

# **Glucose metabolism, genetic factors, vitamin D binding protein and directly measured free 25-hydroxyvitamin D**

Results from a randomized controlled trial with high-dose vitamin D supplementation in subjects with prediabetes

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<b>Contents</b>	<b>page</b>
Acknowledgements.....	4
Norsk sammendrag.....	5
English summary.....	6
List of papers .....	8
Abbreviations .....	9
Definitions.....	10
1. Introduction.....	11
1.1 Prediabetes, T2D and cardiovascular risk factors .....	11
1.2 Vitamin D synthesis .....	13
1.3 Vitamin D status .....	16
1.4 Prediabetes, T2D, hypertension, blood lipids and vitamin D .....	19
1.5 Vitamin D binding protein and free 25(OH)D.....	20
1.6 Genetics and vitamin D.....	22
2. Aims of the thesis .....	25
3. Study population and methods.....	26
3.1 Study population.....	26
3.2 Measurements, questionnaires and safety.....	27
3.3 Blood samples .....	28
3.4 Genotyping, SNPs selection and DBP phenotypes.....	29
3.5 Statistical analyses .....	31
3.5.1 Statistical tests in Paper I.....	31
3.5.2 Statistical tests in Paper II .....	32
3.5.3 Statistical tests in Paper III.....	32
4. Summary of results.....	34
5. General discussion.....	38
5.1 Methodological considerations.....	38
5.1.1 Study design .....	38
5.1.2 Systematic errors .....	41
5.1.3 Random errors, sample size and power calculations.....	42
5.1.4 External validity of the results.....	45
5.1.5 Study medication, dose of study medication and safety .....	45
5.2 Discussion of main results .....	47
5.2.1 Vitamin D and its effect on the glucose metabolism, blood pressure and lipids.....	47
5.2.2 SNPs, baseline serum 25(OH)D concentration and BMI and their effect on the serum 25(OH)D response following vitamin D supplementation.....	51
5.2.3 Free 25(OH)D.....	54
5.2.4 Limitations .....	56

6. Conclusion and implications..... 57  
    6.1 Conclusion ..... 57  
    6.2 Implications ..... 58  
References..... 59

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## Norsk sammendrag

I en klinisk intervensjons studie er 511 personer med prediabetes randomisert til 20,000 IU med vitamin D per uke eller til placebo i 5 år. Denne avhandlingen presenterer 12 måneders resultatene. Fra observasjonsstudier vet vi at lave vitamin D nivåer er assosiert med økt risiko for å utvikle type 2 diabetes (T2D), men om vitamin D tilskudd kan forebygge utviklingen av T2D har ikke vært fullstendig avklart.

Hovedmengden av vitamin D produseres ved sollys eksponering av huden, i tillegg får vi noe vitamin D gjennom kosten. Serum 25-hydroxyvitamin D (25(OH)D) brukes som mål på en persons D vitamin status, men det er ikke fullstendig avklart hva optimale verdier er, og heller ikke om en annen vitamin D metabolitt bedre reflekter vitamin D nivået enn det serum 25(OH)D gjør. Det er kjent at serum 25(OH)D nivået påvirkes av genetiske forhold. Derimot er det lite kunnskap om hvordan genene påvirker serum 25(OH)D responsen etter vitamin D tilskudd. I sirkulasjonen er tilnærmet alt vitamin D sterkt bundet til bindeproteiner, mens fritt 25(OH)D utgjør omtrent 0,1 %. Det viktigste bindeproteinet er vitamin D bindeprotein (DBP). Det er seks ulike DBP fenotyper, disse er ulikt fordelt mellom etniske grupper og har ulik bindings koeffisient for serum 25(OH)D.

Studiedeltagerne hadde ved inkludering serum 25(OH)D nivå på 60.5 nmol/L. Etter 12 måneder var det ingen effekt av vitamin D tilskudd på glukosemetabolismen eller utviklingen av T2D. Vitamin D tilskudd hadde heller ingen effekt på blodtrykk eller lipidverdiene. Vitamin D tilskudd kan på bakgrunn av dette ikke anbefales til personer med adekvate D vitamin nivåer som forebyggende behandling for T2D, og heller ikke for å bedre glukosemetabolismen, blodtrykket eller lipidverdiene. Videre finner vi at genetiske variasjoner sammen med kropps masse indeks og utgangsverdi av serum 25(OH)D medfører store individuelle forskjeller i serum 25(OH)D responsen etter vitamin D tilskudd. Ved anbefaling om vitamin D tilskudd må en derfor ta hensyn til faktorer som påvirker 25(OH)D responsen. Vi finner også at observerte forskjeller i serum 25(OH)D mellom DBP fenotyper, og mellom kjønn, reduseres for serum fritt 25(OH)D, sannsynligvis forårsaket av forskjeller i serum DBP nivåer. På bakgrunn av disse funnene bør serum fritt 25(OH)D vurderes målt ved sykdommer og tilstander som påvirker serum DBP nivået.

## English summary

Subjects with vitamin D deficiency have an increased risk for developing type 2 diabetes (T2D); however, whether vitamin D supplementations can prevent the development of T2D is not known. We have conducted a randomized controlled study with 511 subjects with prediabetes randomized to 20,000 IU vitamin D per week or to placebo for 5 years. In this thesis, the 12 months' results are presented. Annual oral glucose tolerance tests were performed, with the primary endpoint being development of T2D.

T2D is strongly associated with obesity and sedentary lifestyle, in addition, genetic predisposition is important. T2D develops over several years; it is characterized by insulin resistance and also reduced insulin secretion when the pancreatic beta-cells fail. Annually about 10 % of subjects with impaired glucose tolerance and/or impaired fasting glucose, or prediabetes, develop T2D. T2D is associated with increased morbidity and mortality, which again cause major health and economic challenges. The number of people diagnosed with T2D has tripled during the last 30 years, and additional increase is expected in the years to come. Worldwide, at least 382 million have T2D, thus prevention of T2D is of major importance.

Solar exposure of the skin leads to vitamin D production; in addition, some vitamin D is obtained via diet. In the circulation, most vitamin D metabolites are bound with high affinity to the vitamin D binding protein (DBP), while some are more loosely bound to albumin. Serum 25-hydroxyvitamin D (25(OH)D) is used to evaluate a person's vitamin D status; however, it is not settled what the optimal concentration is, or whether another vitamin D metabolite better reflects a person's vitamin D status. Further, the serum 25(OH)D concentration is influenced by genetics; however, little knowledge exists on how genes influence the serum 25(OH)D response following vitamin D supplementation.

We have analysed the effect of vitamin D supplementation on glucose metabolism, blood pressure and lipids. Further, we analysed the relationship between serum 25(OH)D and directly measured free 25(OH)D with the six different DBP phenotypes, and their interrelationship following vitamin D supplementation. Furthermore, the influence of genes on serum 25(OH)D is evaluated, as well as how genes affect the serum 25(OH)D response following vitamin D supplementation.

We found that high dose vitamin D supplementations do not improve glycaemic indices or cardiovascular risk factors in a population with prediabetes. Thus, in subjects with

adequate vitamin D status, vitamin D supplementations cannot be recommended for the prevention of T2D, nor can it be recommended to improve blood pressure or blood lipids. Further, we found that there are large individual differences in the serum 25(OH)D response following vitamin D supplementation, due to genetic, BMI and baseline 25(OH)D differences. These large variations found for the serum 25(OH)D response should be considered when giving advice on vitamin D supplementation. Furthermore, the direct measurements of free 25(OH)D reduces the differences seen in serum 25(OH)D between DBP phenotypes and sexes, most likely caused by the differences seen in DBP concentrations. Thus, in subjects with conditions affecting serum DBP concentrations direct measurements of free 25(OH)D should be considered.

## List of papers

This thesis is based on the following papers:

Paper I: Stina T. Sollid, Moira Y. S. Hutchinson, Ole M. Fuskevåg, Yngve Figenschau, Ragnar M. Joakimsen, Henrik Schirmer, Inger Njølstad, Johan Svartberg, Elena Kamycheva, and Rolf Jorde. **No Effect of High-Dose Vitamin D Supplementation on Glycemic Status or Cardiovascular Risk Factors in Subjects with Prediabetes.** *Diabetes Care* 2014. 37(8): 2123–2131.

Paper II: Stina T. Sollid, Moira Y. S. Hutchinson, Ole M. Fuskevåg, Ragnar M. Joakimsen, and Rolf Jorde. **Large Individual Differences in Serum 25-hydroxyvitamin D Response to Vitamin D Supplementation: Effects of Baseline Concentration, Body Mass Index and Genetic Factors. Results from a Randomized Clinical Trial.** *Hormone and Metabolic Research* 2015. *Epub ahead of print.*

Paper III: Stina T. Sollid, Moira Y. S. Hutchinson, Vivian Berg, Ole M. Fuskevåg, Yngve Figenschau and Rolf Jorde. **Effects of Vitamin D Binding Protein Phenotypes and Vitamin D Supplementation on Serum Total 25(OH)D and Directly Measured Free 25(OH)D.** *European Journal of Endocrinology* 2016. *Epub ahead of print.*



## Abbreviations

BMD	Bone mineral density
BMI	Body mass index
CVD	Cardiovascular disease
CYP2R1	Cytochrome P450, family 2, subfamily R, polypeptide 1. Also known as vitamin D 25-hydroxylase.
CYP24A1	Cytochrome P450, family 24, subfamily A, polypeptide 1. Also known as 1,25-dihydroxyvitamin D <sub>3</sub> 24-hydroxylase.
DBP	Vitamin D binding protein
DHCR7	7-dehydrocholesterol reductase
DRI	Dietary reference intake
D2d study	Vitamin D and Type 2 Diabetes study
ELISA	Enzyme-linked immunosorbent assay
GWAS	Genome-wide association studies
HbA <sub>1c</sub>	Glycated hemoglobin
HDL	High density lipoprotein
HOMA-IR	Homeostasis model assessment of insulin resistance
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IOM	Institute of Medicine
LC-MS/MS	Liquid chromatography-tandem mass spectrometric assay
LD	Linkage disequilibrium
LDL	Low density lipoprotein
NHANES	The National Health and Nutrition Examination Survey
OGTT	Oral glucose tolerance test
PTH	Parathyroid hormone
RANKL	Receptor activator of nuclear factor kB ligand
RCT	Randomized controlled trial
RXR	Retinoic acid X receptor
SNP	Single nucleotide polymorphism
T1D	Type 1 diabetes
T2D	Type 2 diabetes

UVB	Ultraviolet B
VDR	Vitamin D receptor
ViDA study	Vitamin D assessment study
VITAL	<i>VIT</i> amin D and <i>OmegA</i> -3 <i>TriAL</i>
QUICKI	Quantitative insulin-sensitivity check index
1,25(OH) <sub>2</sub> D	1,25-dihydroxyvitamin D
7-DHC	7-dehydrocholesterol
25(OH)D	25-hydroxyvitamin D

## Definitions

The World Health Organization (WHO) diagnostic glucose criteria for:

Type 2 diabetes (T2D) is fasting plasma blood glucose  $\geq 7.0$  mmol/L or a 2-hour plasma glucose after oral glucose tolerance test (OGTT)  $\geq 11.1$  mmol/L;

impaired glucose tolerance (IGT) is fasting plasma blood glucose  $< 7.0$  mmol/L combined with a 2-hour plasma glucose after OGTT  $\geq 7.8$  and  $< 11.1$  mmol/L, and

impaired fasting glucose (IFG) is fasting plasma blood glucose  $> 6.0$  and  $< 7.0$  mmol/L combined with a 2-hour plasma glucose after OGTT  $< 7.8$  (1).

The OGTT consists of a 75-gram glucose load.

The International Expert Committee's diagnostic glycated hemoglobin (HbA<sub>1c</sub>) criterion for:

T2D is HbA<sub>1c</sub>  $\geq 6.5$  % (2).

## 1. Introduction

The number of people diagnosed with type 2 diabetes (T2D) has increased rapidly, and during the last 30 years, the number diagnosed has tripled (3, 4), thus constituting a major challenge to health care systems globally. In 2013, it was estimated that 382 million, equivalent to almost 9 % of the world's adults, had diabetes, half of them not aware of it (5). The estimates for 2035 are an overwhelming 592 million (5). In Norway the numbers of subjects diagnosed with T2D have quadrupled the last 50 years, and annually more than 6,000 individuals are newly diagnosed (6). Overall, it is estimated that more than 350,000 Norwegians have T2D. Accordingly, it is projected that T2D will climb from the 11<sup>th</sup> leading cause of death in 2002 to the 7<sup>th</sup> leading cause of death in 2030 (7). Today most people with T2D live in low- and middle income countries; however, in the years to come the greatest increase is expected in low-income countries (5).

Inverse associations exist between serum total 25-hydroxyvitamin D (hereafter denoted serum 25(OH)D) and several diseases like cardiovascular disease (CVD) (8) and peripheral vascular disease (9), autoimmune diseases like multiple sclerosis (10) and thyroid diseases (11), neurological diseases like Parkinson's disease (12), and several different cancers (13). Similar associations also exist for infections (14), for severe infections and mortality in critical ill patients (15), and also for all-cause mortality (16, 17). Further, this inverse association also exists for gestational diabetes mellitus (18) and T2D (19-21).

### 1.1 Prediabetes, T2D and cardiovascular risk factors

The anabolic hormone insulin, synthesized by the pancreas, is secreted into the blood stream as a response to raised blood glucose obtained after meals containing carbohydrates (22). Insulin helps maintain normoglycaemia through increased glucose uptake in peripheral tissues, mainly muscles and fat, and stimulation of glycogenesis. Simultaneously, insulin inhibits glucagon and thus gluconeogenesis and glycogenolysis (22).

T2D is a chronic disease developing over several years characterized by hyperglycaemia caused either by insulin resistance and/or relative insulin deficiency due to beta-cell dysfunction (23, 24). Impaired fasting glucose (IFG) and impaired glucose tolerance (IGT), both characterized by elevated blood glucose, are known as "prediabetic" stages (25). Recently it has been suggested that the term prediabetes also should be applied for

individuals with a glycated hemoglobin (HbA<sub>1c</sub>) in the range 5.7–6.4 % (23), and it is these individuals that have the greatest risk of developing T2D (2). The conversion rate from IFG and IGT to T2D changes with both populations and diagnostic criteria (26). The Diabetes Prevention Program is a multicentre study with 3,234 participants with IGT randomized to either intensive lifestyle intervention, Metformin or to placebo (the last two groups were double-blinded) (27). The Diabetes Prevention Program found that 11 % of the 1,082 participants receiving placebo, thus representing the normal progression of T2D, developed T2D after 1 year and almost 40 % developed T2D after 5 years of follow-up (27). Correspondingly, in a review article with over 44,000 individuals, the 5-year risk of developing T2D was increased 9–25 % in individuals with HbA<sub>1c</sub> in the range 5.5–6.0 % and 25–50 % in individuals with HbA<sub>1c</sub> in the range 6.0–6.5 % as compared to those with HbA<sub>1c</sub> in the range 5.0–5.5 % (28).

Over time hyperglycaemia causes endothelial dysfunction, impaired angiogenesis and damaged nerves (29). In addition, T2D is associated with hypertension and dyslipidemia, altogether causing increased risk of CVD, retinopathy, foot ulcers, kidney failure and neuropathy (30-33). Although challenging to estimate, mortality in individuals with T2D is higher than in non-diabetic individuals, and also substantially higher compared to individuals with known CVD (34). Positively, a recently published Swedish study reports a 15 % decrease in the overall mortality in patients with T2D (35). Nevertheless, mortality in those under 55 years of age and well-regulated T2D (HbA<sub>1c</sub> < 6.9 %) was twice as high as in individuals without diabetes (35). For peers having poorly regulated T2D (HbA<sub>1c</sub> > 9.7 %), a 200 % increased mortality rate as compared to non-diabetic individuals was seen (35). Furthermore, in estimates from 2011, diabetes is made accountable for over 8 % of deaths worldwide for all age groups (36).

