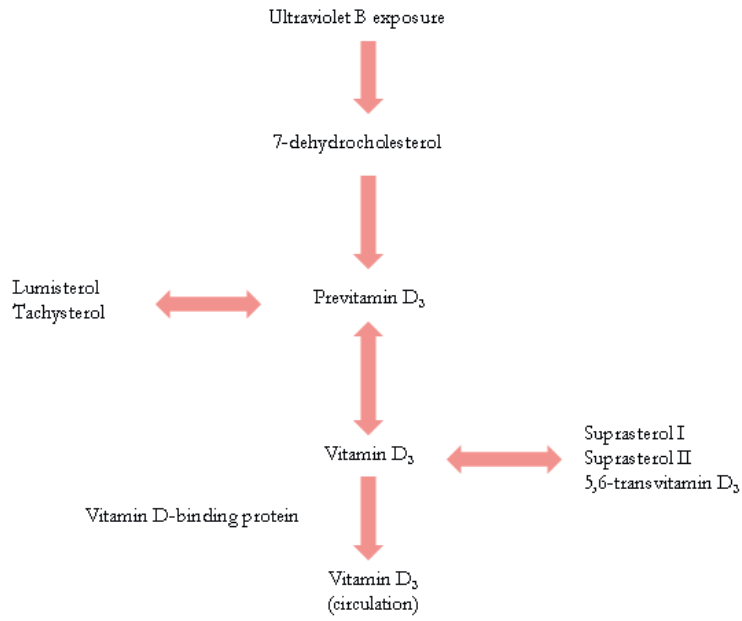

Figure 1. Photosynthesis and of vitamin D in the skin

2.3.2 Vitamin D in foods

Ergocalciferol (vitamin D_2) and vitamin D_3 can both be obtained through diet. Structurally, vitamin D_2 and D_3 differ only in their side chains (Figure 2). In this thesis, the term vitamin D refers to both vitamin D_2 and D_3, although the two forms are distinguished when needed. The amounts of vitamin D in foods and supplements are expressed as micrograms (μg), but International Units (IU) are otherwise also used and 1 μg is equivalent to 40 IU.

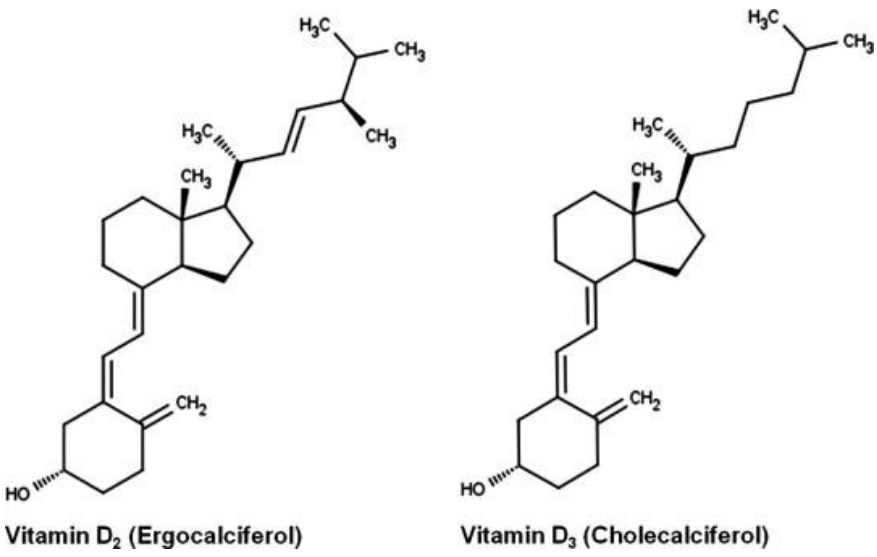

Figure 2. Chemical structure of vitamin D_2 and D_3 (Reproduced with permission, Elsevier)

There are few dietary sources naturally containing vitamin D; the best dietary sources are fish[27] and egg yolks.[28] Some foods are fortified with vitamin D, and together with vitamin D from dietary supplements, these contribute to vitamin D intake. In Sweden, low-fat (fat content ≤1.5%) milk, soured milk, and some yoghurt are fortified with vitamin D_3, as well as margarines (both spreads and cooking fats). Vitamin D_3 is generally thought to be present in primarily fatty types of fish, but studies also report considerable amounts of vitamin D_3 in lean fish types.[27, 29] Also, some vitamin D is present in meat. Ergosterol, the precursor of previtamin D_2, is present in fungi and yeast. When exposed to UVB radiation, the ergosterols in mushrooms are converted to vitamin D_2[30] and vitamin D_2 has been found in chanterelles.[31] In addition, some fortified soy and oatmeal drink products are available that contribute vitamin D_2. Table 2 shows the vitamin D content in some foods.[29, 32] The main dietary sources of vitamin D in Sweden are fish (32%), spreads (14%), and dairy products (12%).[33] Fortification routines differ across the world regarding amount and type of foods fortified. In the United States and Canada, not only are foods such as dairy products fortified, but also bread, cereals, and orange juices as well.[34, 35] In some foods, such as egg yolks and meat, 25-hydroxyvitamin D [25(OH)D] is present and may add to the intake of vitamin D from these foods.[36]

Table 2. Vitamin D content in various foods

Natural sources	μg/100 g
Salmon, fresh wild	12.5
Salmon, fresh farmed	11.3
Salmon, cooked	16.6
Mackerel, fresh	12.8
Mackerel, canned in tomato sauce	1.4
Mackerel, smoked	3.5
Tuna, canned in water	4.2
Cod, fresh	1.8
Herring, fresh autumn	9.4
Herring, fresh spring	7.0
Herring, pickled	12.3
Egg	1.4
Egg yolk	3.8
Chanterelles, fresh	2.5
Chanterelles, canned	15.4
Chicken, fried with no skin	0.63
Beef, fried	0.61
Fortified foods	
Fortified milk (fat content ≤1.5%)	0.45
Fortified margarines	7.5-10.0
Fortified yoghurts (fat content: 0.5%)	0.38
Fortified soured milk (fat content ≤1.5%)	0.38
Fortified soy/oatmeal drink	0.5-0.8

The effect vitamin D intake has on raising circulating 25(OH)D is not totally elucidated. Review studies have shown that 1 μg vitamin D intake from supplements or fortified foods raised the circulating 25(OH)D by approximately 1-2 nmol/L.[37, 38] The effect of vitamin D intake on levels of circulating 25(OH)D seems to be non-linear rather than linear.[39] The effect of vitamin D intake on circulating 25(OH)D is affected by factors such as baseline 25(OH)D and body weight.[38, 40] There is a greater effect of vitamin D intake on levels of

circulating 25(OH)D at low baseline 25(OH)D concentrations, and a lower response in individuals with higher body weight. Some report similar effects of vitamin D_2 and D_3 in raising circulating 25(OH)D concentrations,[41, 42] but some report lower effectiveness of vitamin D_2 compared with vitamin D_3.[37, 43, 44] Results mentioned here are based on studies of vitamin D from supplements or fortified foods. The bioavailability and effect of vitamin D from natural sources is largely unknown. One study has explored this in chanterelles, showing that vitamin D_2 in chanterelles had the same effect on serum 25(OH)D as vitamin D_2 from supplements.[31]

2.3.3 Metabolism

The absorption of vitamin D in the intestine occurs through incorporation into chylomicrons and via the lymphatic system.[45, 46] Recently, facilitated absorption has also been suggested.[47] After exposure to sun or ingestion of vitamin D, the vitamin D molecule being hydrophobic, requires binding to a protein in circulation for the transport to target tissues. During circulation, vitamin D and its metabolites are bound to DBP, which is synthesised in the liver. Vitamin D itself is not biologically active, why two enzymatic hydroxylation processes must take place. Firstly, vitamin D is converted to 25(OH)D in the liver involving the 25-hydroxylase enzymes. Secondly, the 1α-hydroxylase catalyzes further hydroxylation to form the biologically active substance 1α,25-dihydroxyvitamin D [1α,25$(OH)_2$D] (Figure 3). The renal production of 1α,25$(OH)_2$D is regulated by several factors. 1α-hydroxylase is upregulated by increases in serum parathyroid hormone (PTH), and decreases in serum phosphate. While, increases in serum phosphorus and fibroblast growth factor 23 will inhibit the conversion of 25(OH)D to 1α,25$(OH)_2$D.[48] PTH is in turn upregulated by decreasing serum calcium concentrations. 1α,25$(OH)_2$D itself limits the production by inhibiting 1α-hydroxylase. Disposal of vitamin D is a process involving the enzyme 24-hydroxylase. In this multistep catabolic process, both 25(OH)D and 1α,25$(OH)_2$D are degraded to the water-soluble calcitroic acid and subsequently secreted in the bile.[49, 50]

The effects of 1α,25$(OH)_2$D are mediated via its nuclear vitamin D receptor (VDR) that regulates transcription of target genes. VDR has been identified in many cell types and tissues.[51] The 1α,25$(OH)_2$D may also act through a membrane-bound receptor and mediate more immediate non-genomic actions.[51]

Vitamin D plays a central role in calcium and phosphate homeostasis. $1\alpha,25(OH)_2D$ enhances bone resorption and intestinal calcium uptake, leading to serum calcium homeostasis.[48] The enzymes responsible for converting 25(OH)D to $1\alpha,25(OH)_2D$, as well as the VDR, have been found in other tissues, such as the placenta, skin and adipose tissue.[52, 53] This suggests it is possible that vitamin D has an autocrine and/or paracrine mechanism of action that might be involved in the proposed non-skeletal affects.

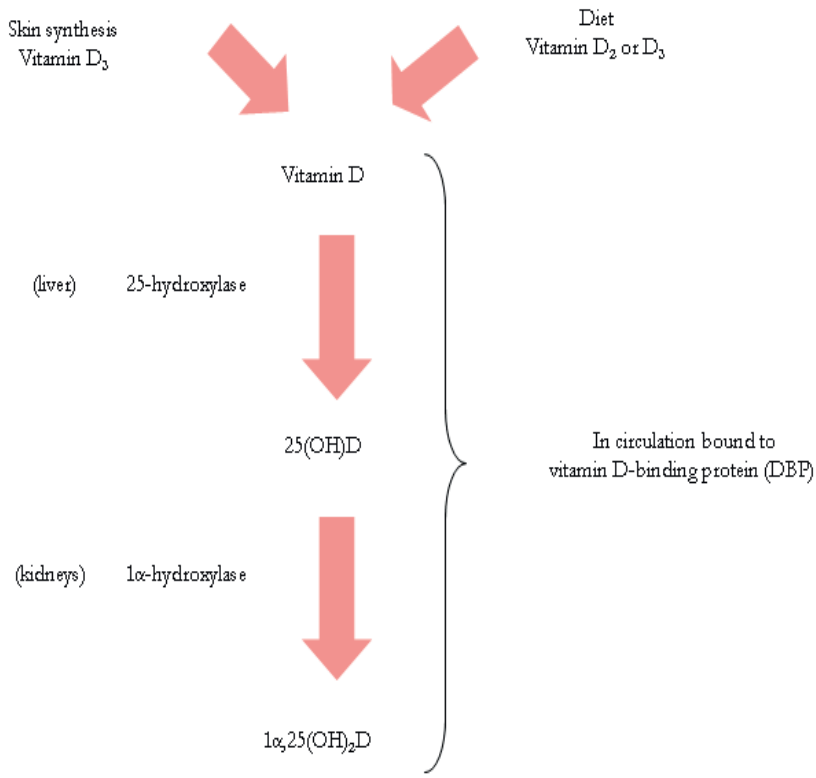

Figure 3. Vitamin D metabolism

2.3.4 Determination of vitamin D status

Circulating 25(OH)D is considered the best marker of vitamin D status, reflecting the contribution of vitamin D from diet and cutaneous synthesis.[54] It

has a long half-life (~2-3 w) and does not withstand tight homeostatic regulation.[55]

Even so, other biomarkers in the vitamin D endocrine system might be of interest when studying function or supply. The renal production of $1\alpha,25(OH)_2D$ is, as earlier mentioned, tightly regulated and has a much shorter half-life than 25(OH)D, and subsequently not a good marker of vitamin D status.[56] PTH has been proposed as a functional marker.[55] The free hormone hypothesis states that the biological activity of a hormone is related to the free portion of the hormone rather than the protein bound hormone.[57] Mendel has proposed that vitamin D could also be classified according to this hypothesis.[57] Therefore, free 25(OH)D and free $1\alpha,25(OH)_2D$ could be biomarkers for supply to and functions in target tissues.

The optimal 25(OH)D concentrations for overall health is under debate. Institute of medicine (IOM) has suggested that concentrations >50 nmol/L are sufficient,[39] while others have suggested that levels >75 nmol/L are optimal.[58] IOM suggests that persons with 25(OH)D concentrations <30 nmol/L are at risk for deficiency with regard to bone health.[39] The assay used in our studies declares deficiency when 25(OH)D level is <25 nmol/L.[59]

2.4 Vitamin D in obesity

2.4.1 Vitamin D status

Circulating 25(OH)D is known to be lower in obese compared with leaner individuals.[60-62] Furthermore, lower $1\alpha,25(OH)_2D$ and higher PTH circulating concentrations have been associated with obesity.[61, 63, 64] In cross-sectional studies in obese individuals, low circulating 25(OH)D has been associated with systemic inflammation,[65] and metabolic syndrome.[66] The mechanisms behind the lower levels of 25(OH)D are not fully understood. There could be several possible explanations, such as lower vitamin D intake or reduced intestinal absorption, reduced UVB exposure or cutaneous synthesis, deposition of vitamin D in the excess adipose tissue, or differences in the metabolism and/or catabolism of vitamin D.[67]

Wortsman *et al.* suggested that vitamin D is sequestered in adipose tissue and subsequently less available to the circulation in obese individuals.[40] This was questioned when Drincic *et al.* showed that the lower circulating 25(OH)D was

fully explained with a volumetric dilution model.[68] Studies on sun exposure behavior are scarce and show inconsistent results. In a study in Estonia, obese individuals were more likely to avoid the sun and expose less skin than individuals with BMI <30 kg/m^2.[69] In contrast, Harris *et al.* did not find a difference in sun habits over quartiles of percentage body fat in older adults.[70]

Few studies have measured vitamin D status in obese individuals or women of reproductive age in Sweden. Most studies have been conducted in elderly,[71, 72] and in mainly normal-weight individuals.[73, 74] One report from Uppsala, Sweden, measured 25(OH)D in obese men and women before undergoing Gastric bypass surgery.[75] But this study did not have a normal-weight group as reference and did not measure dietary intake.

2.4.2 Vitamin D intake

Current recommendation for vitamin D intake in the 2004 Nordic Nutrition Recommendations (NNR) for adults is 7.5 μg/day.[76] At present, a new version of the NNR is pending and an increase to a recommended intake of 10.0 μg/day of vitamin D is suggested. These recommendations are based on the fact that some exposure to sunlight is expected in the general population. Two national dietary intake studies have been conducted, one in 1997-98 and one in 2010-11. The results of these reports both suggest that intake of dietary vitamin D is generally below recommended levels (7.5 μg/day).[33, 77] In Riksmaten 2010-11, dietary vitamin D intake in women (18-44 y) was 5.2-6.2 μg/day. Generally, vitamin D intake is lower in central and southern Europe compared with the Nordic Countries.[78] The use of dietary supplements also contributes to vitamin D intake. In Riksmaten 2010-11, 27% of the women reported usage of some kind of supplements. Multivitamins/minerals, which usually contain vitamin D, together with omega-3 supplements, tend to be the most common supplements used.[33]

A lower intake of vitamin D could be a possible explanation for the lower 25(OH)D in obese individuals. In the European Prospective Investigation into Cancer and Nutrition study no differences in vitamin D intake was found in European individuals with BMI >30 kg/m^2 compared to individuals with BMI 25-30 or <25 kg/m^2.[78] Furthermore, Shapses *et al.* did not find an effect of body weight on total vitamin D intake in women living in the United States.[79] In contrast, two studies have found lower dietary vitamin D intake in obese

individuals.[80, 81] There is, to our knowledge, no study of vitamin D intake in obese compared with normal-weight individuals in Sweden.

2.4.3 Vitamin D and adipose tissue

Vitamin D is deposited primarily in adipose tissue and then in muscle tissue.[82] Vitamin D has been detected in different adipose tissue compartments, such as abdominal subcutaneous, omental, pericardial, and perirenal.[43, 83-85] In a study including obese individuals, vitamin D_3 was measured in adipose tissue and correlated positively with serum vitamin D_3.[83] The content of vitamin D_3 was significantly larger in the adipose tissue than in circulation.[83] There are reports of 25(OH)D in adipose tissue but the concentrations were higher in serum.[82]

Few studies have been conducted comparing the content of vitamin D and its metabolites in the adipose tissue of obese and normal-weight individuals. In a study in women, the expression of vitamin D-metabolizing enzymes was found in adipose tissue in both normal-weight and obese women, with some differences between these groups, as well as differences in the expression between subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT).[53] Furthermore, little is known about the localization of vitamin D and its metabolites at the cellular level.

2.5 Vitamin D in pregnancy

2.5.1 Vitamin D status

Pregnancy is a unique time during life when a growing fetus relies on its mother's nutritional status. In rat placenta, 25(OH)D has been shown to cross the placenta but not $1\alpha,25(OH)_2D$.[86] Vitamin D status in the mother reflects vitamin D status in the neonate. The level of 25(OH)D in the newborn is approximately 75% (range: 50-100%) of that of the circulating concentrations in the mother.[86] Children born to obese women have lower 25(OH)D concentrations compared with children born to leaner women.[87, 88] Circulating concentrations of $1\alpha,25(OH)_2D$ and DBP are raised during pregnancy.[89] $1\alpha,25(OH)_2D$ is raised due to increased activity of renal 1α-hydroxylase and DBP due to increased estrogen levels affecting the hepatic production. Whether circulating 25(OH)D is affected by pregnancy *per se* is somewhat unclear. Studies with a non-pregnant control group have shown no effect,[90] or lower 25(OH)D during pregnancy compared with the non-pregnant control group.[91]

Both the VDR and the enzymes responsible for converting 25(OH)D to 1α,25(OH)$_2$D are present in the placenta, making it at least plausible for autocrine/paracrine effects.[86] In observational studies, lower vitamin D status during pregnancy has been associated with increased risk for pre-eclampsia,[92] GDM,[93] and SGA.[94] Even so, there is currently no strong evidence of beneficial effects of vitamin D supplementation during pregnancy, and the causal link is unproven.[95] Some of the pregnancy complications associated with low vitamin D status are also associated with maternal obesity, such as pre-eclampsia[9] and GDM.[8] If an increased vitamin D status during pregnancy in obese women decreased the incidence of complications, this would be of importance.