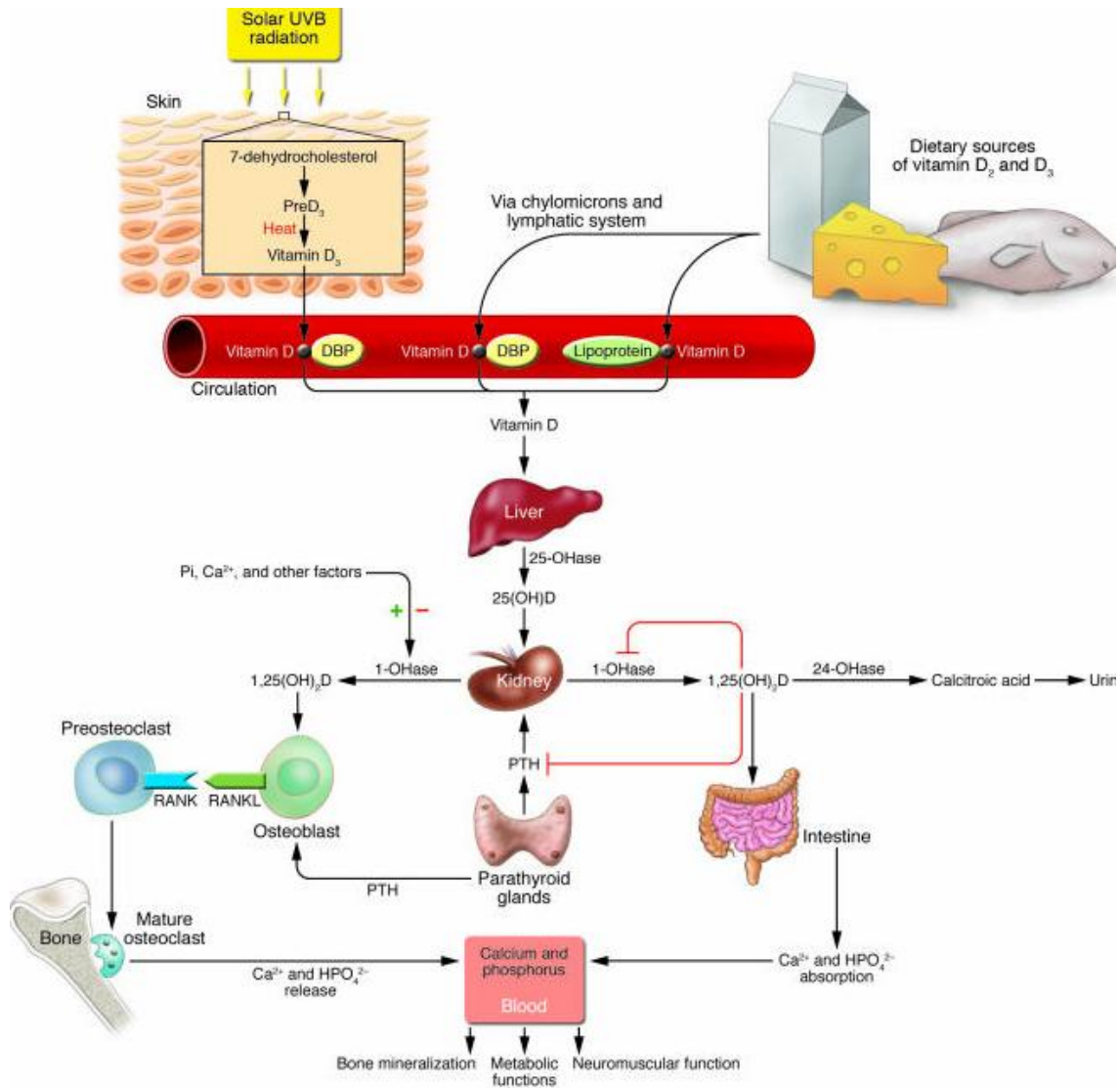
Increasing bodyweight and sedentary lifestyle are the most important risk factors for prediabetes and T2D (23); in addition the risk is affected by family history and genetics (23). Intervention at the “prediabetic” stage with changes in lifestyle, with medication, or with bariatric surgery with resulting weight loss, may prevent progression to T2D (27, 37-42). However, changes in lifestyle are difficult to implement, and in addition pharmacological intervention may be both expensive and have unforeseen side-effects (43-47).

## 1.2 Vitamin D synthesis

Vitamin D and its metabolites are hydrophobic, and in the circulation almost all of the vitamin D metabolites are bound with high affinity to the vitamin D binding protein (DBP) (48). Vitamin D is mainly produced endogenously in the skin or obtained through the diet from fatty fish like salmon, cod liver oil or vitamin D supplementation (13), as shown in Figure 1. Upon solar ultraviolet B (UVB) exposure, 7-dehydrocholesterol (7-DHC) in the skin is converted to vitamin D<sub>3</sub>. Vitamin D<sub>3</sub> formed in the skin together with vitamin D<sub>2</sub> and D<sub>3</sub> obtained via diet are then 25-hydroxylated in the liver to 25(OH)D, the major circulating vitamin D metabolite (13). In the renal proximal tubules the protein megalin acts as an endocytic receptor reabsorbing the vitamin D-DBP complex (49); 25(OH)D is thereafter further 1 $\alpha$ -hydroxylated to 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D) by 1- $\alpha$ -hydroxylase (13). 1- $\alpha$  hydroxylase is found mainly in the proximal tubular epithelial cells of the kidney (50). In addition, several extra-renal cells contain 1- $\alpha$  hydroxylase (51, 52), thus indicating that these cells have the capacity to produce 1,25(OH)<sub>2</sub>D locally (53).

Vitamin D's main function is maintenance of mineral homeostasis and serum calcium concentration (13). Parathyroid hormone (PTH) synthesis and secretion are up-regulated by low serum calcium concentrations and high serum phosphate concentrations, and down-regulated by high serum calcium concentrations and 1,25(OH)<sub>2</sub>D concentrations. PTH exerts its effect through enhanced calcium reabsorption in the kidneys, increased calcium release from the bone, and through enhanced formation of 1,25(OH)<sub>2</sub>D in the kidneys (13, 54). In the small intestine 1,25(OH)<sub>2</sub>D interacts with the vitamin D receptor (VDR) and up-regulates the expression of intestinal epithelial calcium ion channels (13). As a consequence the intestinal calcium absorption is increased about 200 % (13). 1,25(OH)<sub>2</sub>D also interacts with the VDR in osteoblasts of the bone and stimulates the receptor activator of nuclear factor  $\kappa$ B ligand (RANKL) (13). RANKL again interacts with receptor activator of nuclear factor  $\kappa$ B (RANK) which stimulates the formation of osteoclasts that dissolves bonematrix releasing calcium from bone into the circulation (13); thus 1,25(OH)<sub>2</sub>D synergizes with PTH on bone for calcium release (54), shown in Figure 1. 1,25(OH)<sub>2</sub>D in addition helps maintain stable serum calcium concentrations through stimulation of the calcium reabsorption in the renal glomeruli (13).

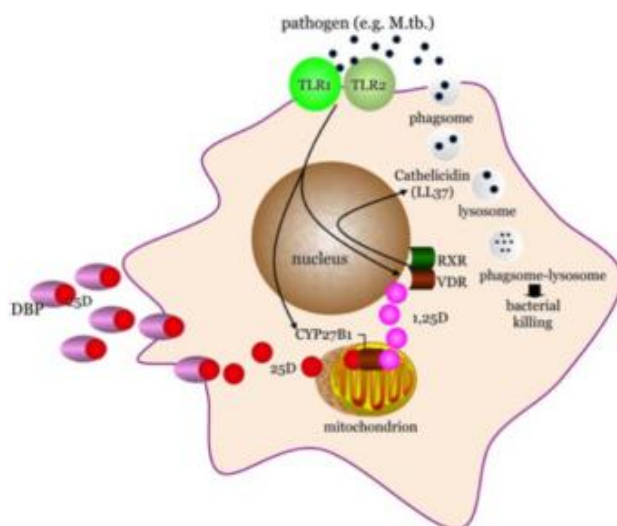




**Figure 1: The photoproduction and metabolism of vitamin D and the various biologic effects of 1,25(OH)<sub>2</sub>D on calcium, phosphorus, and bone metabolism.** Reused with the permission from: American Society for Clinical Investigation and Copyright Clearance Center (Holick MF 2006 Resurrection of Vitamin D Deficiency and Rickets. The Journal of Clinical Investigation 116:2062–2072).

1,25(OH)<sub>2</sub>D's main biological actions are mediated through regulation of gene transcription after binding with the VDR (53). The VDR, encoded by the VDR gene on chromosome 12, is a nuclear receptor present not just in the intestine, bone and kidney, but in most human cells of the immune system, the central nervous system, the endocrine system, in different epithelial cells, in cardiomyocytes and in adipocytes (55). Thus, 1,25(OH)<sub>2</sub>D has effects other than those related to bone metabolism. The VDR is activated by 1,25(OH)<sub>2</sub>D in very low concentrations (56), the liganded VDR binds to retinoic acid X receptor (RXR) which again binds specific nucleotide sequences in the DNA, so-called response elements of the DNA (57), illustrated in Figure 2. In the human genome there are

at least 2,776 VDR binding sites, and over 20 % of them are near the transcriptional start site of vitamin D-responsive genes (58). The VDR-RXR complex is known to affect over 200 genes (59), and it is capable of acting directly upon regulatory regions (60). Consequently, genes can be up- or down-regulated, or the gene expression can be inhibited (59). As a result, the VDR is involved in cell proliferation, differentiation and modulation in relation to cancer (61) and the immune system (59). In the immune system, macrophages are capable of local  $1,25(\text{OH})_2\text{D}$  production, further the  $1,25(\text{OH})_2\text{D}$ -VDR complex stimulates the innate immune response enhancing bacterial killing (62), as shown in Figure 2.  $1,25(\text{OH})_2\text{D}$  is also known to affect the adaptive immune system through modulating lymphocyte proliferation and function, thus reducing inflammation as well as autoimmune disorders (62). It is not known why the VDR is present in different human cells, however, a hypothesis is that the VDR has maintained through evolution in order to protect human cells from bacterial infections (63).



**Figure 2: The VDR binding and actions exemplified by a human macrophage responding to the pathogen mycobacterium tuberculosis.** Reused with the permission from: Endocrine Society and Copyright Clearance Center (Adams JS, Hewison M 2010 Update in Vitamin D. The Journal of Clinical Endocrinology and Metabolism 95:471–478).

Also, the degradation of  $1,25(\text{OH})_2\text{D}$  into inactive metabolites is increased through negative feedback mechanisms via the VDR-RXR complex's activation of gene transcription for the enzyme  $1,25(\text{OH})_2\text{D}$  24-hydroxylase (CYP24A1) (64).

Elevated  $1,25(\text{OH})_2\text{D}$  concentrations are usually seen with vitamin D deficiency since PTH concentrations are high (65). This secondary hyperparathyroidism maintains stable serum calcium concentrations through the increased osteoclast activity, and thus calcium

release, leading to osteopenia and osteoporosis (66). Serious vitamin D deficiency additionally leads to phosphaturia which again causes mineralization defects in the bones leading to the bone-softening diseases rickets, seen in children, and osteomalacia, seen in adults (66).

### 1.3 Vitamin D status

An individual's vitamin D status largely depends on diet and sun exposure. Data from The National Health and Nutrition Examination Survey (NHANES) in the United States of adolescents and adults shows that European Americans, regardless of age group or sex, have the highest serum 25(OH)D concentrations, Mexican Americans the second highest, while the lowest concentrations were seen in African Americans (67). In Africa, studies reveal sufficient serum 25(OH)D concentrations, while in the Middle East, as expected, individuals wearing hijab or niqab have substantially lower serum 25(OH)D concentrations than individuals wearing western clothing (68). In Europe, individuals in the Mediterranean countries Spain, Italy and Greece have lower serum 25(OH)D concentrations than individuals in northern European countries (68). The explanation for differences seen between races, and according to latitude, is most likely that simultaneously with migration from sunny areas to the more northern parts of the world those with lighter skin pigmentation were favoured (69). This is due to the reduced levels of melanin in the skin of individuals with lighter skin pigmentation, causing a more effective vitamin D synthesis (54, 69). Although melanin is an effective sunscreen causing reduced UVB penetration of the skin and thus decreased vitamin D<sub>3</sub> production (70), the capacity of endogenous vitamin D synthesis in the skin is the same regardless of race (71). Latitudes above 33° north and below 33° south have absent solar UVB radiation in the winter months. The same areas also lack ozone (72), a gas absorbing most of solar UVB radiation (73). Thus, together with the lighter skin pigmentation, the lack of ozone contributes to a more effective vitamin D synthesis. In addition, a genome-wide association study (GWAS) in Europeans and Northeast Asians, compared to darker skin pigmented populations, provides evidence for natural positive selection over time of individuals with certain 7-DHC reductase (DHCR7) haplotypes enabling them to have higher serum 25(OH)D concentrations (74).

In spite of having lower serum 25(OH)D concentrations, African-American women and men have fewer osteoporoses, and lower fracture rates, compared to European

Americans (75, 76). This phenomenon has been described as a paradox (77), and will later be discussed in more detail.

Serum 25(OH)D concentrations also vary with season, between sexes, with age, and with body mass index (BMI). On the northern hemisphere, the serum 25(OH)D concentrations are lower in the winter than during the summer months (67), this being caused by decreased and/or disappeared solar UVB radiation during the winter months. Further, in a large meta-analysis of cross-sectional studies from all over the world, women were shown to have higher serum 25(OH)D concentrations than men (78). In addition, with increasing age, lower serum 25(OH)D concentrations are seen; the reasons are thought to be that elderly people have restricted sun exposure (79) and that the dermal 7-DHC concentration is reduced, thus resulting in less vitamin D<sub>3</sub> being produced (80). Also, with increasing age, the conversion of 25(OH)D into 1,25(OH)<sub>2</sub>D is decreased due to reduced kidney function (81). However, North Norwegian elderly have been shown to have higher serum 25(OH)D compared to younger individuals, the reason probably being a traditional diet rich in fatty fish (82). Similarly, there are differences between weight groups, with obese subjects having lower serum 25(OH)D concentration compared to normal weight subjects (83). It is hypothesized that this is due to lower vitamin D intake, less exposure to solar UVB radiation, that adipose tissue degrades vitamin D, and/or that obese subjects' distribution volume is higher compared to normal weight subjects (83).

Several indicators have been suggested as biomarkers for a person's vitamin D status. In 2011, the Institute of Medicine (IOM) decided to use bone health as an indicator for their vitamin D and calcium Dietary References Intakes (DRIs) (84). However, bone mineral density (BMD) measures vary with age (84). For infants, the IOM reports that for the time being no certain association exists between bone mineral content and serum 25(OH)D (84). Further, randomized controlled trials (RCTs) in children and adolescents regarding bone mineral content and serum 25(OH)D have been inconsistent, thus in children and adolescents the IOM concluded that published research so far does not support the use of bone mineral content as a biomarker (84). The strongest association between bone health and serum 25(OH)D was found in postmenopausal women and men; however, only 6 RCTs were the basis of the IOMs decision (84). Serum PTH has also been suggested as a vitamin D biomarker, since vitamin D both indirectly, through increased calcium absorption from the intestines, and directly, through inhibition of PTH synthesis by binding to the VDR in the

parathyroid cells, decreases the serum PTH concentration (85, 86). However, the IOM decided not to use PTH as an indicator for DRIs based on several reasons: most studies supporting PTH as a biomarker have been done in a selected population (elderly European and American Caucasians); the flattening of serum PTH in relation to serum 25(OH)D concentration in various studies has shown great variation, ranging from 37.5 nmol/L to 125.0 nmol/L; it is not uncommon to have normal serum PTH despite having low serum 25(OH) concentrations (84); and also with increasing age, PTH is known to increase (87). Nevertheless, the Endocrine Society decided upon PTH as a biomarker for their “Evaluation, Treatment, and Prevention of Vitamin D Deficiency: an Endocrine Society Clinical Practice Guideline” (65). The Endocrine Society refers to a flattening of PTH at serum 25(OH)D concentration between 75–100 nmol/L when defining vitamin D deficiency (serum 25(OH)D < 50 nmol/L) and insufficiency (serum 25(OH)D between 52.5 nmol/L and 72.5 nmol/L) (65). However, and despite the IOM’s evidence for a wide variation in PTH plateaus, the Endocrine Society cites only two articles used for their definitions for deficiency and insufficiency (65). Consequently, the recommendations from the IOM differ from that by the Endocrine Society by 50 %, with the IOM stating that a serum 25(OH)D concentration of 50 nmol/L covers the requirements of at least 97.5 % of the population.

Recommendations for vitamin D supplementation doses also differ, however, less substantially with recommendations varying between 600–800 IU/day of vitamin D for children and adults (65, 84, 88). Both the Endocrine Society and the IOM recommend increased vitamin D doses for obese individuals, with the Endocrine Society recommending at least a doubled dose compared to recommendations for non-obese individuals (65). However, these recommendations are based solely upon the observed lower serum 25(OH)D concentrations in obese subjects, and there is no evidence for improved bone health with increased intake of vitamin D (89).

Based on the IOM definitions, and even higher if it had been based upon the Endocrine Society definitions, a large proportion of the world’s population is vitamin D deficient, e.g. NHANES reports that 81 % of African Americans and 28 % of European Americans fulfill the deficiency definition (90). Thus, vitamin D deficiency and insufficiency, similar to T2D and prediabetes, constitutes a major global health challenge.



#### 1.4 Prediabetes, T2D, hypertension, blood lipids and vitamin D

A consistent association between low serum 25(OH)D concentrations and an increased risk for T2D has been reported in a number of observational studies and in meta-analyses (19, 20, 91). In the largest meta-analysis by Song et al. including 21 prospective studies with altogether over 76,000 individuals and nearly 5,000 incident T2D cases, an inverse association between serum 25(OH)D and the risk of T2D was found (91). Accordingly, subjects in the highest serum 25(OH)D concentration group, compared to subjects in the lowest serum 25(OH)D concentration group had a 38 % lower risk of developing diabetes. Further, with every 10 nmol/L increase in serum 25(OH)D, the risk for T2D was lowered by 4 % (91). Similarly, a low serum 25(OH)D concentration has been found in some observational studies to increase the risk for both insulin resistance and beta-cell dysfunction (92, 93); however, the findings are not consistent (94, 95). Furthermore, in Tromsø 6, a population-based study, an inverse association between serum 25(OH)D concentration and HbA<sub>1c</sub> was found (96).

Already in 1975, Campbell et al. reported seasonal changes in glucose tolerance in healthy subjects with lower fasting and 2-hour glucose values during the summer months (97), and in rat models in the 1980's, vitamin D deficiency was shown to inhibit insulin secretion (98). The VDR is as described earlier present in almost all human cells, also in the pancreatic beta-cells and adipocytes, both important for glucose metabolism (53). Furthermore, 1 $\alpha$ -hydroxylase is found in pancreatic beta-cells (52) and in adipocytes (51) making local production of 1,25(OH)<sub>2</sub>D possible (53).

Similar associations as for serum 25(OH)D and T2D also exist for CVDs, where lower concentrations of serum 25(OH)D are associated with an increasingly higher number of subjects with hypertension, unfavourable lipid profile, CVD and peripheral vascular disease (99-102). These cardiovascular associations are however not present in African Americans (103). However, supporting an idea that vitamin D might have an effect on the cardiovascular system is the presence of the VDR in vascular smooth muscle, in the endothelium and in cardiomyocytes (53), and findings from several animal models indicate that vitamin D is essential for obtaining a healthy cardiovascular system (102).

Therefore, and to no surprise, Vitamin D supplementations have been suggested as a therapeutic option to prevent T2D and CVD (21, 104-108). However, so far intervention studies with vitamin D supplementations have not shown any convincing beneficial effect on

the glucose metabolism and insulin resistance (8, 107), for prevention of T2D in individuals at risk of developing T2D (109), on improving glycaemic indices in subjects with T2D (21), nor on any of the cardiovascular risk factors (102).

### 1.5 Vitamin D binding protein and free 25(OH)D

DBP is a polymorphic serum protein of 458 amino acids synthesized mainly by the liver (110), a process that is estrogen-dependent (111). Nearly 130 variant DBP alleles exist, however, the three common polymorphisms of DBP in humans, GC1F, GC1S and GC2, gives six allelic combinations yielding three homozygote phenotypes (Gc1F/Gc1F, Gc1S/Gc1S and Gc2/Gc2) and three heterozygote phenotypes (Gc1F/Gc1S, Gc1F/Gc2 and Gc1S/Gc2) (112).

Most of the circulating 25(OH)D and 1,25(OH)<sub>2</sub>D are bound to DBP; about 10 % are bound to albumin, and only about 0.1 % of circulating vitamin D metabolites exists in an unbound, or free, form (113). The reason why most of the circulating vitamin D metabolites are bound to DBP can be explained by the 20 times higher DBP concentrations in the circulation than the concentration of all the vitamin D metabolites together (110). In addition, the genotype non-specific binding coefficient for both 25(OH)D and 1,25(OH)<sub>2</sub>D to DBP is about 1,000 times higher than the binding coefficient to albumin ( $7 \times 10^8 \text{ M}^{-1}$  and  $4 \times 10^7 \text{ M}^{-1}$  compared to  $6 \times 10^5 \text{ M}^{-1}$  and  $5.4 \times 10^4 \text{ M}^{-1}$ ) (114). However, since albumin is much more abundant in the circulation than DBP (650  $\mu\text{M}$  vs. 5  $\mu\text{M}$ ), about 10 % of the vitamin D metabolites end up being bound to albumin (115).

The DBP phenotypes present variations between ethnic groups with Gc1S being most abundant in Caucasians and Gc1F being most abundant in African Americans and black Africans; overall the Gc1 allele is more abundant than the Gc2 allele in all ethnicities (111). The different DBP phenotypes exhibit differences in affinity to 25(OH)D and 1,25(OH)<sub>2</sub>D, with a affinity hierarchy of Gc1F > Gc1S > Gc2 (116), thus affecting the serum 25(OH)D concentrations. In addition to the abovementioned genotype-non-specific binding coefficient for serum 25(OH)D to DBP, genotype-specific binding coefficients for DBP have been identified (116). The binding coefficients for the genotypes Gc1F, Gc1S and GC2 are used as the binding coefficients for the homozygote DBP phenotypes; however, the binding coefficients for the heterozygote DBP phenotypes have been taken as the mean of the two combined haplotypes' binding coefficients (116). Thus, concerns exist as to whether these binding coefficients are correct or not.

DBP's most important function is to stabilize and maintain circulating concentrations of vitamin D and its metabolites (111). In addition, DBP plays a role in the renal synthesis of 1,25(OH)<sub>2</sub>D through reabsorption and endocytosis of the DBP-vitamin D metabolite complex from the glomerular filtrate in the proximal tubules (49). This endocytosis is mediated by the transmembrane protein megalin, which also has the capacity to internalize several other proteins in addition to DBP (48). The "free hormone hypothesis" states that the biologically active metabolite is the unbound, or free, hormone (115). For vitamin D, the concentration of free hormone is extremely low, and therefore the term bioavailable vitamin D, meaning both the free and that loosely bound to albumin, has been hypothesized as the biologically active one (115). As all other vitamin D metabolites, the unbound 25(OH)D is also highly lipophilic, thus making passive diffusion across cell membranes possible (115).

In order to calculate free 25(OH)D concentrations, serum DBP concentrations are needed. Serum DBP concentrations are stable over time in the same individual (117). However, some diseases are associated with low DBP concentrations, such as liver cirrhosis due to affected synthesis (118) and nephritic syndrome due to protein loss in the urine (111). It is also known that DBP changes under conditions such as pregnancy (119) and estrogen therapy (120), both causing higher serum DBP concentrations. Also, serum DBP concentrations have been shown to be influenced by two SNPs located in the gene encoding DBP (rs7041 and rs705117) (121). Recently it has been possible to measure free 25(OH)D directly, using a commercially available kit, thus eliminating the error that conditions affecting the serum DBP concentration present.

In a study by Powe et al., African Americans were found to have significantly lower DBP concentrations due to different DBP phenotypes than European Americans, hence the free 25(OH)D concentrations were the same for African Americans as for European Americans despite the differences seen in serum 25(OH)D concentrations (122). This finding may at least partially explain why African Americans have better bone health than European Americans despite their lower serum 25(OH)D concentrations, and perhaps also the absence of an inverse association between CVDs and 25(OH)D concentrations. Further, Powe et al. showed that 80 % of the variation in serum DBP concentrations can be explained by the genetic variants (122).

Serum DBP concentrations can be measured by a wide range of analytical methods, and relatively high serum concentrations permit measurements by simple immunochemical

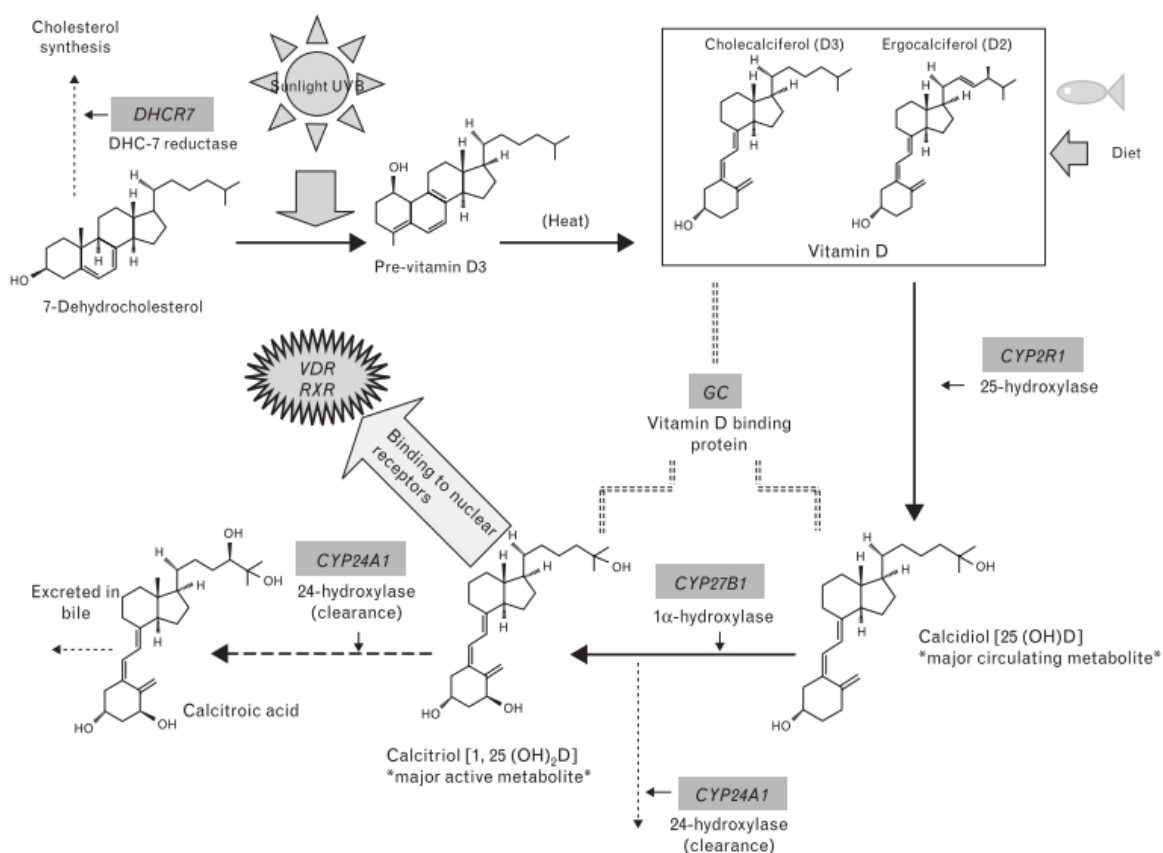
techniques or by enzyme-linked immunosorbent assay (ELISA) (111). Due to DBP's polymorphic nature, a polyclonal antibody is preferred, since different DBP assays recognize the DBP phenotypes differently (123). The Powe et al. study has been criticized for using a monoclonal immunoassay sandwich for the measurement of serum DBP; however, the Powe et al. findings with almost identical free 25(OH)D concentrations in African Americans and European Americans have been confirmed in a study using both direct measurements of free 25(OH)D and a polyclonal antibody assay for the measurement of serum DBP (77). An alternative analytic method using liquid chromatography-tandem mass spectrometric assay (LC-MS/MS) analysing serum DBP in 187 subjects, has revealed similar results as the polyclonal assay, while the monoclonal assay showed lower concentrations for Gc1F phenotypes (124), thus supporting the use of polyclonal assays. The question of how to measure serum DBP concentrations is however not yet settled, since another recently published study using LC-MS/MS found similar serum DBP concentrations regardless of race; however, the serum DBP concentrations for the six DBP phenotypes are not presented in the article (125).

## 1.6 Genetics and vitamin D

Genetics is the most important factor for phenotype variation. The human genome comprises 23 chromosomes with 3.2 billion nucleotides (126), and the DNA sequences in human genomes are almost identical, with less than 1 % of genome variations, or polymorphisms, existing between individuals (127). A single nucleotide polymorphism (SNP) is a variation at a single nucleotide; SNPs can occur throughout the whole genome, e.g. the DNA base guanine (G) is substituted by cytosine (C), and take place in more than 1 % of the world's population (128). Less than 1 % of all SNPs have an impact on protein function (129). Most genetic variations in humans are due to SNPs which are either linked or causative (128). Linked SNPs do not reside within the gene, but are located in the intergenic regions of the DNA, thus the protein function remains unaffected. On the other hand, causative SNPs occur on coding or non-coding genes and therefore lead to affected protein function; coding SNPs can affect the protein sequence directly, while the non-coding SNPs may affect the gene splicing. Thus, SNPs can correlate with specific diseases and may also affect a person's response to various drugs (127, 128). The term linkage disequilibrium (LD) is used to describe the nonrandom association of alleles of different loci, thus it measures the degree

to which two loci are associated with each other (130). Measured LD compares the observed and expected frequency of haplotypes (131). The deviation between these two values tells something about genetic evolution of humans and the effects of natural selection, hence isolated populations have larger LD (131). Further, GWAS, studying common genetic variation across the entire human genome, is contributing to the understanding and improving the diagnostics of genetically associated diseases (126).

The synthesis of the active vitamin D metabolite 1,25(OH)<sub>2</sub>D occurs through several enzymatic steps as shown in Figure 1 and more thoroughly in Figure 3 below (132).



**Figure 3: Vitamin D metabolism and closely related genetic variations.** Reused with the permission from: Lippincott Williams & Wilkins (Berry D, Hypponen E 2011 Determinants of Vitamin D Status: Focus on genetic Variations. Current Opinion in Nephrology and Hypertension 20:331–336).