Not many studies have explored vitamin D status during pregnancy in Sweden. Brembeck *et al.* measured serum 25(OH)D in late pregnancy in fair-skinned, mostly normal-weight women. Circulating 25(OH)D in this study was found to be determined by season, travel abroad and supplement use and 65% had insufficient (defined as serum 25(OH)D <50 nmol/L) levels during wintertime. Sääf *et al.* measured 25(OH)D in women of Somali origin compared to women of Swedish origin.[96] They found that vitamin D deficiency was common in the Somali women but not in the women of Swedish origin. No studies in Sweden have explored the vitamin D status in obese women during pregnancy and longitudinal studies are lacking. In studies at similar latitudes as Sweden, 25(OH)D has been negatively associated with BMI during pregnancy.[97, 98]

2.5.2 Vitamin D intake

The current national recommendation for vitamin D intake during pregnancy is 10.0 μg/day.[76] Few studies have explored vitamin D intake during pregnancy in Sweden. Two studies have reported vitamin D intake, one in mid-gestation and one in late pregnancy.[99, 100] The mean dietary vitamin D intake in the study in late pregnancy was 6.1 μg/day.[100] Åden *et al.* measured vitamin D intake in 50 women at mid-gestation and found that dietary intake of vitamin D was 5.6 μg/day.[99] 65 and 66% of the pregnant women in these two studies used supplements containing vitamin D or vitamins/minerals, respectively.[99, 100] There are, to our knowledge, no previously published studies using a longitudinal approach during pregnancy or studies that have compared vitamin D intake between pregnant obese and normal-weight women in Sweden.

A systematic review of micronutrient intakes during pregnancy conducted by Blumfield *et al.* reported vitamin D intake to be at 5.7 μg/day in USA/Canada,

2.2 μg/day in United Kingdom, 3.6 μg/day in Europe, and 1.3 μg/day in Australia/New Zealand.[101] In Norway, Finland, Iceland, and Denmark, dietary intake of vitamin D during pregnancy was reported at between 3.5 and 8.0 μg/day.[102-105] When vitamin D from supplements is added to the dietary intake, the intake in supplement users increases but varies largely depending on type and amount of supplement used. In Norway and Iceland, where fish liver oil is commonly used, vitamin D from supplements can be especially large. In a study including Norwegian pregnant women, the intake of vitamin D was reported at 13.6 μg/day in supplement users and 3.5 μg/day in non-users.[102] In an Icelandic study, the vitamin D intake from fish and fish liver oil alone was 14.0 μg/day.[106] In contrast, in one Swedish and one Finnish study, the contribution of vitamin D from supplements in users was 5.8 and 1.7 μg/day respectively.[100, 107] Thus, vitamin D intake in pregnant women varies and may largely depend on if vitamin D-containing supplements are used or not.

Regarding vitamin D intake in obese women during pregnancy, both higher and lower vitamin D intake in obese compared to non-obese has been reported. In a New Zealand study, dietary vitamin D intake increased with increasing BMI.[108] In contrast, in a large Norwegian cohort, overweight/obese pregnant women had a lower total vitamin D intake compared with normal-weight women.[102] In this Norwegian study the use of supplements was lower in obese compared to normal-weight women, perhaps explaining the lower total vitamin D intake found in obese women. If also the dietary vitamin D intake was affected by BMI was not reported.

3 Aim

The aim of this thesis was to investigate vitamin D status and intake in obese and normal-weight women living in Sweden who are of reproductive age and during pregnancy.

3.1 Specific aims

Paper I

- Compare vitamin D status and intake between obese and normal-weight women of reproductive age
- Explore vitamin D status according to different cut-off levels in normal-weight and obese women of reproductive age
- Explore factors associated with circulating 25(OH)D

Paper II

- Compare vitamin D status and intake in obese and normal-weight women during pregnancy
- Explore vitamin D status according to different cut-off levels in normal-weight and obese women during pregnancy
- Explore factors associated with circulating 25(OH)D

Paper III

- Explore the possibility to use the TOF-SIMS (time-of-flight secondary ion mass spectrometry) technique to measure vitamin D and its metabolites in small samples of adipose tissue from normal-weight and obese individuals

4 Subjects and Methods

4.1 Subjects

Table 3 gives an overview of the three papers.

Table 3. Overview of the study designs

Paper	I	II	III
Design	Cross-sectional Vitamin D study	Longitudinal PONCH study	Cross-sectional Vitamin D study
Participants (*n*)	86 43 obese 43 normal-weight	105 25 obese 80 normal-weight	9 6 obese 3 normal-weight
Inclusion year	2009-2011	2009-2012	2010-2011
Measurements	Blood sample Anthropometry Body composition Dietary questionnaire Fish and shellfish FFQ	Blood sample Anthropometry Body composition Dietary questionnaire Fish and shellfish FFQ	Adipose tissue biopsy Blood sample Anthropometry Body composition Dietary questionnaire

Abbreviations: FFQ, food frequency questionnaire; PONCH, Pregnancy obesity nutrition & child health.

4.1.1 Vitamin D study

The vitamin D study was a cross-sectional study with recruitment between 2009 and 2011. The women in this study were intended to be comparable with the population in the Pregnancy Obesity Nutrition & Child Health (PONCH) study described below, except that they were not pregnant. Obese women were invited to participate in the study from referrals to the Obesity Unit, at Sahlgrenska University Hospital (Figure 4). In addition, obese women were also recruited through postings at public billboards and advertisements in a local newspaper (Figure 5). The normal-weight women were recruited through postings at public billboards and advertisements in a local newspaper (Figure 5). Exclusion criteria were diseases and use of medications known to affect vitamin D status, severe psychiatric disorder, non-European descent, pregnancy, smoking, and vegan diet. Inclusion criteria were age 20-45 years, BMI 18.5-24.9 or >30 kg/m^2.

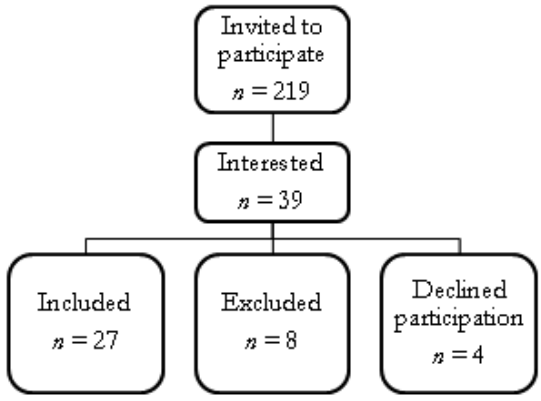

Figure 4. Recruitment of participants invited from the Obesity Unit

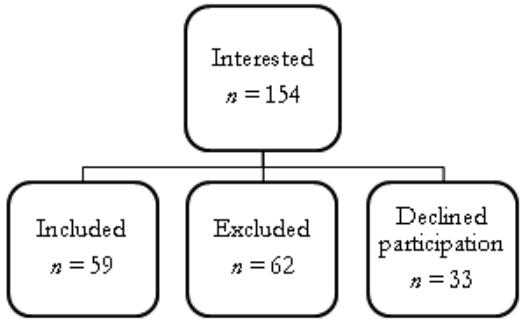

Figure 5. Recruitment through postings at public billboards and advertisements in newspaper

In addition to six of the subjects from the vitamin D study (paper I), three subjects who had undergone gastric bypass surgery at Sahlgrenska University Hospital were used in paper III.

4.1.2 PONCH study

In paper II, subjects from the PONCH study were included. This is an ongoing randomized controlled trial (RCT) with the purpose of studying the health of normal-weight and obese mothers and their children. The recruitment to PONCH started in 2009. The women were randomized to a dietary intervention group or to a control group. The women's first visit was in gestational weeks 8-

12. The second and third visits during pregnancy were conducted during the second (gestational week 24-26) and third (gestational week 35-37) trimesters. The women were also followed postpartum, with the first visit taking place at six months postpartum (Figure 6). Exclusion criteria were the use of neuroleptic drugs, non-European descent, smoking, diabetes, twin pregnancy, and vegan diet. Inclusion criteria were age >20 years and BMI 18.5-24.9 or >30 kg/m^2.

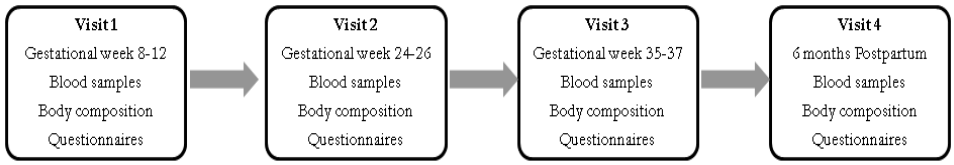

Figure 6. Study visits in the PONCH study

4.1.3 Ethics

The studies were approved by the Ethics Committee at the University of Gothenburg. Oral and written information was given to each participant, and written informed consent was obtained from the participants before entering the study.

4.2 Methods

4.2.1 Dietary intake

Dietary intake was measured using a self-administered dietary questionnaire. The purpose of the questionnaire is to assess the habitual intake over the past three months, originally designed for the Swedish Obese Subjects study.[109] The questionnaire consists of 49 questions with a food frequency questionnaire (FFQ) design and considers portion sizes for hot meals, sandwiches and candies. Daily micro- and macronutrient intake were calculated using the food database of the Swedish National Administration, Version 04.1.1; Uppsala, Sweden. Additionally, the daily intake of nutrients was divided into 15 different food groups. This questionnaire has been validated in normal-weight, overweight and obese non-pregnant subjects, giving valid estimates of energy intake.[109]

Also, an FFQ mirroring fish- and shellfish intake over the past three months was given to all participants. The weekly intake of fish- and shellfish was asked for, as well as the type of fish and shellfish consumed.

In paper I, the supplement use during the past six months was asked for. In the PONCH study (paper II), the pregnant women were asked during all study visits about their supplement use. At the first trimester visit, the women were asked about the use of supplements taken before the beginning of pregnancy and since the start of pregnancy. During the later visits, during pregnancy and postpartum, the women were asked about their supplement use since the previous visit.

4.2.2 Sun exposure

Time spent outdoors (between 9:00 AM and 6:00 PM), travelling abroad and use of sunbeds were asked for. Travel to locations below latitude 35°N, where there is UVB exposure all year round, was considered travelling to a sunny country.[110] In order to explore vitamin D status according to season, the calendar year was divided into two (Paper I and II) or four periods (Paper II). The two periods consisted of October- March ("winter") and April-September ("summer"). Four periods, on the other hand, included January-March, April-June, July-September, and October-December.