The SNPs in the enzymes necessary for activation or degradation of vitamin D and its metabolites have been shown to affect serum 25(OH)D concentration (133-135). Thus, the serum 25(OH)D concentration is related to SNPs in the DHCR7 gene, responsible for the availability of vitamin D precursor 7-DHC in the skin, in the vitamin D 25-hydroxylase (CYP2R1) gene involved in the conversion of vitamin D into 25(OH)D in the liver, and in the



CYP24A1 gene involved in the degradation of 25(OH)D (132). Further, since serum 25(OH)D reflects both the DBP-bound, the albumin-bound, and the free 25(OH)D (65), polymorphisms in the DBP gene could also certainly affect the serum 25(OH)D concentration.

Since the VDR is present in most cells of the human body (55), it is not surprising that VDR polymorphisms are associated with several conditions and diseases other than those related to bone health and calcium metabolism (136). In a large meta-analysis including 126 studies, associations between several VDR polymorphisms and cancer were found (137). For the VDR BsmI polymorphism an increased risk for overall cancer was found for subjects with the b allele, with further subgroup analyses revealing that the association was most prominent for colorectal cancer and skin cancer in Caucasians (137). Also, meta-analyses have found associations between VDR polymorphisms and the risk of asthma (138) and multiple sclerosis (139). Regarding diabetes, reports are conflicting (140, 141). While Wang et al. in a meta-analysis found associations for the BsmI polymorphism with type 1 diabetes (T1D) in Asians (140), another meta-analysis found no associations between VDR polymorphism and T1D (141). Wang et al. also found an association in Asians for the FokI polymorphism with T2D (140), and these findings have been confirmed in another meta-analysis (142).

In spite of the abovementioned associations, an association between polymorphisms in the VDR and serum 25(OH)D concentration is not expected, since the VDR does not directly influence the synthetic pathway of 25(OH)D (132). Accordingly, most previous studies have not shown an association with 25(OH)D concentration (143-146). However, an SNP near the VDR (rs7968585) has been reported to modify associations between low serum 25(OH)D concentrations and major clinical outcomes (147), and also to affect the serum 25(OH)D concentration when given vitamin D supplementations (148).

## 2. Aims of the thesis

The main goal of this study was to investigate whether high doses of vitamin D supplementation can affect the glucose metabolism and prevent the development of T2D in a population of subjects with prediabetes.

Specifically, the aims of the subprojects were:

- to investigate the effect of vitamin D supplementation on blood pressure, serum lipid concentrations and on hs-CRP in subjects with prediabetes;
- to investigate whether SNPs related to vitamin D synthesis affect baseline serum 25(OH)D and whether these SNPs affect the 25(OH)D response following vitamin D supplementation;
- to investigate whether sex, BMI and baseline serum 25(OH)D affect the 25(OH)D response following vitamin D supplementation, and
- to investigate the relationship between serum 25(OH)D, calculated free 25(OH)D, directly measured free 25(OH)D and DBP in regard to DBP phenotypes, sex, BMI, age and season and their interrelationship following vitamin D supplementation.

### 3. Study population and methods

#### 3.1 Study population

The RCT (Prevention of type 2 diabetes with vitamin D supplementation in subjects with reduced glucose tolerance) was approved by the Norwegian Medicines Agency and by the Regional Committee for Medical Research Ethics. All participants gave written informed consent prior to the examinations.

Subjects were recruited to the study mainly from the sixth survey of the Tromsø Study that took place in 2007–2008 (149). The Tromsø study is a longitudinal population-based study where the first survey took place in 1974, and its main focus was on CVDs. For the sixth survey, all residents in the municipality of Tromsø between 40–42 years of age and between 60–87 years of age, a 10 % random sample of men and women between 30–39 years of age, and a 40 % random sample of men and women between 43–59 years of age were invited. In addition, if not already invited, all men and women that had attended the second phase of Tromsø 4 were invited (149). Altogether 19,762 men and women were invited, with an attendance rate of 66 % (149).

HbA<sub>1c</sub> was measured in 12,771 subjects (149). Since HbA<sub>1c</sub> was not established as a diagnostic criterion at time of study start in 2008, individuals without known diabetes and with HbA<sub>1c</sub> in the range 5.8–6.9 % (39.9 mmol/mol–51.9 mmol/mol), as well as a random sample of subjects with lower HbA<sub>1c</sub> values, were invited to an oral glucose tolerance test (OGTT) with 75 gram glucose dissolved in 300 ml water (150). In a fasting state in the morning, the OGTTs were performed by a trained nurse. Among the 4,393 subjects invited, 3,476 completed the OGTT and of these 713 were diagnosed with IFG and/or IGT (150). In addition, some participants were diagnosed with IFG/IGT and recruited to the study based on OGTTs performed at the outpatient clinic at the University Hospital of North Norway, from a former RCT with vitamin D supplementation in overweight and obese subjects (151), and from the Renal Iohexol-clearance Survey that had OGTTs as part of its study (152).

All subjects were invited to a baseline visit at the Clinical Research Unit at the University Hospital of North Norway within 1-2 weeks after their OGTTs. At the same time, the hospital's pharmacy was informed, and the subject was assigned the next randomization number on the randomization list. The randomization list was computer-generated by the Randomization Unit at the hospital's Clinical Research Unit in a 1:1 ratio between vitamin D

and placebo, and a copy of the randomization list was kept at the hospital's pharmacy. The code was only known to the pharmacy and the Clinical Research Unit at the hospital; everyone else was blinded. The randomization was not stratified.

Subjects with primary hyperparathyroidism, sarcoidosis or other granulomatous disorders, urolithiasis, cancer the last five years, allergies to nuts (the placebo capsules contain peanut oil), unstable angina pectoris or acute myocardial infarction or stroke the last year, or reduced kidney function with creatinine above 125  $\mu\text{mol/L}$  in men and 105  $\mu\text{mol/L}$  in women were excluded. For women exclusion criteria in addition included pregnancy, lactation, and fertile age and no use of contraception.

### **3.2 Measurements, questionnaires and safety**

At the baseline visit, the participants' medical histories were taken and they had a brief clinical examination. The participants were informed that results from the first visit, as well as future visits, would be a part of their hospital record. Further, they were informed that all results from the blood tests would be stored in the hospital's computer system. Height and weight were measured while the subjects wore light clothing and no shoes. BMI was calculated as weight (kilogram) divided by squared height ( $\text{m}^2$ ). Blood pressure was measured on the upper right arm with the subject in a sitting position with an automatic device (AND, A & D Medical, Tokyo, Japan), three times with 2-minute intervals. The mean of the two last blood pressures was used in the statistical analyses. The participants were informed orally and in writing about physical activity and healthy food habits. Information about the importance of weight loss was given if needed. Questionnaires regarding current use of medicines, prescription drugs and calcium and vitamin D supplementations, smoking, and traveling to sunny areas were filled in at baseline and at the 12-month visit.

The next visit at the Clinical Research Unit was six months later, when the participants returned unused study medication, were supplied with new study medication, and had their serum calcium and serum creatinine measured for safety reasons. After 12 months, the same examinations as at the baseline visit were done, and a new OGTT was performed. Also, study medication was returned, counted, and new study medication was supplied to participants continuing in the study. Registrations of adverse events were made after 6 and 12 months.

Study medication: capsules with cholecalciferol (vitamin D<sub>3</sub>, 20,000 IU [Dekristol, Mibe, Jena, Germany]) or identical-looking placebo capsules containing arachis oil (Hascolek, Wroclaw, Poland) were provided for six months with information that one capsule was to be taken each week. The subjects were not allowed to take vitamin D supplements (including cod liver oil) exceeding 400 IU per day.

All participants were informed about the risk and symptoms of hypercalcemia, and if experiencing such symptoms they were instructed to contact the Clinical Research Unit at the University Hospital of North Norway. Further, they were informed that they were covered by the Norwegian patient injury compensation, and in addition that they would have separate liability insurance in connection with clinical trials of drugs. All participants were informed that they would receive written information about their results, and recommendations of treatment and follow-up when leaving the study. At the end of the study, all participants will receive information about the conclusions.

To keep all investigators blinded, all data were sent directly to the hospital's Clinical Research Unit where the data files were merged and coupled to the randomization code. The Clinical Research Unit then sent the final file without person identification to the principal investigators (S.T.S. and R.J.).

### 3.3 Blood samples

Fasting blood samples for glucose, insulin and lipids were collected at the baseline OGTT visit, while non-fasting blood samples were drawn at the baseline visit. At the 12-month visit, fasting blood samples were drawn.

Plasma glucose, serum HbA<sub>1c</sub>, serum insulin, serum C-peptide, serum total cholesterol, serum triglycerides, serum high density lipoprotein (HDL) cholesterol, serum low density lipoprotein (LDL) cholesterol, serum apolipoprotein A1, serum apolipoprotein B, serum albumin, serum calcium, serum PTH and serum high-sensitivity C-reactive protein (hs-CRP) were measured at the Division of Diagnostic Services at the University Hospital of North Norway (149). Plasma glucose was measured by an enzymatic photometric assay (Glicoquant Glucose/HK, Roche Diagnostics, Mannheim, Germany); serum insulin and c-peptid were measured by immunometry using an electro-chemiluminescence immunoassay (ECLIA) (Roche Diagnostics, Mannheim, Germany); HbA<sub>1c</sub> was measured by high performance liquid chromatography (HPLC) using an automated analyser (variant II, BIO-Rad laboratories Inc.,



Hercules, California, USA); serum total cholesterol and triglycerides were measured with an enzymatic colorimetric assay using an automated clinical chemistry analyser (Modular P, Roche Diagnostics, Mannheim, Germany); serum HDL cholesterol was measured by a homogeneous enzymatic colorimetric method; serum albumin was measured by a colorimetric method (bromocresol green) using an automated analyser, Cobas 8000 (c702, Roche Diagnostics, Mannheim, Germany), and serum calcium was measured by an automated analyser (Modular P, Roche Diagnostics, Mannheim, Germany) with reagents from Boehringer Mannheim (149). Serum 25(OH)D was measured by an in-house LC-MS/MS; the limit of detection was < 4 mmol/L, and the between day coefficient of variation (CV %) < 9 % (153). Direct measurements of serum free 25(OH)D were done using competitive ELISA assay kits from Diasource Diagnostics based on patented monoclonal antibodies developed by Future Diagnostics (154); the range was 0.2 pmol/L–87.4 pmol/L, the limit of detection was 7.0 pmol/L, and the precision was < 10 %. Serum DBP was measured by an in-house competitive radioimmunoassay (RIA) using purified group specific-globulin (Sigma, St.Louis, USA) and a polyclonal antibody (DakoCytomation, Glostrup, Denmark), at the Hormone Laboratory, Aker University Hospital (155).

Estimates of insulin sensitivity were calculated with homeostasis model assessment of insulin resistance (HOMA-IR)  $[(\text{insulin (pmol L}^{-1}) \times (\text{glucose (mmol L}^{-1})))/135]$  (156) and with the quantitative insulin sensitivity check index (QUICKI)  $[1/\log (\text{insulin mU mL}^{-1}) + (\log (\text{glucose mg dL}^{-1}))]$  (157).

### 3.4 Genotyping, SNPs selection and DBP phenotypes

Blood samples for SNP analyses were sent to KBiosciences (158) and all genotyping was performed with a competitive allele-specific polymerase chain reaction assay (KASpar) that enables highly accurate scoring of SNPs.

For Paper II, we selected SNPs that in GWAS reports have been related to serum 25(OH)D concentrations (133, 134); rs2282679 in the DBP gene, rs3829251 in the DHCR7 gene, rs10741657 in the CYP2R1 gene, and rs6013897 in the CYP24A1 gene (132). The SNPs with the highest differences in serum 25(OH)D between the major and minor homozygote genotype for the genes in question were chosen to avoid problems with multiple testing, as reported in our previous studies (143). In addition, two SNPs in the DBP gene (rs7041 and

rs4588) were included, since references to these two SNPs are frequently made in relation to serum 25(OH)D concentrations (133, 134, 159).

For Paper III, the DBP haplotypes were identified based on two SNPs, rs7041 and rs4588, in the DBP gene. A, C, G, and T are abbreviations for the nucleotides adenine, cytosine, guanine and thymine respectively. Diplotype GA does not occur in humans and is marked as 0 in the table below.

		rs7041		
		GG	TG	TT
rs4588	AA	0	0	TA/TA = 6
	CA	0	GC/TA = 3	TC/TA = 5
	CC	GC/GC = 1	GC/TC = 2	TC/TC = 4

From the table above the DBP diplotypes and six DBP phenotypes listed in the table below were identified:

Diplotype	DBP Phenotype
GC/GC	Gc1S/Gc1S
GC/TC	Gc1S/Gc1F
GC/TA	Gc1S/Gc2
TC/TC	Gc1F/Gc1F
TC/TA	Gc1F/Gc2
TA/TA	Gc2/Gc2

For serum 25(OH)D the binding coefficient for albumin is  $6 \times 10^5 \text{ M}^{-1}$  and for DBP the non-specific binding coefficient is  $7 \times 10^8 \text{ M}^{-1}$  (114). Calculations of free- and bioavailable serum 25(OH)D concentrations were performed by using an equation for free testosterone (160), adapted for calculating free 25(OH)D (161); in the equations below serum 25(OH)D is in nmol/L, serum albumin is in g/L, and serum DBP is in  $\mu\text{mol/L}$ :

- Calculated free 25(OH)D (pmol/L) = serum 25(OH)D / (((binding coefficient albumin x ([albumin]/66437)) + (binding coefficient DBP x ([DBP]/ $1 \times 10^6$ ))) x 1000),
- Calculated albumin bound 25(OH)D (nmol/L) = ([free 25(OH)D] x binding coefficient albumin x [albumin/66437])/1000, and
- Calculated bioavailable 25(OH)D (nmol/L) = free 25(OH)D + Albumin bound 25(OH)D.

In addition, we performed analyses using the DBP phenotype-specific binding coefficients listed in the table below.

<b>Diplotype</b>	<b>Phenotype</b>	<b>Binding coefficients</b>
GC/GC	Gc1S/Gc1S	$6 \times 10^8$
GC/TC	Gc1S/Gc1F	$8.6 \times 10^8$
GC/TA	Gc1S/Gc2	$4.8 \times 10^8$
TC/TC	Gc1F/Gc1F	$11.2 \times 10^8$
TC/TA	Gc1F/Gc2	$7.4 \times 10^8$
TA/TA	Gc2/Gc2	$3.6 \times 10^8$

### 3.5 Statistical analyses

Normal distribution was evaluated with visual inspections of histograms and by kurtosis and skewness in Papers I and III, as well as by means of the Kolmogorov–Smirnov test and Q-Q plots in Paper II. Data are presented as mean  $\pm$  standard deviations (*SD*) for normally distributed variables. Triglycerides, insulin, HOMA-IR, QUICKI and PTH were non-normally distributed and are presented as median (2.5<sup>th</sup>, 97.5<sup>th</sup> percentile). Level of significance was set at  $P < 0.05$  (two-tailed). Statistical analyses were performed using the IBM Statistical Package for Social Sciences (SPSS) version 21 for Papers I and II and version 22 for Paper III.

The main endpoint for the five year intervention was development of T2D defined as fasting glucose  $> 6.9$  mmol/L or 2-h glucose  $> 11.0$  mmol/L at OGTT. Our null hypothesis was that the development of T2D would be equal in the vitamin D and in the placebo group.