4.2.3 Background and lifestyle variables

Interviews took place at all study visits where questions on education, physical activity, medication and sun exposure were answered.

In paper I, physical activity was assessed by a method earlier described by Bouchard *et al.*[111] Physical activity was recorded for every 15 minutes during three consecutive days. An individual physical activity level (PAL) for every participant was calculated.

4.2.4 Dietary intervention

The subjects in paper II received a dietary intervention during pregnancy with the main purpose of improving dietary quality according to the NNR.[76] Emphases were put on increased fish, fruit and vegetable intake and decreased intake of sucrose. The normal-weight women received information on additional intake of energy during their second (+350 kcal/day) and third (+500

kcal/day) trimesters. In addition to increasing dietary quality as earlier mentioned, the obese women were advised to restrict energy intake by 20%. Their energy requirement was calculated using the Harris-Benedict equation to calculate basal metabolic rate with the addition of a 1.4 PAL. The intervention participants meet with a dietician at every study visit, and eight additional telephone calls were conducted between the first trimester and six months postpartum.

4.2.5 Anthropometry and Body composition

Body weight was measured on a calibrated digital scale and height on a wall-mounted height scale. For the three additional subjects undergoing gastric bypass in paper III, weight and height were collected from medical journals.
In papers I and III, quantitative magnetic resonance equipment (EchoMRI-AH™ by EchoMRI, Houston, TX) was used to measure body composition. The nuclear magnetic signals generated differ depending on the tissues from which the signal originate.[112] Subsequently, fat mass (FM) can be established.
In paper II, the BodPod® (Cosmed Inc., Rome, Italy) was used to measure body composition during pregnancy. The BodPod® uses the air displacement plethysmography method to measure body volume. With the combination of body volume and body mass, body density was derived.[113] Using the equation by Siri,[114] the BodPod® software calculated FM.

4.2.6 Laboratory analyses

All venous blood samples were collected after an overnight fast. Serum (S) 25(OH)D and plasma (P) 1α,25$(OH)_2$D were measured in a laboratory taking part in the Vitamin D external quality assessment scheme. S-25(OH)D was measured using a competitive two-step chemiluminescent immunoassay (CLIA), LIAISON® (DiaSorin, Saluggia, Italy), and P-1α,25$(OH)_2$D was analysed using a radioimmunoassay (RIA) (DiaSorin, Saluggia, Italy). S-DBP was measured with a commercial enzyme-linked immunosorbent assay (ELISA) (R&D Systems®, Minneapolis, USA).

Serum PTH, calcium and albumin were measured in an ISO 15189 accredited laboratory (Biochemistry laboratory at Sahlgrenska University Hospital, Gothenburg, Sweden).

4.2.7 Calculation of free 25(OH)D

To calculate free 25(OH)D the following equation was used[115]

$$\text{Free 25(OH)D} = \frac{\text{Total 25(OH)D}}{1+(6\times10^5\times[\text{albumin}])+(7\times10^8\times[\text{DBP}])}$$

The percentage free 25(OH)D was calculated using the ratio of free 25(OH)D to total 25(OH)D × 100.

4.2.8 Adipose tissue biopsy

In paper I, SAT needle biopsy at the women's umbilical level was obtained under local anesthesia. Additionally, in paper III, adipose tissue samples from three subjects undergoing gastric bypass were examined. SAT and VAT (omental) samples were collected during surgery.

4.2.9 Time-of-flight secondary ion mass spectrometry

The TOF-SIMS technique is a surface-sensitive analytical method that uses a pulsed ion beam which bombards the sample surface. This will start a reaction where ions (secondary ions) from the sample surface will detach and travel towards a detector. TOF-SIMS uses the time (i.e. time-of-flight) it takes for the ions to travel to the detector to separate molecules on the basis of mass over charge. A mass spectrometry as well as images can be produced with this method.[116]

In paper III, small pieces (2 mm diameter) of the samples of adipose tissue were frozen under high pressure (2000 bar) at -196°C. Samples were thereafter freeze-fractured in a liquid nitrogen bath. Spectra and images were produced from at least three samples from each participant's adipose tissue biopsy. The samples were analysed with a TOF-SIMS V instrument (ION-TOF, Münster, Germany) equipped with a Bi_3^+-liquid metal ion gun at the University of Gothenburg.[117]

4.2.10 Goldberg cut-off

In order to identify energy misreporting in the participants used in paper I, the Goldberg cut-off method was used both at the group and individual level.[118, 119] Confidence limits of 95% were used in the calculation.

To calculate individual estimated energy expenditure, basal metabolic rate (BMR) and their individual PAL was added. BMR was calculated using the equation by Mifflin-St Jeor,[120] and the subjects' individual PAL were calculated from the 3-day physical activity record described earlier. Data on PAL was missing for one normal-weight and four obese women with dietary intake information. For these subjects, the mean PAL for the entire group of normal-weight and obese, respectively, was imputed.

4.3 Statistics

All statistical analyses were performed using SPSS for windows versions 18.0, 19.0 or 20.0 (IBM, Armonk, NY, USA), except for calculation of the principal component analysis (PCA) in paper III where Matlab R2012a (MathWorks®, Natick, MA, USA) was used. A two-tailed *P*-value below 0.050 was considered statistically significant.

In papers I and II, means and standard deviations (SD) are given for continuous variables. Non-parametric tests were used due to limited sample size and the fact that several variables were skewed. The Mann-Whitney U Test or the Wilcoxon Signed-Rank test was used to calculate quantitative data, and Chi-square test was used for analysing proportions between groups. When exploring correlations, the Spearman Rank Order Correlation was used. When evaluating factors associated with circulating 25(OH)D, multiple regression analysis was performed. In paper II, a linear mixed model was used to analyse the effect of the intervention on dietary vitamin D intake and supplement use. To subdivide the subjects circulating 25(OH)D into groups according to cut-offs, levels of 25, 50 and 75 nmol/L were considered.[39, 58, 121, 122]

In paper III, poisson-corrected summed intensities of the peak areas were used in the PCA, and the PCA model is in the paper presented as scores and loadings plots. PCA is a mathematical method used to explore patterns in data and detecting differences and similarities between groups. To analyse differences between groups, one-way analysis of variance (ANOVA) was used.

5 Results

Table 4. Baseline characteristics of study participants in the vitamin D study (paper I) and the PONCH study (paper II)

	Paper I		Paper II	
	Normal-weight *n* = 43	Obese *n* = 43	Normal-weight *n* = 80	Obese *n* = 25
Age (years)	32.3 ± 6.7	34.7 ± 5.7	31.4 ± 4.0	32.0 ± 3.2
Weight (kg)	60.4 ± 6.4	110.3 ± 15.2	62.9 ± 6.1	96.6 ± 12.5
BMI (kg/m²)	21.5 ± 1.8	39.1 ± 4.6	22.0 ± 1.4	33.9 ± 3.3
FM (kg)	15.2 ± 4.8	55.4 ± 11.7	16.7 ± 4.0	43.3 ± 9.0
Education, n (%)				
Compulsory school	1 (2.3)	2 (4.8)	0 (0.0)	0 (0.0)
Upper secondary school	11 (25.6)	30 (71.4)	14 (17.5)	9 (36.0)
< 3 y at university	2 (4.7)	1 (2.4)	8 (10.0)	4 (16.0)
≥ 3 y at university	29 (67.4)	9 (21.4)	58 (72.5)	12 (48.0)

Abbreviations: BMI, body mass index; FM, fat mass; PONCH, pregnancy obesity nutrition & child health.
Values are means ± SD or *n* (%).
Missing data, paper I: FM (*n* = 6), education (*n* = 1), paper II: FM (*n* = 3).

5.1 Paper I

The mean circulating DBP was higher in obese women (320 ± 121 μg/mL) compared with normal-weight women (266 ± 104 μg/mL) (P=0.02), and calculated free 25(OH)D concentrations were lower (Table 5). Obese women had 20.1 nmol/L lower mean 25(HO)D concentration compared to normal-weight women after controlling for season of blood sampling, total vitamin D intake, travelling to a sunny country, and age (P<0.001). Fifty-six per cent of obese women and 12% of normal-weight women had 25(OH)D concentrations ≤50 nmol/L (Table 6). The obese women reported spending more time outdoors compared with the normal-weight women (Table 5).

Table 5. Vitamin D status, dietary intake and sun exposure in 43 normal-weight and 43 obese women

	Normal-weight	Obese	*P*-value[1]
Winter season, *n* (%)	17 (39.5)	19 (44.2)	0.83
Sun exposure			
Time spent outdoors (min/day)	111 ± 72.4	148 ± 87.6	0.04
Sunbed use, *n* (%)[2]	5 (11.6)	1 (2.3)	0.20
Travelling to sunny climate, *n* (%)[3]	8 (18.6)	3 (7.0)	0.20
Vitamin D status			
Serum 25(OH)D (nmol/L)	76.9 ± 25.1	52.2 ± 19.6	<0.001
Plasma 1α,25$(OH)_2$D (ng/L)	68.0 ± 19.5	50.7 ± 17.1	<0.001
Serum DBP (μg/mL)	266 ± 104	320 ± 121	0.02
Free 25(OH)D (pmol/L)	23.7 ± 10.7	13.3 ± 5.5	<0.001
Dietary intake			
Dietary vitamin D intake (μg/day)	7.2 ± 2.8	7.9 ± 2.4	0.21
Dietary vitamin D intake, median (25^{th}-75^{th} percentiles)	6.8 (4.7-9.3)	7.6 (6.4-9.6)	
Total vitamin D intake (μg/day)	13.7 ± 15.6	8.5 ± 3.1	0.40
Total vitamin D intake, median (25^{th}-75^{th} percentiles)	8.4 (5.8-12.9)	7.8 (6.4-9.7)	
Supplement use, *n* (%)[4]	15 (34.9)	6 (14.0)	0.04
Fish and shellfish intake (meals/w)	2.3 ± 1.5	1.7 ± 1.1	0.10
Fatty fish (meals/w)	1.3 ± 1.1	0.7 ± 0.7	0.01

Abbreviations: DBP, vitamin D-binding protein; FM, fat mass; PAL, physical activity level; 25(OH)D, 25-hydroxyvitamin D; 1α,25-dihydroxyvitamin D.
Values are means ± SD or *n* (%).
[1]Means were compared using the Mann-Whitney *U* test, and proportions using Fisher's exact test.
[2]Women stated using sunbed within two months prior to blood sampling.
[3]Travelling to a country below latitude 35°N within six months prior to blood sampling.
[4]Supplements containing vitamin D.