#### 3.5.1 Statistical tests in Paper I

To compare the vitamin D and the placebo groups the independent samples *t*-test was used for continuous variables and chi-square test for categorical variables. To evaluate relations between serum 25(OH)D and glucose metabolism, blood pressure, serum lipid concentrations and hs-CRP at baseline, linear trend across four serum 25(OH)D groups (serum 25(OH)D  $< 30$  nmol/L, 30 – 49 nmol/L, 50 – 74 nmol/L and  $> 74$  nmol/L), using linear regression with sex, age and BMI as covariates or by chi-square linear by linear association were used. To evaluate the effects of vitamin D and placebo on glucose metabolism, blood pressure, serum lipids and hs-CRP general linear regression models adjusting for baseline values were used (162).

### 3.5.2 Statistical tests in Paper II

The genotype frequencies were examined for compliance with Hardy-Weinberg equilibrium using  $\chi^2$  analysis (163). The LD between SNPs was evaluated with  $r^2$  using CubeX calculations with  $r^2 \geq 0.4$  as a cut off for LD (164).

To compare the vitamin D and the placebo group the independent samples *t*-test was used. To evaluate trends across the genotypes, linear regression with age, sex and BMI as covariates was used. Season (summer [May–September]/winter [October–April]) and intake of vitamin D supplements (including cod liver oil), were also included as covariates for baseline values.

To predict change in serum 25(OH)D concentration based on baseline 25(OH)D concentration, baseline BMI, and genotype, a regression equation with randomization status, baseline BMI and baseline serum 25(OH)D and the three SNPs that in the model were significant (rs2282679, rs7041, and rs10741657) and interaction terms between each of the three SNPs with the randomization status was set up:

- $\Delta 25(\text{OH})\text{D} = \text{intercept} + (\beta\text{-randomization status} \times \text{randomization status}) + [\beta\text{-baseline } 25(\text{OH})\text{D} \times \text{baseline } 25(\text{OH})\text{D}] + (\beta\text{-baseline BMI} \times \text{baseline BMI}) + (\beta\text{-rs2282679} \times \text{rs2282679}) + (\beta\text{-rs10741657} \times \text{rs10741657}) + (\beta\text{-rs7041} \times \text{rs7041}) + [\text{randomization status} \times \text{baseline BMI} \times (\beta\text{-randomization status} \times \text{rs2282679})] + [\text{randomization status} \times \text{baseline BMI} \times (\beta\text{-randomization status} \times \text{rs10741657})] + [\text{randomization status} \times \text{baseline BMI} \times (\beta\text{-randomization status} \times \text{rs7041})]$ .

Since sex and age did not significantly influence delta serum 25(OH)D (12 months value minus baseline value), they were not included in the equation.

### 3.5.3 Statistical tests in Paper III

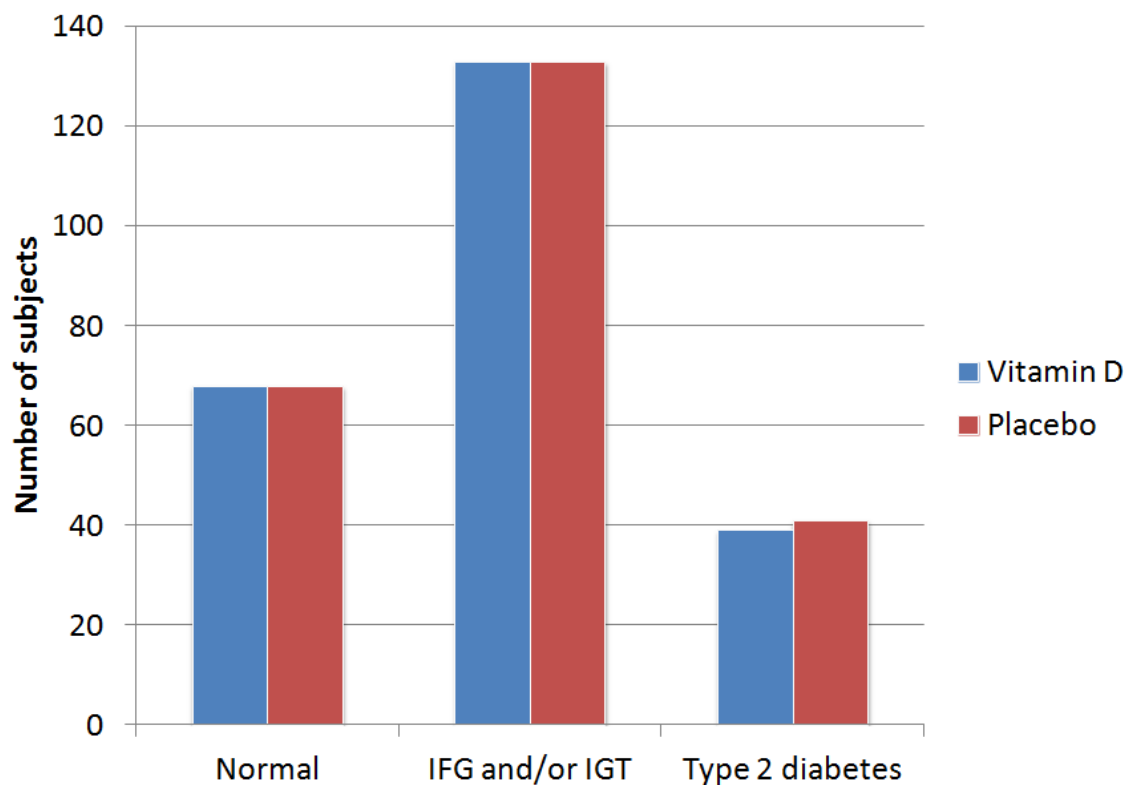
To compare the vitamin D group and the placebo group the independent samples *t*-test was used. To compare serum calculated free 25(OH)D and directly measured free 25(OH)D paired samples *t*-tests were used. To determine differences in distribution between DBP phenotypes, a chi-square test was used for sex, and one-way analysis of variance (ANOVA) was used for age and BMI. To examine the DBP phenotype-vitamin D parameters associations, a general linear model was used with sex, BMI, age and season as covariates. The Bonferroni procedure was used for post hoc analyses. To evaluate differences between

sex and season, the independent samples *t*-test was used, while linear trend analyses were used across BMI groups and age groups. Univariate correlations were assessed by calculating Pearson correlation coefficient for normally distributed variables and Kendall's tau-b for non-normally distributed variables.

#### 4. Summary of results

***Paper I: Sollid ST, Hutchinson MYS, Fuskevåg OM, Figenschau Y, Joakimsen RM, Schirmer H Njølstad I, Svartberg J, Kamycheva E and Jorde R. No Effect of High-Dose Vitamin D Supplementation on Glycemic Status or Cardiovascular Risk Factors in Subjects With Prediabetes. Diabetes Care 2014. 37(8): 2123–2131.***

Low serum 25(OH)D concentrations have been associated with increased plasma glucose and increased insulin resistance in several observational studies. Risk factors for CVD have also been associated with low serum 25(OH)D. In Paper I, we aimed to investigate whether high-dose vitamin D supplementation could improve glucose metabolism in a population with IFG and/or IGT. We used data from an RCT with 511 subjects with IFG and/or IGT who were randomly assigned to 20,000 IU of vitamin D<sub>3</sub> per week ( $n=256$ ) or placebo ( $n=255$ ) for 12 months. OGGTs were performed annually with the main outcome after 12 months being changes in glucose tolerance. Mean baseline serum 25(OH)D concentrations were 59.9 nmol/L and 61.1 nmol/L in the vitamin D and the placebo groups, respectively, and serum 25(OH)D increased by  $45.8 \pm 24.2$  nmol/L and  $3.4 \pm 11.9$  nmol/L after 12 months, respectively. There were no differences between the groups regarding fasting blood glucose, 2-hour blood glucose after OGTT, HbA<sub>1c</sub> concentration, insulin secretion and sensitivity or the development of T2D after 12 months, see Figure 4 below. There were no differences between the groups in blood pressure or lipid concentration. The major findings in Paper I were that high dose vitamin D supplementations do not improve glycaemic indices or cardiovascular risk factors in a population with prediabetes.



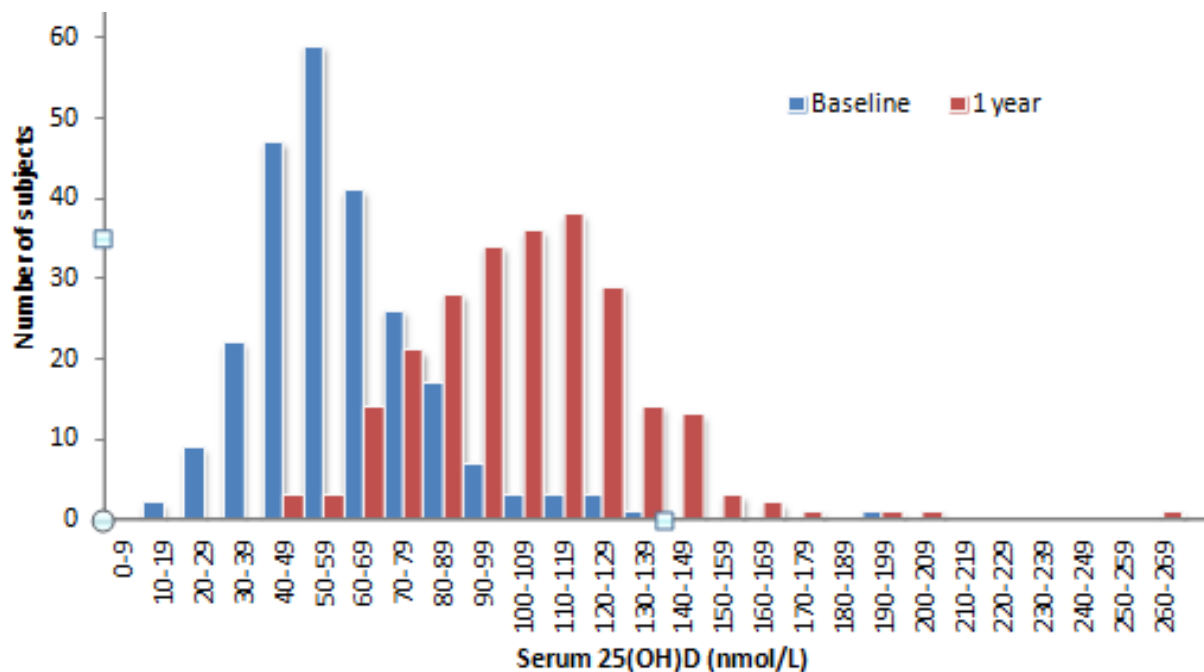
**Figure 4: Glycaemic status after 12 months in subjects randomized to vitamin D and to placebo.**

***Paper II: Sollid ST, Hutchinson MYS, Fuskevåg OM, Joakimsen R and Jorde R. Large Individual Differences in Serum 25-Hydroxyvitamin D Response to Vitamin D Supplementation: Effects of Genetic Factors, Body Mass Index, and Baseline Concentration. Results from a Randomized Controlled Trial. Hormone and Metabolic Research 2015. Epub ahead of print.***

Since the vitamin D synthesis depends upon several enzymatic steps, genetic differences between individuals most likely affect a person's vitamin D status. In Paper II, we aimed to determine the influence of genetic and other factors on the serum 25(OH)D response following vitamin D supplementation. Four hundred eighty-four subjects from an RCT in individuals with prediabetes assigned to 20,000 IU of vitamin D<sub>3</sub> per week or placebo for 12 months were included in the analyses and genotyped for SNPs in the DBP, DHCR7, CYP2R1 and CYP24A1 genes. We found that SNPs from all four selected genes were significantly related to baseline serum 25(OH)D concentrations with differences between major and minor homozygote genotypes ranging from 4.4 to 19.2 nmol/L. In the vitamin D group, subjects having the genotypes with the highest baseline 25(OH)D concentration also had the highest 25(OH)D concentration after 12 months. The increase in serum 25(OH)D was



significantly related to three of the SNPs. We also found that the increase in serum 25(OH)D was higher in non-obese versus obese subjects, and higher in those with the lowest baseline 25(OH)D concentrations. We combined these three factors (genetics, BMI and baseline serum 25(OH)D) in a linear regression model. We found that the predicted (and observed) difference in 25(OH)D increase between high and low responders to the supplementation was approximately 60 nmol/L. Our major finding in Paper II was that there are large individual differences in the serum 25(OH)D response following vitamin D supplementation, as shown in Figure 5, due to genetic, BMI and baseline 25(OH)D differences. The large variations found for the serum 25(OH)D response should be considered when giving advice on vitamin D supplementation.



**Figure 5: The distribution in baseline and in 12 months serum 25(OH)D concentration in subjects randomized to vitamin D.** Reused with the permission from: Hormone and Metabolic Research and Copyright Clearance Center (Sollid ST et al. 2015 Large Individual Differences in Serum 25-Hydroxyvitamin D Response to Vitamin D Supplementation: Effects of Genetic Factors, Body Mass Index, and Baseline Concentration. Results from a Randomized Controlled Trial. Epub ahead of print).

***Paper III: Sollid ST, Hutchinson MYS, Berg V, Fuskevåg OM, Figenschau Y, Thorsby PM and Jorde R. Effects of DBP Phenotypes and Vitamin D Supplementation on Serum Total 25(OH)D and Directly Measured Free 25(OH)D. European Journal of Endocrinology 2016. Epub ahead of print.***

Serum 25(OH)D is the metabolite used to evaluate a person's vitamin D status. Recently it has also been possible to measure free 25(OH)D directly. In Paper III, we have examined the relationship between serum 25(OH)D, directly measured free 25(OH)D, and calculated free 25(OH)D in regard to DBP phenotype groups, sex, BMI, age and season, and their interrelationship to vitamin D supplementation. Included in the analyses are the 472 subjects from an RCT in subjects with prediabetes who at baseline had complete datasets for serum albumin, serum 25(OH)D, directly measured free 25(OH)D and DBP. We evaluated baseline serum concentrations and increases in serum 25(OH)D, directly measured free 25(OH)D, calculated free 25(OH)D and DBP. Subjects with the DBP phenotype Gc2/Gc2 had significantly lower baseline serum 25(OH)D and DBP concentrations compared to DBP phenotypes with the Gc1S allele; similarly, males had lower baseline serum 25(OH)D and DBP concentrations than females. For directly measured free 25(OH)D, the differences between the DBP phenotype groups and sexes diminished. Calculated free 25(OH)D and bioavailable 25(OH)D concentrations were overestimated compared to the directly measured free 25(OH)D. Using the specific binding coefficients for the six different DBP phenotypes, thus adjusting for DBP genotype, did not improve the results. Serum parathyroid hormone was inversely correlated with all vitamin D parameters analysed. All vitamin D parameters had similar increases after 12 months of vitamin D supplementation regardless of DBP phenotype, sex or age. Serum DBP concentrations were not affected by vitamin D supplementation. The major finding in Paper III was that direct measurements of free 25(OH)D reduces the differences seen in serum 25(OH)D between DBP phenotypes and sexes, most likely caused by the differences seen in DBP concentrations. Direct measurements of free 25(OH)D should be considered in subjects with conditions affecting serum DBP concentrations.

## 5. General discussion

### 5.1 Methodological considerations

#### 5.1.1 Study design

RCTs, considered the gold standard for establishing causality, are ranked at the top among methods used to acquire evidence-based medicine (165). RCTs have together with high quality observational studies during the last 20 years been the foundation for treatment recommendations for different conditions and diseases (165). Furthermore, the yearning for new knowledge has led to medical advances and benefits with the result that both morbidity and mortality have decreased (165). RCTs are limited by several ethical concerns, e.g. no doubt should exist that any of the treatment arms is better than another (166). Further, exposing participants to a treatment or drug believed to be inferior to the current treatment would be considered unethical by most people, however, not performing experimental trials may result in a harmful treatment or drug being used (167). For the participants, RCTs can be justified with that a new drug, or treatment, benefits the individual.

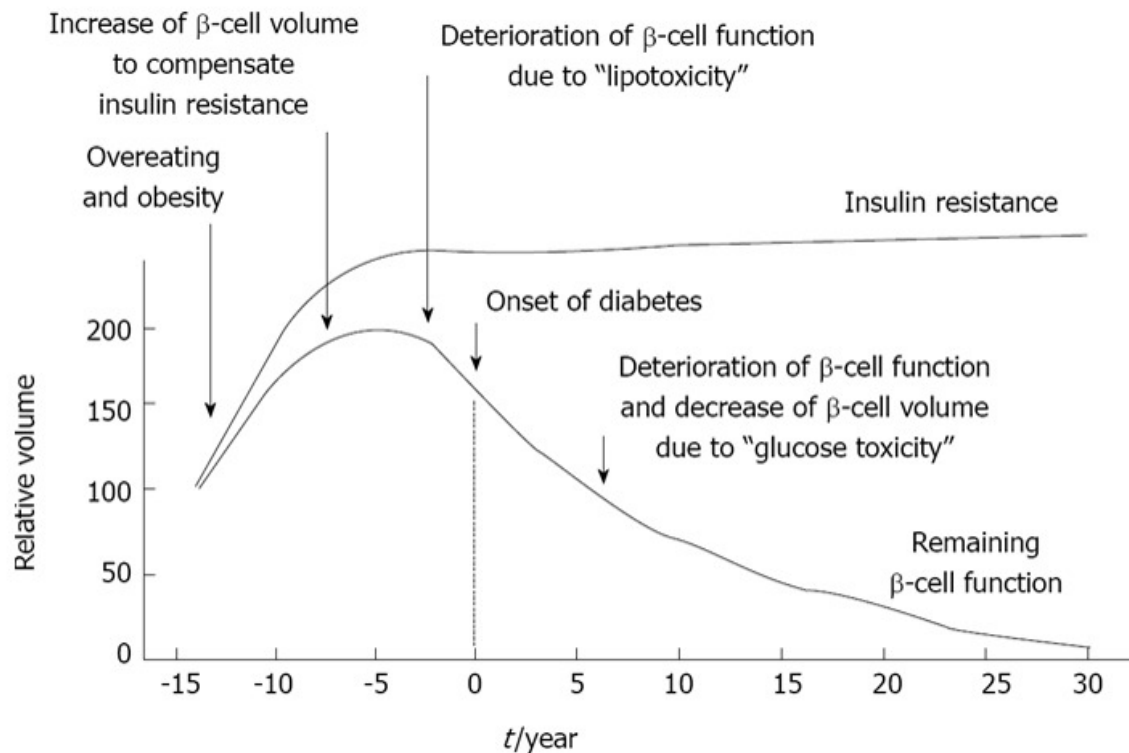
RCTs, and with it evidence-based medicine, have been criticized for the large number of studies, some with large numbers of participants, and the use of surrogate endpoints which in turn leads to statistical significance being achieved without it having clinical consequences (165). In addition, the dominant use of algorithms and treatment recommendations are criticized, consequently overlooking individual treatment (165). Besides, it can lead to clinicians failing to see their different patients as individuals (165). Further, since many studies are financed by the pharmaceutical industry this might lead to secret holding of all or parts of the results, so called outcome-reporting bias (168, 169), especially if no statistically significant effect for the treatment was found. Also, publicists are more prone to publish results reaching statistical significance, so-called publication bias (168). Furthermore, publication bias will lead to an overestimation of treatment effects (168). Additionally, RCTs are often carried out under strictly controlled conditions, thus do not necessarily represent a true clinical setting.

Our study was a double-blinded RCT, and although receiving grants from the Novo Nordisk Foundation, an international pharmaceutical company, the study was not dependent upon the pharmaceutical industry. In addition, the study received grants from

the North Norway Regional Health Authority, the Norwegian Diabetes Association, the University of Tromsø, and the Research Council of Norway.

Observational studies provide ideas about incidence, prevalence, and prognosis of the disease studied, thus associations from these studies help to generate hypotheses to be tested in RCTs (170). Observational studies also formed the basis for our main null hypothesis; that the development of T2D would be equal in the vitamin D and in the placebo group. In addition to the null hypothesis, several other hypotheses were to be tested after 12 months: there is no difference in the glucose metabolism, in blood pressure, in blood lipid concentrations, and in hs-CRP between the vitamin D and the placebo group. Further, we wanted to investigate whether SNPs related to the vitamin D synthesis, BMI, sex or age affect the response following vitamin D supplementation, whether polymorphisms in DBP affect the serum free 25(OH)D concentration, and whether the serum free 25(OH)D was more strongly related to known 25(OH)D effects than serum 25(OH)D.

Since T2D is a disease that develops slowly over several years, illustrated in Figure 6, the study was designed to run over 5 years in order to detect any differences between the groups. It was approved by the Regional Committee for Research Ethics and by the Norwegian Medicines Agency in 2007 (EUDRACTNR. 2007-002167-27). The trial is registered at ClinicalTrial.gov (NCT00685594). This thesis is based upon the first 12 months of study duration.

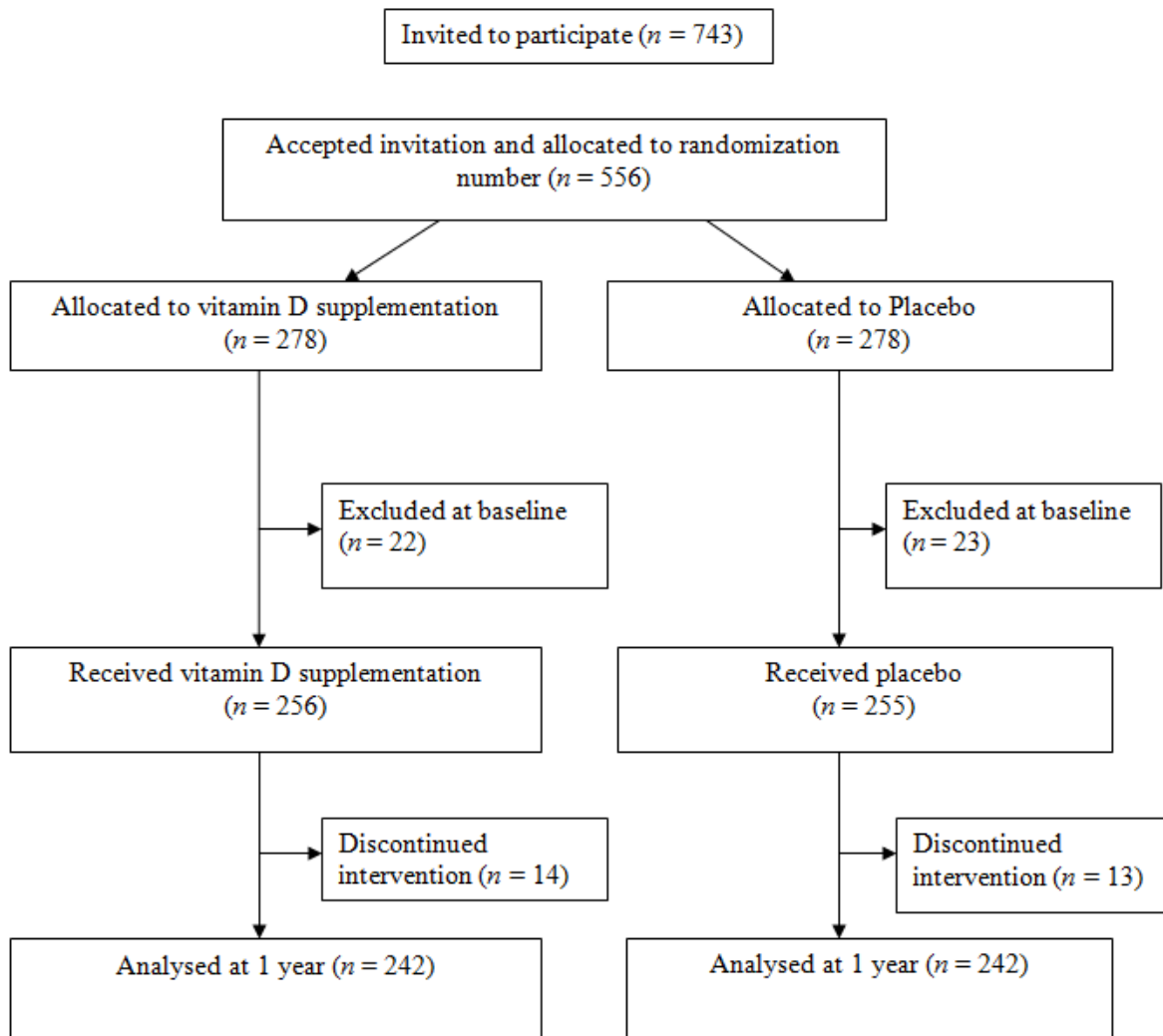


**Figure 6: The stages from normal glucose metabolism to T2D.** Reused with permission from (Kaneto H, Matsuoka TA 2013 Down-Regulation of Pancreatic Transcription Factors and Incretin Receptors in Type 2 Diabetes. World Journal of Diabetes 4:263–269), 2013 Copyright © Baishideng Publishing Group Co.

The first participants were recruited in 2008; the last was included in 2010, resulting in the last participant ending his/her 12-month visit in 2011, while the five-year study lasted until the spring of 2015. In order to limit the risk of bias, the randomization was double-blinded, meaning that both the participants and the researchers remained unaware of which treatment was given until the study was completed, and this also ensured that all participants were treated identically independent of receiving intervention or placebo. Further, the participants were randomly allocated to either the intervention or the control group. Thus, if the intervention did not have an effect, the observed result would be the same in both groups.

The flow chart of the 12-month study can be seen below in Figure 7. Seven hundred forty-three individuals were invited to participate; 556 accepted the invitation, and were randomly allocated to vitamin D or placebo for 5 years. We do not have any data on the 187 subjects not accepting invitations, thus representing a limitation of the study. The reasons for exclusions at the baseline visit did not differ between the vitamin D and placebo group; two subjects did not meet the inclusion criteria and 43 had one or more of the exclusion

criteria. Further, the number of subjects excluded at baseline was 22 and 23 subjects allocated to the vitamin D and placebo group, respectively. Also, during the first 12 months of the study, 13 in the vitamin D group and 14 in the placebo group dropped out. The most common reasons were withdrawals and having received a new diagnosis of CVD or cancer, and there were no differences between the vitamin D and placebo group. Thus, this should not influence the results.



**Figure 7: Flow chart of the study**

### 5.1.2 Systematic errors

Selection bias and confounding are systematic errors which may reduce the validity of the study results (171). Systematic errors occur more often in observational studies than in

experimental studies since the randomization process in RCTs prevents the occurrence of systematic differences between study groups (171).

Selection bias occurs when subjects recruited to a study differ consistently from those not being included. At inclusion of the participants in RCTs, selection bias is likely to occur based on the researchers' advance knowledge of the treatment (172). This is especially true for subjective assessments, and an argument for blinding of randomized trials. Thus, having performed a double-blinded RCT, selection bias should not be a problem, since the allocation is concealed for both participants and researchers. In Table 2 in Paper I and Table 1 for Papers II and III, the baseline characteristics of the study participants have been presented, and as expected none of the variables evaluated differed significantly between the vitamin D and the placebo group.

Another systematic error, confounding, occurs if there are systematic differences between the groups being compared, which is related to the variable being studied (171). Thus, in order not to draw the wrong conclusions confounders need to be taken into account. However, some confounders will remain unknown and still represent a problem in non-randomized trials. However, the randomization process in RCTs minimizes confounding, thus confounding is most likely not a problem in our study.

### **5.1.3 Random errors, sample size and power calculations**

Upon testing hypotheses, two out of four events are desirable: that an accepted null hypothesis is correctly accepted or that a rejected null hypothesis is correctly rejected. The two other possible outcomes, denoted random errors, are not desired. Random errors are caused by unknown and unpredictable changes in the experiment, and could happen by chance due to statistical principles. Random errors cannot completely be avoided, but may however be prevented by designing an as optimized study as possible and by having adequately sized study samples. Random errors are divided into Type 1 and Type 2 errors. Type 1 error denotes rejecting a true null hypothesis and Type 2 error denotes accepting an untrue null hypothesis.

With an increasing number of analyses being performed, the number of  $p$ -values calculated increases, thus the risk for a Type 1 error also increases. We have in all papers set the level of significance at  $p < 0.05$ , which means that there is less than a 5 % chance that results are found by chance. The best strategy to avoid Type 1 errors is to reduce the



number of end points to a minimum. Another way to reduce the probability of a false positive finding is by using Bonferroni correction. In doing Bonferroni correction every  $p$ -value is multiplied by the number of statistical tests performed, e.g. when performing three statistical tests every  $p$ -value is multiplied by three. However, Bonferroni corrections are considered conservative; therefore, they might overcorrect, especially if the different endpoints are correlated. We have performed several subgroup analyses, thus making our results susceptible to Type 1 error. In Paper I, subgroup analyses were done for subjects with serum 25(OH)D concentrations  $< 50$  nmol/L and  $< 40$  nmol/L, however, these subgroups consisted of respectively 87 and 37 participants in the vitamin D group, and of respectively 83 and 35 participants in the placebo group. In addition, several subgroup analyses regarding blood pressure and lipid concentrations were performed in Paper I. Further, for the post hoc analyses in Paper III, the Bonferroni procedure was used, thus the chances for Type 1 errors were limited. Nevertheless, the use of subgroup analyses obviously represents a short-coming of this thesis; therefore, especially results from the subgroup analyses may be unreliable and should not be over-interpreted.

Type 2 errors are most often seen in studies with an inadequate number of participants, in studies lasting too briefly to show an effect, or when the treatment given is not optimal. RCTs are exposed to Type 2 errors if the sample size is too small and can be prevented by optimal power calculations ahead of study start. Further, when planning a trial, it is important to decide upon a level of significance and on how large the difference in effect between the study groups needs to be in order for it to be of clinical relevance. A power calculation prevents too many subjects being exposed to the treatment, and hence also reduces the risk of side effects. The less difference desired to reveal, the more subjects need to be included.

Our power calculation was done for the 5-year study, where we had to include 505 subjects to have a strength of 0.80 and an alpha value of 0.05. The prerequisites were: that there were equal numbers of participants in both the intervention and the control groups; that 20 % of the participants would withdraw from the study, equally distributed between the groups; that 10 % of the participants in the placebo group annually would progress to T2D; that vitamin D supplementations annually would reduce the development of T2D by 30 %; in other words that 7 % of participants in the vitamin D group would annually progress to T2D.

Like most trials, our study was not powered for subgroup analyses, thus representing another short-coming. Nevertheless, we did a post hoc power analysis where we assumed that the differences found at baseline between those with low versus high serum 25(OH)D concentrations ( $\sim 0.2\%$  for HbA<sub>1c</sub>,  $\sim 0.15$  mmol/L for HDL cholesterol, and  $\sim 1$  mmHg for systolic blood pressure) were correct, and that these differences represented what could maximally be improved by vitamin D substitution in subjects with vitamin D deficiency. If we had wanted a power of 0.8 and a significance level of 0.05 we would have needed to include, 120, 160 and  $> 2,000$  subjects, respectively, with serum 25(OH)D  $< 50$  nmol/L. Therefore, we did have the power to disclose such effects regarding HbA<sub>1c</sub> and HDL cholesterol, but not blood pressure.