DBP did correlate positively, but not statistically significant, to FM% when obese and normal-weight women were combined (r=0.14, P=0.24). When obese and normal-weight were analysed separately, the correlation became inverse in the normal-weight group but did not reach statistical significance (r=-0.28, P=0.08).

Table 6. Vitamin D distribution

	Normal-weight n = 43	Obese n = 43
<25 nmol/L	0 (0.0)	1 (2.3)
25-50 nmol/L	5 (11.6)	23 (53.5)
51-75 nmol/L	17 (39.5)	14 (32.6)
>75 nmol/L	21 (48.8)	5 (11.6)

Values are n (%). Fisher's exact test P<0.001.

There were no differences in dietary or total vitamin D intake between normal-weight and obese women (Table 5). Sixty-one per cent of the women had a total vitamin D intake ≥7.5 μg/day, which is the current national recommendation for vitamin D in Sweden. Total fish and shellfish intake did not differ between the groups, but normal-weight women had a higher intake of fatty fish compared to obese women (1.3 vs. 0.7 times/week) (P=0.01). Women eating fish and shellfish 2-3 times or more per week were more likely to have dietary intake of vitamin D ≥ 7.5 μg/day (P=0.006).

A higher proportion of the normal-weight women used vitamin D-containing supplements compared with obese women (Table 5). Multivitamins/minerals were the most common supplement used. In supplement users, the median (25^{th}-75^{th} percentiles) total vitamin D intake was 15.0 (9.9-24.9) μg/day, and the intake of vitamin D from supplements was 6.3 (3.4-19.5) μg/day. Dietary supplement use, classified as use of any kind of dietary supplements during the last three months, was 41.9% in obese and 53.5% in normal-weight women (P=0.051).

FM, time spent outdoors, sunbed usage, and travelling to a sunny country were all statistically significant associated with 25(OH)D concentrations. 34% of the variance in serum 25(OH)D was explained by FM alone.

The Goldberg cut-off method was used to explore misreporting of energy intake. At a group level, the obese women under-reported energy intake while the normal-weight women did not, and at an individual level, 7.5% and 23.7% of normal-weight and obese women, respectively, under-reported energy intake. Furthermore, 7.5% of normal-weight and 15.8% of obese women over-reported energy intake.

5.2 Paper II

Baseline characteristics are shown in Table 4. Participants who did not complete the study (drop-outs) had shorter education. Drop-outs and study completers did not differ in age, BMI, parity, energy intake, dietary vitamin D intake, use of supplements or randomization group.

Vitamin D status during pregnancy is shown in Table 7. Compared to normal-weight women, obese women had lower circulating 25(OH)D in the first trimester ($P<0.001$). Obese women had lower S-25(OH)D also in the second and third trimester compared with normal-weight women, but this did not reach statistical significance. After controlling for supplement use, travelling to a sunny country, and season of blood sampling, obese women had 11.4, 8.2, and 5.7 nmol /L lower mean 25(OH)D concentrations in the first, second, and third trimesters, respectively. In the summer season, 88% (normal-weight) and 50% (obese) had S-25(OH)D >50 nmol/L in the first trimester ($P<0.01$). While in the winter season, 60% of the normal-weight and 33% of obese women had circulating 25(OH)D >50 nmol/L ($P=0.21$). In Figure 7, the distribution of 25(OH)D (all year) in the first trimester is shown.

In the first trimester, women with S-25(OH)D concentrations ≥50 nmol/L were more likely to be supplement users ($P=0.017$), and had a tendency to have higher mean intake of low-fat (fat content ≤1.5%) milk, soured milk and yoghurt ($P=0.075$).

Table 7. Vitamin D status and intake during pregnancy

	1st trimester			2nd trimester			3rd trimester		
	Normal weight *n* = 76-80[a]	Obese *n* = 22-25[a]	*P*	Normal weight *n* = 54-56[a]	Obese *n* = 16-20[a]	*P*	Normal weight *n* = 51-54[a]	Obese *n* = 15-16[a]	*P*
Energy intake (kcal/day)	2252 ± 617	2529 ± 817	0.14	2316 ± 571	2448 ± 636	0.31	2354 ± 597	2337 ± 518	0.93
Dietary vitamin D intake (µg/day)	7.2 ± 2.5	8.8 ± 3.3	**0.024**	7.9 ± 2.7	8.2 ± 2.7	0.69	7.9 ± 2.3	8.1 ± 2.3	0.72
Dietary vitamin D intake ≥ 10 µg/day, *n* (%)	7 (9.2)	8 (33.3)	**< 0.01**	13 (23.6)	5 (29.4)	0.75	10 (19.2)	3 (20.0)	1.00
Supplement use, *n* (%)[b]	49 (62.0)	15 (60.0)	1.00	25 (44.6)	10 (47.6)	1.00	30 (55.6)	7 (46.7)	0.57
Fish- and shellfish intake (cooked meals/w)	2.5 ± 1.4	2.0 ± 1.1	0.18	2.9 ± 1.6	2.7 ± 1.5	0.71	2.8 ± 1.4	2.2 ± 1.6	0.074
Use of fortified spread, *n* (%)	64 (84.2)	22 (91.7)	0.51	49 (89.1)	16 (88.9)	1.00	44 (84.6)	16 (100.0)	0.18
Use of fortified fats in cooking, *n* (%)	12 (15.8)	8 (33.3)	0.080	7 (12.7)	8 (47.1)	**< 0.01**	6 (11.5)	8 (53.3)	**< 0.01**
S-25(OH)D (nmol/l)	64.2 ± 18.3	49.7 ± 11.5	**< 0.001**	58.2 ± 18.3	49.7 ± 18.9	0.17	51.7 ± 18.3	47.7 ± 18.3	0.78

Values are means ± s.d or *n* (%).
Data in bold indicate significance.
[a]The range of *n* is due to missing data (predominantly dietary questionnaires and FFQs).
[b]Vitamin D containing supplements.

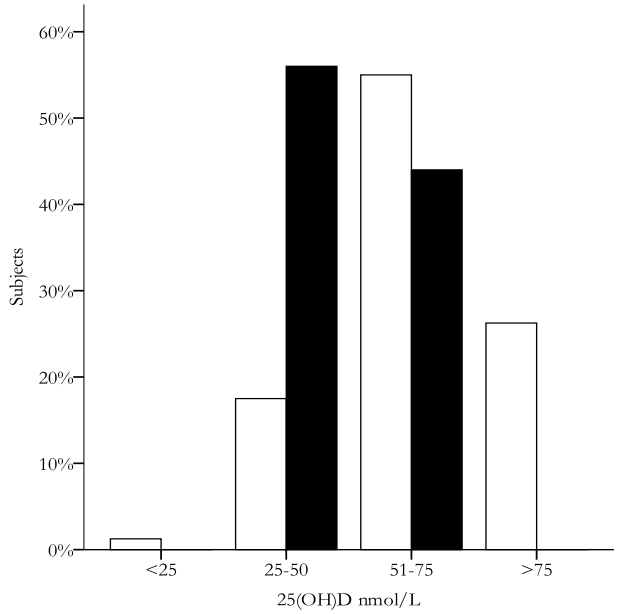

Figure 7. Vitamin D distribution in the first trimester in 80 normal-weight (open bars) and 25 obese (filled bars) women (P<0.001)

In 39 women (26 normal-weight and 13 obese) who had measurements at both the first trimester and six months postpartum, we found no difference in mean S-25(OH)D between the first trimester (55.8 ± 14.5 nmol/L) and at six months postpartum (61.5 ± 17.9 nmol/L) (*P*=0.062). First trimester circulating 25(OH)D was positively correlated with S-25(OH)D at six months postpartum (*rho*=0.51, *P*=0.001).

Dietary intake during pregnancy is shown in Table 7. Dietary vitamin D intake was higher in obese women compared to normal-weight women (*P*=0.024) in the first trimester, and a larger proportion of the obese women had a vitamin D intake above the national recommendation (*P*<0.01). Nine and 32% of normal-weight and obese women, respectively, had a dietary vitamin D intake above the national recommendation for pregnant women (10.0 μg/day) in the first trimester (Table 7). Distribution of dietary vitamin D intake in the first trimester is shown in Figure 8.