Furthermore, variables known a priori to be moderately or strongly associated with the primary outcome should be included in the statistical analyses. Such variables, known as covariates, can affect the outcome of a study. Therefore, by adding covariates, the statistical analyses of the treatment differences should become more accurate. In our linear regressions, sex, age and BMI are covariates in all papers. In Papers II and III, season (winter/summer), and in Paper III intake of vitamin D supplements (including cod liver oil), were also included as covariates when appropriate. In Paper I, when evaluating the effect of vitamin D on the different outcome variables, we adjusted for baseline values (162), thus most likely eradicating a possible association between baseline covariates and the outcome measure.

There are two main strategies to use when selecting which participants should be included in a statistical analysis: a “per protocol” or an “intention to treat” analysis. A “per protocol” analysis includes only the compliant participants. However, many participants do not take study medications as prescribed, and when these participants are included the analysis is denoted an “intention to treat analysis”. An intention to treat analysis reduces the difference in adherence between the study participants and the clinical patients, thus, reducing bias and strengthening the conclusions. In Paper I, intention to treat analyses were done in addition to per protocol analyses; however, they did not change the results.

In statistics, regression toward the mean denotes a phenomenon where an extreme variable, large or small, tends to be closer to average when the measurement is repeated. Regression toward the mean should always be considered as a possible cause of an observed change. In Paper II, subjects receiving placebo who had either fairly low ( $< 40$  nmol/L) or

high serum 25(OH)D concentrations (> 75 nmol/L) most likely showed a regression toward the mean with an increase in 8.6 nmol/L and a decrease in 7.9 nmol/L, respectively. Further, subjects in the vitamin D group with low baseline serum 25(OH)D had the largest 25(OH)D response, also most likely due to regression toward the mean. Thus, randomization status is included in the regression equation to predict the 25(OH)D response following high dose vitamin D supplementation in order to account for regression toward the mean.

#### **5.1.4 External validity of the results**

The generalizability of study results largely depends upon the study population being representative of other populations or not. Our study consisted exclusively of subjects with IFG and/or IGT; almost all were Caucasians, and all live in Northern Norway at a latitude of 69° with low solar UVB exposure.

For the main outcome T2D, the study population is representative since they were at high risk for developing T2D. Further, for blood pressure and lipid concentration, they were most likely also representative. However, for the 25(OH)D response following vitamin D supplementation in Paper II and the relationship between serum 25(OH)D and directly measured free 25(OH)D in Paper III, they represent a skewed population, since both genetics and DBP phenotypes differ with different populations.

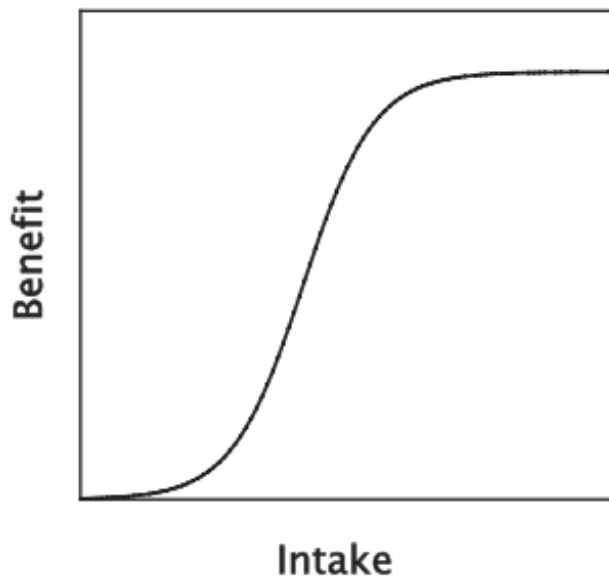
#### **5.1.5 Study medication, dose of study medication and safety**

The participants of the intervention groups received 20,000 IU per week of vitamin D while the placebo group received identical looking placebo capsules. The compliance rates in the vitamin D group were 0.86 and 0.84 for the first and the last 6 months, respectively, and in the placebo group 0.86 and 0.83, respectively. The compliance rates were calculated as the ratio between study capsules used and study capsules supplied for that time period.

To prevent hypercalcemia, participants at risk of developing this condition were excluded at baseline. Further, serum calcium, as well as side effects, were measured/registered every sixth month; if serum calcium was above 2.60 nmol/L or serum creatinine was above 130 µmol/l new blood samples were taken, and if still considered elevated, the participant was excluded. In addition, the participants were carefully informed, orally and in writing, about the symptoms of hypercalcemia. At the 6-month visit, one

participant in the vitamin D group had a serum calcium concentration of 2.63 mmol/L, thus was excluded; after 5 months, serum calcium had normalized.

As are most nutrient studies today, our study was based on a pharmaceutical drug model. Heaney has described a sigmoid-shaped nutrient response curve which probably better takes into account the basal nutrient status of individuals, shown in Figure 8 below (173).



**Figure 8: A typical, sigmoid-shaped dose-response curve relating nutrient benefit to nutrient intake within the physiological range of intakes.** Reused with permission from: Nutrition Review and Copyright Clearance Center (Heaney RP 2014 Guidelines for Optimizing Design and Analysis of Clinical Studies of Nutrient Effects. Nutrition Reviews 72:48–54).

Heaney proposes five rules for nutrient trials, which includes the following three: that basal nutrients status should be used as an inclusion criterion; that the change in a person's nutrient status must be large enough for effects to be measurable; and that the study hypothesis is that the nutrient response causes the desired effect (173). For glucose metabolism and T2D with regard to serum 25(OH)D the nutrient response curve is not known. Since our subjects had a basal serum 25(OH)D concentration of 60.5 nmol/L, they most likely had too high baseline serum 25(OH)D concentrations for an effect to be found. On the other hand, an insufficient dose of Vitamin D<sub>3</sub> is not likely since the vitamin D group experienced a mean increase in serum 25(OH)D of 45.8 nmol/L after 12 months of vitamin D supplementation. However, if the study subjects had had a pre-specified serum 25(OH)D concentration at the outset, and in addition the response would have been tailored after vitamin D supplementation, this probably could have improved the study.

## 5.2 Discussion of main results

### 5.2.1 Vitamin D and its effect on the glucose metabolism, blood pressure and lipids

In Paper I, we published results showing that 12 months of vitamin D supplementations did not have any beneficial effects on glucose metabolism nor did it appear to prevent the development of T2D in a population with prediabetes. At the time of publication, Paper I was by far the largest RTC on vitamin D supplementation in individuals with prediabetes.

Ahead of the publication of Paper I, several RCTs with vitamin D supplementation in subjects with obesity, prediabetes, and T2D had been published (174-177). However, and in spite of the inverse association between low serum 25(OH)D and both insulin resistance and T2D, the results were inconclusive (174-177). Furthermore, several review articles had recently been published in which subjects with normal glucose tolerance, subjects at risk for developing T2D, and subjects with T2D had been included (8, 21, 107). Vitamin D supplementations did not seem to have a beneficial effect in subjects with normal glucose tolerance (107), and vitamin D supplementations had at best only a slight effect in patients with T2D (8, 107, 178). However, in subjects at risk of developing T2D, the results were more uncertain (109, 176, 177). In a study by von Hurst et al. including 81 insulin-resistant South Asian women with vitamin D deficiency with a mean baseline serum 25(OH)D concentration of 21 nmol/L, insulin resistance was significantly less pronounced after receiving 4,000 IU/day of vitamin D supplementation for 6 months compared to placebo (176). On the other hand, and most likely due to an adequate vitamin D status, a study by Davidson et al. including 117 individuals with prediabetes with a mean baseline serum 25(OH)D concentrations of 55 nmol/L found no effect on glucose metabolism following one year of high dose vitamin D supplementation as compared with placebo (109). The results remained unchanged for those having a baseline serum 25(OH)D < 50 nmol/L (109), however, only 15 subjects had such low serum 25(OH)D concentrations.

After 12 months of vitamin D supplementation the increase in serum 25(OH)D in our study was 45.8 nmol/L, thus the increase was substantial and therefore the dose given should have been high enough in order for an effect to be seen. However, the subjects included had a mean serum 25(OH)D at baseline of 60.5 nmol/L. They probably therefore represent a too vitamin D sufficient population for an effect to be found similar to that seen

in the Davidson et al. study. Also similar to the Davidson et al. study, no effect was found in subgroups of subjects with serum 25(OH)D < 50 nmol/L and < 40 nmol/L. On the other hand, since T2D is a slowly developing disease, the follow-up time might have been too short; but results after 5 years of follow-up were similar to the 12 months results (unpublished data). However, in both the 12-month analyses and the 5-year analyses regarding glucose metabolism, we did not have the power to disclose differences in the subgroups with the lower serum 25(OH)D concentrations.

Further, a recently published review article of 35 RCTs with altogether over 40,000 patients found no evidence that vitamin D supplementation prevents T2D in subjects without T2D (179). Also, there was no evidence for reduced insulin resistance or reduced hyperglycaemia in subjects with prediabetes or T2D (179), thus supporting our findings. Therefore, for the time being, we lack evidence to recommend vitamin D supplementation for prevention of T2D in subjects at risk of developing T2D (180).

Despite the so far negatively published results regarding prediabetes and vitamin D supplementation for prevention of T2D, a large RCT named “Vitamin D and type 2 diabetes study” (D2d) started inclusion of participants in 2013 (181). The aim of D2d is to include 2,382 subjects with prediabetes and allocate participants to either 4,000 IU of vitamin D<sub>3</sub> per day or placebo for 2–4 years (181). D2d’s primary goal is similar to ours: to evaluate whether the progression from prediabetes to T2D can be prevented or reduced (181). In addition, and also similar to our study, several glycaemic indices will be evaluated. Positively, D2d has a pre-specified serum 25(OH)D response of 75–125 nmol/L, however, how tailoring of this response is intended to be carried out is not described. Not using serum 25(OH)D concentrations as an inclusion criterion is probably the largest limitation of D2d (181), since one could argue, based on our study, together with other studies, that the question in subjects with adequate vitamin D already has been settled. Nevertheless, with such a large number of participants, they hopefully will obtain large enough power for subgroup analyses to establish whether vitamin D supplementations have an effect at least in vitamin D insufficient or deficient subjects.

In our study, no favourable effect was seen regarding blood pressure after 12 months of vitamin D supplementations. As for glucose metabolism, the results remained unchanged after subgroup analyses. For blood pressure, subgroup analyses were done in individuals with vitamin D deficiency, in individuals with hypertension, and in individuals with both

vitamin D deficiency and hypertension. On the other hand, statistically significant reductions were found for total cholesterol and LDL cholesterol in the vitamin D group compared to the placebo group. However, the effects were marginal, and since we also found a reduction in HDL cholesterol, the benefit is questionable. For subgroups not using statins, similar effects were found. Furthermore, for subjects not using statins, or on unchanged statin doses, and with baseline serum total cholesterol > 7.8 mmol/L and/or baseline serum LDL > 4.9 mmol/L, no beneficial effects regarding lipid concentrations were found in the vitamin D group as compared to the placebo group. Also, no effect was seen in the total cohort or in various subgroups for the inflammation marker hs-CRP following vitamin D supplementation.

For blood pressure and the cardiovascular system, an effect of vitamin D could be expected since the VDR is present in vascular smooth muscle, endothelium and cardiomyocytes (53). Also, experimental data have shown beneficial effects of VDR activation on the renin-angiotensin-aldosterone (RAAS) system, most likely caused by inhibition of the synthesis of renin in the juxtaglomerular cells by vitamin D (182). Further, vascular smooth muscle cell proliferation and vascular calcification and inflammation are also inhibited by VDR activation (102). Despite this, intervention with vitamin D supplementation does not seem to have a beneficial effect on cardiovascular risk factors (102). Supporting this are the results from a recently published large meta-analysis including 52 studies where no evidence supporting vitamin D supplementation for lowering blood pressure was found (183). This finding was similar in patients with hypertension or T2D (183). Similarly, RCTs have not been able to show a positive effect of vitamin D supplementation on lipid concentrations (184). Despite vitamin D appearing not to have any beneficial effects on blood pressure or lipid concentrations, arterial stiffness improved in 47 individuals with T2D recruited from an outpatient hypertension clinic following vitamin D supplementation (185). However, the number of subjects was small and the clinical consequence questionable. Further, for subjects without T2D, no such effect on arterial stiffness has been found: in an RCT in 130 northern Europeans without T2D receiving 3,000 IU per day of Vitamin D<sub>3</sub> for 20 weeks (186); and in a RCT in 98 native American women receiving either 400 IU or 2,500 IU per day (187). However, another interesting finding in the vitamin D group was increased adiponectin concentrations, adiponectin being a protein known to be decreased in obesity, which is associated with inflammation and atherogenesis (188).



Based on a Mendelian randomization study by Afzal et al. (189) a hypothesis that perhaps lifelong endogenous vitamin D is what is needed to prevent T2D was proposed (190). The Afzal et al. study included 96,423 subjects and found a non-significant association between genetic variants in the DHCR7 gene associated with low serum 25(OH)D concentration and the risk of T2D (189). Mendelian randomization studies use observational data and incorporate genetic information, thus the exposure is defined based on the presence or absence of the risk allele (191). Also, several biases known to limit observational studies, such as confounding and reverse causality are much less an issue in Mendelian randomization studies (191), thus these could offer an explanation for any differences found between observational studies and RCTs. However, Ye et al. in a larger, recently published, Mendelian randomization study examining four SNPs near the DHCR7 gene, the CY21R gene, the CYP24A1 gene and the DBP gene, found no association for serum 25(OH)D concentrations with the risk of T2D, or with the glycaemic indices fasting glucose, 2-h glucose, fasting insulin and HbA<sub>1c</sub> (192). On the other hand, for blood pressure, a Mendelian randomization study found that increased serum 25(OH)D concentrations were associated with reduced systolic blood pressure, but not diastolic blood pressure (193). Thus, even though Mendelian randomization studies support findings from RCTs that vitamin D supplementations do not reduce the risk for T2D, they might play a role for blood pressure.

At the present time, two large RCTs with vitamin D supplementation are running (194, 195). The largest, *VITamin D and OmegaA-3 Trial* (VITAL), started inclusion in 2010 and plans to include over 20,000 healthy men and women above the age of 50 from all across the United States (194). The VITAL study has a 2 x 2 factorial design with a mean treatment period of at least five year, and its main goal is to investigate whether the risk of cancer, heart disease, and stroke can be reduced by 2,000 IU of Vitamin D<sub>3</sub> per day and 1 gram of omega-3 fatty acids per day (194). In addition, the study will also evaluate the effect on several other diseases including T2D and hypertension (194). The other large RCT running, the *Vitamin D assessment (ViDA)* study, is based in New Zealand (195). The ViDA study is a 4-year RCT investigating whether vitamin D prevents CVD, respiratory disease, falls, and fractures or not, and the 5,100 participants between 80–84 years of age are receiving either 100,000 IU per month of vitamin D supplementation or placebo (195). Despite the large number of participants in both VITAL and ViDA, similar to D2d, their major limitation is not using baseline serum 25(OH)D concentrations as an inclusion criterion. Further, in VITAL it is

optional to have serum 25(OH)D measured at the beginning of the study, thus representing another weakness of the study.

In conclusion, based on a growing pool of evidence from intervention studies, vitamin D supplementation does not have an effect on blood pressure (183), on lipid concentrations (184), for the prevention of T2D or on improving glucose metabolism (179) in individuals with an adequate vitamin D status. However, for subjects with real deficiency, the effect of vitamin D on at least the glucose metabolism still remains unanswered. The discrepancy between observational studies and RCTs is probably due to a reverse causality; thus those living healthy lives with healthy diet probably also spend more time outdoor in the sun, thus their higher serum 25(OH)D concentrations. Therefore, a person's vitamin D status can be regarded as a marker for good health rather than an option for intervention.