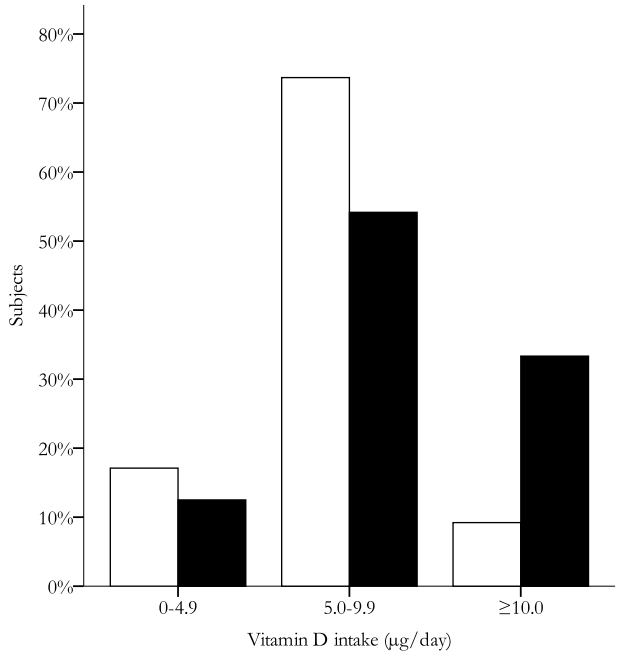

Figure 8. Distribution of dietary vitamin D intake in 76 normal-weight (open bars) and 24 obese (filled bars) women in the first trimester (P=0.016)

The use of fortified spreads was high, with 86% of all the women using fortified spreads in the first trimester. Compared to normal-weight women, the obese women had a greater tendency to use fortified fats during pregnancy (Table 7). There was a tendency in the intervention group to have a higher dietary vitamin D intake during pregnancy compared with the control group (P=0.060). There were no differences between normal-weight and obese women in the intake of dairy products and fortified spreads during pregnancy.

Sixty-one per cent of the pregnant women used vitamin D-containing supplements during the first trimester. The intake of supplements declined from the first trimester to the second and third trimesters. There were no differences in the use of vitamin D-containing supplements between normal-weight and obese women, and multivitamins/minerals were the most common type of supplement used. Although the dietary intervention did not include any advice on use of dietary supplement, the potential effect of being in the intervention group was examined regarding supplement use. No effect of the intervention was found on supplement use during pregnancy (P=0.70).

Table 8 shows the results of multiple regression analysis in the first trimester. Season, FM%, supplement use, and travelling to a southern latitude were associated with circulating 25(OH)D. This model explained 31.1% of the variance in S-25(OH)D concentrations, and FM% alone explained 11.3%.

Table 8. Multiple linear regression results of selected factors on serum 25(OH)D in the first trimester (n =100)

	B[1]	Std. err	*P*	R^2
Fat mass (%)	-0.5	0.17	< 0.01	31.1
Season of blood sampling[2]	6.2	1.53	< 0.001	
Travel to southern latitude[3]	10.6	3.90	< 0.01	
Supplement use[4]	8.7	3.15	< 0.01	

[1]B coefficents.
[2]Jan-March=1, Oct-Dec=2, April-June=3, July-Sep=4.
[3]Travelling to a country below latitude 35°N within six months prior to blood sampling.
[4]Supplements containing vitamin D (0=no, 1=yes).

5.3 Paper III

We were able to measure vitamin D and its metabolites in adipose tissue using the TOF-SIMS technique. Neither vitamin D_2 nor its metabolites were found in adipose tissue, and vitamin D_2 could not be detected in serum. This suggests a very limited intake of vitamin D_2 in these subjects even though 25(OH)D_2 and 25(OH)D_3, as well as 1α,25(OH)D_2, and 1α,25(OH)D_3 cannot be separated from each other with the assays used.

Vitamin D_3 and its metabolites were distributed across the lipid droplet in the adipocyte. In the images produced, the secondary ion signalling from 25(OH)D_3 and 1α,25$(OH)_2D_3$ was lower than signals from vitamin D_3, which suggests lower levels of these metabolites in adipose tissue. We could not detect any major differences regarding vitamin D or its metabolites in the images of adipose tissue between normal-weight and obese individuals.

The relative peak ion intensities for vitamin D_3 and 25(OH)D_3 were higher in SAT from normal-weight compared with SAT from obese women (Table 9), but no differences were found between the normal-weight and the obese

women for 1α,25(OH)D_3 (P=0.443). This might suggest that the content of vitamin D_3 and 25(OH)D differs in SAT from obese women compared with normal-weight women. Also, in the three obese subjects undergoing gastric bypass, relative peak ion intensities of vitamin D_3 were higher in SAT compared with VAT (P=0.005).

Table 9. Relative peak intensities in abdominal subcutaneous adipose tissue in normal-weight (n = 3) and obese (n = 3) women

	m/z	PI, normal-weight	PI, obese	P value[1]
Vitamin D_3	367.1	0.033 ± 0.002	0.022 ± 0.003	0.006
25(OH)D_3	383.2	0.034 ± 0.003	0.023 ± 0.003	0.018

Abbreviations: m/z, mass over charge; PI, relative peak intensities; 25(OH)D_3, 25-hydroxyvitamin D_3.
Values are mean ± SEM.
[1]Means were compared using one-way ANOVA.

The PCA showed that the lipid composition of the adipocyte was the most important contributor in explaining the properties of the adipose tissue in normal-weight and obese subjects, and vitamin D had some influence in the model.

6 Discussion

6.1 Methodology

6.1.1 Vitamin D status measurements

Due to the dual exposure to vitamin D, diet and solar radiation, measurement of dietary intake alone is not adequate to determine vitamin D nutritional status. Measurement of circulating 25(OH)D is currently regarded as the best indicator of vitamin D status.[54] On the other hand, measuring DBP and the free fraction of 25(OH)D might give additive information on vitamin D status with regards to the supply to target tissues.

Several different methods for measuring 25(OH)D are available. Liquid chromatography mass spectrometry (LC-MS) can currently be considered a golden standard,[123] but different immunoassays are also available.[54] Immunoassays have the overall advantage of being more simply automated and therefore more easily integrated to the core laboratory. In the present study, a CLIA from DiaSorin® was used which was the method of choice in the clinical laboratory at the Sahlgrenska University hospital at the time of study initiation. Snellman *et al.* demonstrated that the CLIA method gave lower concentrations compared with RIA and high-performance liquid chromatography (HPLC).[124] If values produced in our study are generally lower compared with other studies using these methods, this would indicate that deficiency and insufficiency rates could be overestimated in the present studies in comparison. Because of the different methods used, as well as the differences demonstrated between laboratories,[125] comparisons of vitamin D status between studies are difficult.

In paper I, DBP was measured with an ELISA method, because of the possibility to carry out this method in our own laboratory, as well as the fact that this method is commonly used in other published studies. Serum DBP was measured in duplicate reducing technical errors. The mean coefficient of variation between duplicates was low (2.6%), suggesting that the performance of the assay was satisfactory. The free 25(OH)D in paper I was explored by calculation. The calculated free 25(OH)D has been shown to be well correlated with measured free 25(OH)D (r=0.925) indicating that the calculated values can be used.[115]

6.1.2 Dietary intake assessment

The assessment of dietary intake is challenging. Commonly used methods to measure dietary intake are prospectively estimated or weighted dietary records and retrospectively, FFQ and 24-h recalls.[126] All these methods are imposed with different errors. One commonly shared problem for all these dietary intake assessment methods is that they are self-reported, and therefore rely on the individual's ability to correctly describe their dietary intake.

The dietary questionnaire used in our study, which has an FFQ design, was originally developed to measure dietary intake in the Swedish Obese Subjects study.[127] It has been validated for energy and protein intake in obese and non-obese individuals giving valid results.[109] However, it has not been validated for vitamin D intake. Validating dietary vitamin D intake is difficult since circulating vitamin D molecules are not good indicators of vitamin D intake due to the additional source of vitamin D from skin synthesis. Twenty-four hour recalls or food records is often used as reference methods to FFQs, but this is only a relative validation because the reference methods are not in any sense a golden standard method for measuring vitamin D intake. Vitamin D occurs only in few natural sources, and the best of these are fish, egg yolks and in Sweden fortified dairy products. Some vitamin D is also found in meat. Questions of the consumption of these foods are included in the questionnaire used in the present studies, making it possible to measure vitamin D intake. There are some limitations to the dietary questionnaire, foremost being assumptions done in calculations, such as to the type of fish and shellfish consumed, as well as portion sizes to some extent being estimated. One might argue that a weighed food record could have been added to the study protocol, but the addition of one more questionnaire was considered to be too much of a workload to the participants. Also, a food record describes only the recent dietary intake and is necessarily not a reliable tool for habitual intake assessment. In addition, because vitamin D is not a widespread vitamin in many different foods, the day-to-day variation is anticipated to be high and a shorter food record (3-4 days) could possibly underestimate vitamin D intake.

Several validation studies using food records as the reference method show that the FFQ generally seems to produce higher mean/median intakes of vitamin D,[128-133] yet some have not.[134, 135] Correlation coefficients between 0.26 and 0.61 have been reported.[128-133, 135] On the other hand, food records have been found to produce under-eating during the recorded time, and therefore might produce

under-reporting of the habitual dietary intake.[136, 137] Our questionnaire is of an FFQ design and might produce higher intake values compared to food records and 24-h recalls, and that needs to be taken into consideration when comparing our dietary data to other studies.

Under-reporting of energy intake may potentially affect the reported intake of micronutrients such as vitamin D. Reported energy intake should approximate energy expenditure in a weight-stable person. However, under-reporting of energy intake is common and increases with increasing BMI.[138] In order to exclude extreme values of reported dietary intake in paper I, five questionnaires were excluded due to unlikely high or low energy intake. In paper I, under-reporting of energy intake was higher in the obese women compared to the normal-weight women. This could potentially underestimate vitamin D intake in the obese women. The under-reporting of energy intake was similar or maybe slightly lower compared to other studies.[139] Exploring under-reporting in pregnancy is more difficult due to the fact that pregnancy is a time of growth. Therefore, the assumption of energy balance is not correct. Also, BMR is difficult to estimate in pregnancy and subsequently also estimating energy expenditure. In addition, our study includes energy restriction for the obese pregnant women in the intervention group, making assumptions when calculating energy expenditure even more prone to potential faults. Therefore, no attempt to calculate under-reporting of energy during pregnancy was made in the present study. Other studies have attempted to estimate energy under-reporting by adding assumed energy usage during pregnancy, and under-reporting of energy intakes between 24-45% have been found.[100, 140] Studies show that social desirability may affect reported intake, and that foods such as sugars, cakes and pastries may be under-reported more frequently.[141-143] One might argue that the foods that contain vitamin D such as milk, eggs and fish are generally desirable to eat and therefore might be less underreported. In a few studies, the reported intakes of fish, eggs and dairy/milk products were not different between energy under-reporters and non-under-reporters.[141, 144, 145] In a study in non-pregnant obese and normal-weight women, intake of vitamin D was not different in low-energy reporters compared with non-low-energy reporters.[146] Altogether, reported dietary vitamin D intake might not be extremely influenced by under-reporting of energy intake.

In addition to food intake, when assessing dietary intake, the use of dietary supplements must be considered. Collecting information on supplement use

may be difficult, as it is for food intake, and also depends on the ability of the subject to correctly report supplement usage. Information of frequency, dose and type/brand of the supplement are crucial to be able to account for amount consumed. Unfortunately, in the study during pregnancy (paper II), the information collected from the women was not sufficient enough to use absolute intake from supplements in the analysis. Information on brand, dose and frequency were poor in quality and therefore only the use of supplements was considered adequate to use in analysis. In the non-pregnant women in paper I, the information on supplements taken was considered sufficient and included in paper I.

In summary, to measure dietary intake is difficult and all methods available incorporate errors. The foods that contain vitamin D are included in the dietary questionnaire used, and values produced are not extensively different from other studies on vitamin D intake. This together suggests that the used dietary questionnaire is adequate enough in measuring the intake of dietary vitamin D.

6.1.3 Study population

Difficulties in recruiting obese pregnant women made the groups uneven in size. The lower rate of recruitment of obese women might depend on the fact that fewer women are obese compared to normal weight. Also, pregnancy might possibly be a time when obese women do not want to deal with their weight or that midwives might feel reluctant to bring up weight and therefore fewer women get asked to participate.[147]

The education level in our women, both the pregnant and the non-pregnant women, was higher than the average in the general Swedish population.[148] Additionally, measurement of dietary intake and body composition in our studies might have attracted women who were more health conscious. All women were fair skinned and vitamin D status results in the present studies do not apply to women with darker skin types. These things taken together impair the generalizability of the results.

6.1.4 TOF-SIMS

Vitamin D has previously been measured in adipose tissue with LC-MS and HPLC.[43, 82-85] However, these methods require a relatively large quantity of adipose tissue samples and they do not produce information on localization at a cellular level of the molecule measured. TOF-SIMS is an imaging mass

spectrometry technique which is able to give information on the distribution of biomarkers in cells and tissues as well as a semiquantitative measurement of content. It has successfully been used for measurement and semiquantitative analysis of biomarkers in small amounts of tissue.[117, 149, 150] The TOF-SIMS technique has previously been used for the characterization of vitamin E and cholesterol in cells and tissues.[151-153]

The TOF-SIMS technique has its strengths and limitations. One limitation with this method is the non-quantitative measurement; therefore it is not possible to produce numbers of the total amount of vitamin D molecules deposited in tissues. In order to further validate the ability to compare groups regarding vitamin D, the TOF-SIMS should be validated against methods able to measure vitamin D quantitatively. However, with the advantage of only needing small tissue sample sizes as well as producing images it could be a valuable tool in studies on vitamin D in adipose tissue, as well as other tissues of interest.

6.2 Main findings

6.2.1 Vitamin D status

We found higher circulating DBP concentrations in obese women of reproductive age compared with normal-weight women. Other studies exploring circulating DBP in association with body weight or body composition measures have shown conflicting results. In keeping with our results, Oberbach *et al.* found a higher mean serum DBP in obese men and women,[154] and Taes *et al.* found a positive correlation between DBP and FM in elderly men.[155] In contrast, negative association between DBP and BMI has been shown in pregnant women,[156] and in young men and women.[157] Additionally, several studies have shown no association between DBP and BMI, body weight, fat free mass or FM.[158-162] The populations in the mentioned studies differ to some extent with regard to age, gender, pregnancy, and that most populations being more likely to trend to overweight rather than obese, and this might explain differences in results between the studies.

A reason for the higher circulating DBP in the obese women can only be speculated on. We found a positive correlation between DBP and FM when obese and normal-weight women were analysed together, but a negative correlation between DBP and FM in the normal-weight women when analysed

separately. This suggests that DBP is not easily explained by linear association to body composition measures. Both pregnancy and oral estrogen administration increases circulating DBP concentrations.[163-165] Estrogen metabolism might be impaired in obese women[166] and this could potentially affect the hepatic production of DBP and should be further studied. Circulating concentrations of DBP are affected by DBP phenotype,[155, 167] and an association between DBP gene polymorphism and FM has been shown.[168] Whether genetic differences in obese subjects affect circulating DBP, however, is unknown. In an *in vitro* study, interleukin-6 (IL-6) increased the expression of DBP messenger ribonucleic acid (mRNA) in, and the DBP secretion from, hepatic cells.[169] Higher levels of circulating IL-6 in obese women have been found and might potentially affect the hepatic production of DBP.[170] In rats, the expression of the DBP mRNA was moderately expressed in fat tissue.[171] Whether DBP is, to some degree, produced by human adipose tissue is unknown at present. Interestingly, DBP concentrations were positively correlated to serum lipids and high sensitive C-reactive protein (hs-CRP).[161, 172] Whether the altered serum lipids and/or the elevated CRP in obese subjects[173] affects DBP concentrations needs further investigation. Certainly, these proposed possible explanations for higher circulating DBP are highly speculative and the regulation of DBP synthesis needs further study. Because the potential role of DBP in several biological functions, apart from vitamin D binding, such as actin scavenging, fatty acid transport, macrophage activation and chemotaxis,[174, 175] our findings are of interest and further studies of DBP are warranted.

Unlike the higher circulating DBP, calculated free 25(OH)D was lower in obese compared to normal-weight women of reproductive age. In circulation, approximately 88% of 25(OH)D is bound to DBP, 12% to albumin and only a very low amount (0.03%) is unbound.[115] According to the free hormone hypothesis, the free fraction of a hormone is more biologically active than the total amount.[57] A few studies have shown that the free rather than total 25(OH)D concentrations were associated with differences in bone mineral density, osteoporosis, and serum calcium.[157, 176, 177] Therefore, it may be important, in future studies to include DBP concentrations and free levels of 25(OH)D and not only total circulating 25(OH)D in relation to vitamin D status and its effect on health outcomes.

We found lower 25(OH)D and 1α,25$(OH)_2$D concentrations in obese women of reproductive age, and lower 25(OH)D early in pregnancy in obese women

compared with normal-weight women. This is in accordance with earlier reports.[60, 61, 63, 87, 178] There is, to our knowledge, only one report of vitamin D status in obese individuals in Sweden,[75] but this study did not include a normal-weight comparison group. Hulthin *et al.* found that 70.4% of obese men and women had circulating 25(OH)D between 25 and 75 nmol/L, compared to 86.1% in the obese women of reproductive age in the present study.[75] A larger proportion of the obese women in the present study, both pregnant and non-pregnant, had 25(OH)D concentrations <50 nmol/L compared with the normal-weight women. Presently, there are no suggested cut-off levels specifically for obesity or during pregnancy. We found few measures of circulating 25(OH)D concentrations below 25 nmol/L (defined as severe deficiency or deficiency depending on chosen cut-off level) either in normal-weight or obese women of reproductive age or during pregnancy. Additionally, some studies have found that not only low but also high circulating 25(OH)D was correlated with a higher risk of total mortality,[179-181] prostate cancer,[182] and total cancer mortality.[180] One study performed during pregnancy found that children to women with 25(OH)D concentrations >75 nmol/L compared with <30 nmol/L in late pregnancy had a higher risk for asthma at age 9 years.[183] An RCT in older women found higher risk for fractures and falls in the vitamin D supplemented group.[184] Even though the studies are few, the possible adverse effects with high 25(OH)D should be considered when setting cut-off levels for optimal circulating 25(OH)D concentrations.

We found that obese women had lower circulating 25(OH)D compared with normal-weight women in the first trimester but not in the second and third trimester. Controlling for supplement use, travelling to a sunny country and season did not change the results. In accordance with our results, Josefsson *et al.* found that there was no difference in third trimester serum 25(OH)D in 33 lean compared with 15 obese women.[88] In contrast, Bodnar *et al.* found that obese women (based on pre-pregnancy BMI) had lower mean serum 25(OH)D concentrations at gestational week 4-22 compared with normal-weight women.[87] The lower serum 25(OH)D in the first, but not in the second and third trimester is somewhat unexpected and difficult to explain. A plausible explanation might be that differences in gestational weight gain between normal-weight and obese women might have an effect on vitamin D status in late pregnancy. Drincic *et al.* found that volumetric dilution explained the differences in circulating 25(OH)D between obese and non-obese individuals,[68] and generally obese women gain less weight during pregnancy compared to normal-weight women.[185] The obese

women in our study gained less body weight compared to normal-weight women, possibly contributing to decreased differences in circulating 25(OH)D in the second and third trimester. Due to dropout, the sample size decreased and it could simply be the loss of power explaining the loss of difference in the second and third trimesters. Other dietary intervention studies have reported drop-out rates of 15-24%.[186-188]