### **5.2.2 SNPs, baseline serum 25(OH)D concentration and BMI and their effect on the serum 25(OH)D response following vitamin D supplementation**

In Paper II, we published results regarding the influence of genetic factors on baseline serum 25(OH)D concentration, and also how genetics, together with BMIs and baseline serum 25(OH)D concentrations, predicts the serum 25(OH)D response following vitamin D supplementation.

SNPs related to genes in synthesis, binding and degradation of the vitamin D metabolites were all shown to have significant differences between the major and minor homozygotes regarding serum 25(OH)D concentration. This was in line with earlier reports (133, 134, 143, 159, 196-199). We did not reveal any relationship between serum 25(OH)D and SNPs related to the VDR, also this in line with most previous studies (143-146). The largest difference between the major and minor homozygotes were found for the SNP rs228279 in the DBP gene; the major homozygotes had a 19.2 nmol/L higher serum 25(OH)D concentration than those with the minor homozygote genotype. Despite this difference, no relationship between serum PTH and rs228279 was found. Nevertheless, SNPs in the DBP gene are also related to the serum DBP concentration and/or DBP phenotypes (121, 122), and since the DBP phenotypes have different binding coefficients for 25(OH)D (116, 200), the serum 25(OH)D may not accurately reflect the concentration of serum free or bioavailable 25(OH)D (115). However, we did find a highly significant association between serum PTH and the degradation enzyme CYP24A1, both at baseline and in subjects

randomized to vitamin D supplementation after 12 months. A similar finding has previously been reported in 9,471 subjects from our region (196), thus this association is unlikely to be due to chance.

There has been a general assumption that an intake of 100 IU per day leads to an increase in serum 25(OH)D of approximately 2.5 nmol/L (201). However, we found a wide variation in the 25(OH)D response following vitamin D supplementation, with the most important factor for the observed variation being genetics. For three of the SNPs; rs2282679 and rs7041 in the DBP gene and rs10741657 near the CYP2R gene, we found a significant relation between genotype and increase in serum 25(OH)D concentrations after vitamin D supplementation. Ahead of the publication of Paper II, few studies had been published regarding SNPs and the 25(OH)D response following vitamin D supplementation (143, 148, 202-204). In the Barry et al. study including 1,787 European Americans, an SNP near CYP2R1 (rs107661979), an SNP near CYP24A1 (rs6013897), and also an SNP near VDR (7968585) were found to be associated with the 25(OH)D response (148). The same SNP near CYP2R1 was also in a study in 644 older Australians found to be associated with the serum 25(OH)D response (204). Thus, the genetic effects on the serum 25(OH)D response are most likely caused by differences in the vitamin D metabolism, which as described earlier has numerous enzymatic steps. On the other hand, Mendelian randomization studies have not found any significant association with CYP2R1 genotypes associated with low serum 25(OH)D concentration and T2D, thus low serum 25(OH)D caused by impaired conversion of vitamin D into 25(OH)D in the liver does not seem to be associated with increased risk of T2D (189, 192).

For subjects randomized to vitamin D, the largest serum 25(OH)D response was found in subjects with high baseline serum 25(OH)D concentration, also in line with earlier studies (143, 204). This was to be expected since genetic differences in all steps of the vitamin D synthesis are probably responsible for differences in baseline serum 25(OH)D concentrations, and therefore most likely also affect the handling of the additional vitamin D supplements. However, in subjects with the DHCR7 SNP rs3829251, the serum 25(OH)D response was greatest in those with the lowest baseline levels. An explanation for this finding might be that DHCR7 is not involved in the exogenous vitamin D metabolism, but upon solar UVB radiation is involved in the conversion of 7-DHC into vitamin D<sub>3</sub> in the skin (132). Further, we found that obese subjects had a worse 25(OH)D response following

vitamin D supplementation compared to non-obese. Also other studies have shown a reduced increase in serum 25(OH)D concentration in obese subjects compared to non-obese (205, 206). Thus, these findings support the idea that vitamin D metabolites are stored and/or degraded in adipose tissues (83).

Genotype together with the two other response factors; BMI and baseline serum 25(OH)D concentration, were integrated in a regression equation to predict the serum 25(OH)D response to a weekly dose of 20,000 IU vitamin D<sub>3</sub>. In addition, we included the randomization status in the equation to account for regression toward the mean. For subjects with a BMI of 22 kg/m<sup>2</sup> and the “best” genetic status, meaning those with the highest 25(OH)D response, all would reach a serum 25(OH)D concentration > 75 nmol/L; however, hardly any of the morbidly obese subjects with BMI  $\geq$  35 kg/m<sup>2</sup> and the “worst” genes, meaning those with the lowest serum 25(OH)D response, and a baseline serum 25(OH)D < 50 nmol/L would reach the same serum 25(OH)D concentration. In paper II, 12.4 % had a serum 25(OH)D concentration < 75 nmol/L after 12 months of vitamin D supplementations, and worldwide millions of people have these “worst” genotypes. Additionally, a “too” good serum 25(OH)D response was seen for 9.1 % of our study participants reaching a serum 25(OH)D > 140 nmol/L after 12 months, a concentration which may not be favourable (207-209). Importantly, the regression equation above is only applicable in the setting of this intervention study, and cannot be used without problems in other cohorts.

Unfortunately, no consensus exists on what the optimal, or the sufficient, serum 25(OH)D concentration is. A U- or J-shaped relation between serum 25(OH)D concentrations and health effects has been reported (207-209); some suggest that the optimal serum 25(OH)D concentration is 50–60 nmol/L (209), others that possible adverse effects appear with serum 25(OH)D > 220 nmol/L (210). However, these relations with possible harmful effects in the higher 25(OH)D concentrations are based on association studies only.

Regardless, when giving recommendations for vitamin D supplementation to the general public, it is important to know not only if there are subgroups in need of higher doses than average, but also if there are subgroups where the increase in serum 25(OH)D will be particularly high. Consequently, further RCTs with vitamin D supplementations need to take into account the wide variations in serum 25(OH)D response.

### 5.2.3 Free 25(OH)D

In Paper III, we found that serum 25(OH)D concentrations were significantly lower for the DBP phenotype Gc2/Gc2 compared to phenotypes with the Gc1S allele, as previously reported (211). Although the serum calculated free 25(OH)D, bioavailable 25(OH)D and directly measured free 25(OH)D also were lowest in subjects with this phenotype, the differences were greatly diminished and no longer significant. This finding is in line with the almost identical free 25(OH)D concentrations seen in European and African Americans despite great differences in serum 25(OH)D (77, 122), due to different distribution of DBP phenotypes (111). Further, this could also partly explain why African Americans have better bone health than European Americans despite lower serum 25(OH)D concentrations (76). In the Powe et al. study, 2,085 European and African Americans were included; DBP was measured by a monoclonal antibody test, and the calculated free 25(OH)D and bioavailable 25(OH)D concentrations were validated by directly measured free 25(OH)D concentrations in a subgroup of homozygous participants.

In our study, in addition to the abovementioned differences, there were also differences between males and females in serum 25(OH)D concentrations, with males having significantly lower concentrations than women. These differences diminished for both calculated free 25(OH)D and calculated bioavailable 25(OH)D, and for the directly measured free 25(OH)D the concentrations were equal. The explanation for our finding is most likely differences in serum DBP concentrations between males and females, which previously also have been reported by others (212). These differences seen between the sexes are probably caused by the estrogen-dependent DBP synthesis (111).

Schwartz et al. in 106 subjects with either liver cirrhosis or pregnancy (second or third trimester) compared to healthy controls found that serum 25(OH)D was lower in patients with liver cirrhosis (213). However, the directly measured free 25(OH)D concentrations were significantly higher, most likely due to reduced synthesis of DBP in patients with liver cirrhosis. Further, and despite increased serum DBP concentration during second and third trimester, no differences for serum 25(OH)D or for directly measured free 25(OH)D were found for pregnant women compared to healthy controls (213). However, DBP's affinity to different vitamin D metabolites has been shown to decrease during pregnancy (214).

Further, we found that the calculated serum free 25(OH)D concentrations as compared to the directly measured free 25(OH)D differed, with the highest concentrations

found for the calculated levels. This is similar to what was found in the Schwartz et al. study (213). Since different DBP assays recognize the DBP phenotypes differently (123), and different methods measure significantly different concentrations (77), this will certainly also affect the calculated free 25(OH)D concentrations. Furthermore, the validity of the equation for calculating free 25(OH)D concentration, which is derived from an equation for free testosterone (114), has been questioned (123). Recently results measuring serum DBP, which is needed for calculations of free 25(OH)D concentrations, using LC-MS/MS instead of by mono- or polyclonal immunoassays are conflicting. The largest study, including 187 participants, reports similar serum DBP concentrations with the polyclonal immunoassay and with LC-MS/MS, and as expected, as previously described, lower concentrations when using the monoclonal assay (124). On the other hand, the smaller study reports that the differences between races in serum DBP concentration disappear using LC-MS/MS (125). In addition, and as expected, they report significantly higher DBP concentrations using LC-MS/MS as compared with the monoclonal immunoassay (125). Thus, what the correct serum DBP concentrations are is still unknown. Also worth mentioning is that DIASources ELISA assay for measurements of free 25(OH)D is the only commercially available one (154), with many researchers and clinicians hoping for the methodology used to be proven by a mass spectrometric assay.

Similar to DBP for the vitamin D metabolites, several ligand-specific carrier proteins exist for other hormones; thyroid hormone binding globulin (TBG) for thyroid hormone; corticosteroid-binding globulin (CBG) for glucocorticoids and mineralocorticoids; and sex hormone binding globulin (SHBG) for testosterone (215). Also in the same manner as for free 25(OH)D, it is the unbound fraction of the abovementioned hormones that are considered the biologically active metabolites (215). Serum measurements are most widely used for the measurement of hormones. However, for the measurements of free hormones in serum, ultra-filtration is needed, thus making the methods expensive and laborious (216). In saliva, a fluid which contains little plasma proteins due to their size and thus incapability to pass over membranes, it is possible to measure some free hormones using LC-MS/MS (217). This is a method used routinely for cortisol in the diagnosis of Cushing's disease (218). Also, for salivary free 25(OH)D, a method using LC-MS/MS has been described by Higashi et al. (219); however, this method has not been confirmed by others. A major limitation for salivary free

25(OH)D is that the free fraction is very low (216). Thus, the saliva measurements may not reflect the serum concentrations accurately.

It is not settled which of the vitamin D metabolites, e.g. the mother compound vitamin D, serum 25(OH)D, serum free 25(OH)D or bioavailable 25(OH)D, one should measure to evaluate a person's vitamin D status. Serum PTH concentrations have been suggested as a vitamin D biomarker (86), and based on PTH correlations with different 25(OH)D metabolites, one could get an indication which vitamin D metabolite is the preferred one. However, as mentioned before, the use of PTH is questionable (84). Further, one should keep in mind that the receptor-mediated endocytosis of the DBP/serum 25(OH)D complex in the proximal renal cells (49), and probably also in a number of other cells (48), could favour serum 25(OH)D as an indicator of vitamin D status. We found that the analysed 25(OH)D metabolites all had a similar and negative correlation with PTH, while previously studies shows variation as to which vitamin D metabolite best correlates with PTH (77, 220, 221). Thus, also this question remains unsettled. If instead we had used BMD as a biomarker, the question of which vitamin D metabolite best reflects a persons' vitamin D status could perhaps have been answered. Or, it might be that the question will remain unanswered until we have a new, and better, biomarker available.

The increases after 12 months of vitamin D supplementation were similar for all vitamin D metabolites analysed regardless of DBP phenotype, sex or age. Further, serum DBP concentrations were not affected by vitamin D supplementation.

Based on our findings, measurement of free 25(OH)D should not be recommended for the general population. However, it should be considered in situations where DBP phenotypes differ between ethnic groups, as seen between European and African Americans, and also in conditions affecting serum DBP concentrations like liver cirrhosis and nephritic syndrome.

#### **5.2.4 Limitations**

The main limitation of our study is that the participants had an adequate baseline serum 25(OH)D concentration (mean baseline serum 25(OH)D concentration of 60.5 nmol/L). Further, our study population is homogeneous consisting almost exclusively of Caucasians, and for serum DBP the consequence is a skewed distribution of DBP phenotypes. Also, the results cannot be generalized as all participants had IGT and/or IFG and live in Northern



Norway at a latitude of 69° with low solar UVB exposure, and we did not have BMDs that could have given us additional information regarding which 25(OH)D metabolite best reflects a person's vitamin D status. Furthermore, we did not include data on sun exposure, skin pigmentation and physical activity. However, it is not likely that having the abovementioned information, except lower baseline serum 25(OH)D concentrations, would have changed our results.

Despite these limitations, our study has considerable strength and importance as it at the present time is by far the largest published RCT with vitamin D supplementation in subjects with prediabetes. Another strength is the inclusion of a large group of subjects; furthermore, we had predefined a limited number of SNPs to evaluate; and we were able to create an applicable regression equation for predicting the serum 25(OH)D response following vitamin D supplementation. Additionally, we had direct measurements of serum free 25(OH)D, and could also examine the effects of vitamin D supplementation on various vitamin D metabolites and on serum DBP, which has not so far been thoroughly studied.

## **6. Conclusion and implications**

### **6.1 Conclusion**

In conclusion, vitamin D supplementation in subjects with prediabetes appears to have no beneficial effect on the glucose metabolism. This probably does not mean that vitamin D is without importance for glucose regulation, but at least in subjects with adequate serum 25(OH)D concentrations, vitamin D supplementation should not be recommended.

Further, vitamin D supplementation did not affect the study subjects' blood pressure or lipid concentrations. However, and similarly as for the glucose metabolism, this does not mean that vitamin D is without importance for cardiovascular health. However, vitamin D supplementation should not be recommended in subjects with adequate serum 25(OH)D concentrations for these purposes either.

We also found that all five SNPs investigated influence the serum 25(OH)D concentration at baseline, in addition two SNPs in DBP and one in CYP2R1 gene affect the serum 25(OH)D response following vitamin D supplementation. Furthermore, in order to have similar serum 25(OH)D responses, obese subjects need higher vitamin D doses as compared to non-obese. Therefore, subgroups in need of higher doses than average and

subgroups expected to have a higher than average serum 25(OH)D response must be given special considerations when giving recommendations for vitamin D supplementation.

In addition, we have found that the serum 25(OH)D concentration differs between, and depends upon, DBP phenotypes. Further, the differences seen in serum 25(OH)D between DBP phenotypes, and between sexes, diminished for the directly measured free 25(OH)D concentrations. This finding is most likely caused by differences in DBP concentrations between DBP phenotypes, thus, direct measurements of free 25(OH)D should be considered in subjects with conditions or diseases known to affect the serum DBP concentrations.

## 6.2 Implications

Based on our study, vitamin D supplementation cannot be recommended for prevention of T2D in subjects with prediabetes, in addition it cannot be recommended in order to better hypertension or improve blood lipid concentrations in subjects with adequate vitamin D status.

Both baseline serum 25(OH)D concentration and BMI are inexpensive to measure and need to be taken into account when giving recommendations for vitamin D supplements. However, it is costly to predict the serum 25(OH)D response following vitamin D supplementation based on genotyping, therefore the simplest way to tailor the response is by measuring serum 25(OH)D concentrations.

Further, serum 25(OH)D should still be the metabolite used to evaluate a person's vitamin D status. However, in subjects having conditions or diseases associated with low or high DBP concentrations direct measurement of serum free 25(OH)D should be considered.

Vitamin D constitutes an important factor for bone health. However, what the sufficient serum 25(OH)D concentration is still remains an open question (65, 84, 89). In the future guidelines may need to consider subgroups with regard to threshold serum 25(OH)D concentrations (222).

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