Observational studies have shown an association between vitamin D status and obesity, as well as an association between vitamin D status and diseases such as diabetes and cancers, but causal associations are less proven. Additionally, the effects of lower 25(OH)D concentrations on long-term health in obese subjects are largely unknown. Moreover, one study found that the circulating PTH in obese compared to non-obese subjects was suppressed at lower serum 25(OH)D concentrations, suggesting that circulating 25(OH)D levels in obese individuals might have different physiological affects compared with that in non-obese individuals.[189] Some vitamin D supplementation trials in obese populations have been conducted. An RCT study from Norway, where overweight and obese individuals were supplemented with either vitamin D (500 or 1000 μg/week) or placebo for one year, did not find any beneficial effects on inflammation markers,[190] cardiovascular risk factors,[191] thrombotic markers,[192] or on weight or body composition.[193] They did, however, find a reduction in symptoms of depression in the supplemented groups.[194] Additionally, Wamberg *et al.* did not find any effect of vitamin D supplementation (225 μg/day for 26 weeks) on weight, body composition measures, serum lipids, inflammation markers, blood pressure or homeostasis model assessment of insulin resistance in obese individuals.[195] Some studies have also considered vitamin D supplementation in conjunction with a weight loss program, but no additional effects of vitamin D have been found on weight loss measures.[196,197] In contrast, Zittermann *et al.* found an additional effect of vitamin D supplementation during a weight loss program on triglycerides and tumor necrosis factor alpha, but also an increase in low-density lipoprotein.[198] Rosenblum *et al.* found that vitamin D and calcium given in orange juice did not have an effect on total weight loss compared with control groups, but the supplemented groups reduced their VAT more compared with the non-supplemented group.[199]

In summary, there is a lack of proven causality and limited evidence that vitamin D supplementation has any positive effects on weight measures or other health outcomes in obesity. Furthermore, currently there is not enough evidence for a

widely spread vitamin D deficiency in Swedish women. Altogether, based on the current knowledge, a general screening of healthy fair-skinned women during pregnancy or in obese women in the clinical setting cannot be recommended at present.

6.2.2 Vitamin D intake

One possible reason for lower 25(OH)D in obese individuals would be a lower vitamin D intake. However, we found no evidence of lower vitamin D intake in the obese women. The self-reported dietary intake of vitamin D in the present studies was generally low, both in women of reproductive age and during pregnancy, and a majority of the women reported intakes below national recommended levels. Additionally, considering that the vitamin D intake recommendation in the coming 5th edition of the NNR (meant to be published during autumn 2013) for non-pregnant adults most certainly will be increased to 10.0 μg/day, an even higher proportion of women than found in the present study would not attain recommended intake levels.

The mean dietary vitamin D intake in the women of reproductive age in our study was 7.2-7.9 μg/day, and in the pregnant women 7.2-8.8 μg/day. In the Swedish national dietary intake survey (Riksmaten 2010-11), the dietary intake of vitamin D in women in the age groups 18-30 years and 31-44 years were 5.2 and 6.2 μg/day, respectively.[33] In two Swedish studies of predominantly normal-weight pregnant women, dietary vitamin D intake was found to be 5.6-6.1 μg/day.[99, 100] The higher dietary intake in the women found in our study may be due to a higher reported fish intake.[33, 200] Also, we used a dietary questionnaire with FFQ design, and FFQs are often found to produce higher mean intakes of vitamin D compared with other methods.[128-133] The obese women, both pregnant and non-pregnant, did not have lower dietary intake of vitamin D compared to normal-weight women. In the first trimester, the obese pregnant women reported higher vitamin D intake compared to normal-weight women. Earlier studies have shown both lower,[80, 81] or no difference in vitamin D intake in obese compared with overweight or normal-weight individuals.[78, 79] In pregnancy, higher[108] and lower[102, 201] intake in obese compared with normal-weight women has been shown. Thus, it is not likely that a lower dietary vitamin D intake would contribute to the lower 25(OH)D concentrations in the obese women in the present study.

In paper I, the use of vitamin D-containing dietary supplements was higher in the non-pregnant normal-weight (35%) compared with obese (14%) women. A more limited use of dietary supplements in obese individuals compared with non-obese individuals has been shown.[202, 203] In Riksmaten 2010-11, 27% of the women reported use of dietary supplements.[33] This included all kinds of supplements suggesting that the participants in our study, at least the normal-weight women, had a higher dietary supplement use than the general population. In contrast, we found no difference of vitamin D-containing supplement use between normal-weight and obese individuals during pregnancy, 61% using vitamin D-containing dietary supplements in the first trimester. From the first trimester, the usage of dietary supplements decreased to 46 and 53% to the second and third trimester respectively. A Swedish study reported a use of vitamin D-containing dietary supplement of 48% and the usage was lower in women with BMI ≥25 kg/m^2.[204] Brembeck *et al.* reported that 56% of the women used supplements containing vitamin D in late pregnancy.[100] Thus, the use of vitamin D-containing dietary supplements is relatively common during pregnancy. Multivitamins/minerals were the most commonly used supplement contributing vitamin D. There is limited evidence of any benefit of a general multivitamin supplementation during pregnancy,[205] and specifically for vitamin D, a Cochrane review published in 2012 concluded that the benefits of vitamin D supplementation during pregnancy as a part of routine antenatal care were yet to be determined.[95]

In summary, obese women did not report lower vitamin D intake compared with normal-weight women, thus suggesting that a low vitamin D intake is not a major contributing factor to the lower circulating 25(OH)D concentrations in obese individuals. A majority of the women did not reach the national dietary recommendations and actions on a national level should be taken to increase vitamin D intake in women of reproductive age and during pregnancy.

6.2.3 Vitamin D and adipose tissue

In paper I, FM% was the most important factor explaining serum 25(OH)D concentrations in the multiple regression analysis. In the pregnant women (paper II) FM% was negatively associated with circulating 25(OH)D in the first trimester. Other studies have found 25(OH)D to be negatively associated with increased body weight and FM, both during pregnancy and non-pregnancy.[97, 178, 206] The lower 25(OH)D in obese populations has been explained by a dilution model.[68]

In paper III, a new method for measuring vitamin D in adipose tissue was described. We found vitamin D_3 and its metabolites to be localized to the lipid droplet in the adipocyte. However, we did not find any obvious differences in the localization between normal-weight and obese women. To our knowledge, this is the first image showing localization of vitamin D or its metabolites on a cellular level. Also, we found that the ion peak intensities of vitamin D_3 and 25(OH)D_3 were lower in SAT from the obese compared with the normal-weight individuals. A dilution model has shown to completely explain the lower circulating 25(OH)D in obesity.[68] The lower vitamin D_3 and 25(OH)D_3 in SAT in obese individuals in the present study might be explained by dilution of these metabolites in the higher amount of FM in the obese individuals. Additionally, we found higher ion peak intensities from SAT compared to VAT in obese subjects. This is in contrast with the results of Beckman *et al.* showing the opposite.[207] The low number of subjects in our study limits the possibility of drawing conclusions on differences in the vitamin D content in adipose tissue compartments between normal-weight and obese subjects or differences in site-specific contents.

In summary, TOF-SIMS can be used to measure vitamin D and its metabolites in adipose tissue, and we have produced images of the localization of vitamin D and its metabolites. The TOF-SIMS could be a useful tool in future studies elucidating vitamin D stores and localizations in tissues.

7 Conclusions

Obese pregnant women and obese women of childbearing age had lower circulating 25(OH)D concentrations compared with normal-weight women. Interestingly, obese women of reproductive age had higher vitamin D-binding protein compared with normal-weight women. A higher proportion of obese women had circulating 25(OH)D concentrations that could be considered insufficient. In the healthy normal-weight women, a majority had sufficient circulating 25(OH)D concentrations.

We found no evidence of lower dietary vitamin D intake in obese compared with normal-weight, suggesting small effects of vitamin D intake on the lower 25(OH)D found among the obese women.

A majority of the women, both pregnant and non-pregnant, had vitamin D intakes below Swedish national dietary recommendations. This should be considered as a public health issue and attempts should be made to increase vitamin D intake in women of reproductive age and during pregnancy.

We found vitamin D and its metabolites to be localized in the lipid droplet of the adipocyte. Also, there might be differences between normal-weight and obese women in the content of vitamin D molecules in adipose tissue.

8 Future perspectives

Low circulating 25(OH)D in obesity is established knowledge, but still several questions remain to be answered. Large prospective studies on vitamin D status, with special emphasis on the obese state should be carried out, elucidating the effect of vitamin D and its effects on long-term heath. Also, studies exploring if vitamin D supplementation could be an effective treatment of obesity itself or in conjunction with other obesity treatment regimes is warranted. Few studies have examining the effect of vitamin D supplementation during pregnancy, especially in pregnancy further complicated by obesity. Additionally, large studies on 25(OH)D concentrations in different populations in Sweden to examine the need for increasing vitamin D status or not is needed.

Vitamin D intakes are generally low and many have intakes below the national recommendations. National efforts should be made in order for the general population to reach dietary intake recommendations and the means to reach this should be addressed. Also, there is a lack of studies on vitamin D absorption and the effect of vitamin D in foods on vitamin D status, as well as studies on content of vitamin D in foods to improve the data in nutritional databases. This will further strengthen de ability to measure dietary vitamin D intake correctly.

Future studies should address:

- The effect of the lowered vitamin D status in obesity on long-term health outcomes, and the possible different vitamin D metabolism in obesity, including DBP, and its effects on the mechanism of action of vitamin D in obesity.
- Whether specific cut-off levels should be set for obese individuals, and what doses are needed for raising circulating 25(OH)D to these levels. The effect of long-term intake of high vitamin D doses (supplement use) and possible adverse effects.
- The fact that mechanisms of release of vitamin D from adipose tissue are largely unknown, both in normal-weight and obese individuals.

- What the most effective way to increase vitamin D intake in the general population might be, and how this would affect 25(OH)D concentrations.
- The use of the TOF-SIMS technique in measuring vitamin D in tissues in larger groups, and by using quantitative methods validate the ability of TOF-SIMS to compare vitamin D content in tissues between groups.

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