The role of vitamin D in glycaemic control

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THE ROLE OF VITAMIN D IN GLYCAEMIC CONTROL

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THE ROLE OF VITAMIN D IN GLYCAEMIC CONTROL

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geboren te Graft-De Rijp

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

This thesis highlights the role of vitamin D in glycaemic control in different populations. Type 2 diabetes represents a worldwide epidemic with significant co-morbidity and mortality (1). In the Netherlands, approximately 800.000 inhabitants had been diagnosed with diabetes up till 2011 and estimations have been made that this number will increase to 1.3 million in 2025 (2). Insulin resistance and decreased beta cell function are major contributing factors in the onset of type 2 diabetes. Although therapies for type 2 diabetes have improved over the last few decades, new insights in the prevention and management remain necessary.

In the first part of this thesis the link between vitamin D and gestational diabetes will be described in a systematic review and meta-analysis. The second part of this thesis will focus on the role of vitamin D on metabolic disturbances in patients with polycystic ovary syndrome (PCOS). The third part of this thesis describes the effect of vitamin D supplementation on glycaemic control in patients with type 2 diabetes mellitus, the association between vitamin D and advanced glycation endproducts, and the effect of vitamin D supplementation on quality of life.

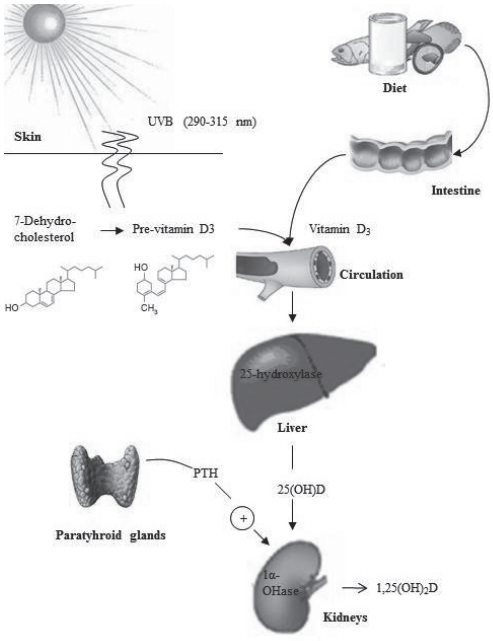
Vitamin D – historical review

Vitamin D has been originally termed 'D' as the 4th known vitamin, after the discovery of vitamin A, B and C. The first scientific description of rickets or "English disease" (Morbus Anglorum) was provided in the 17th century by both Dr. Daniel Whistler (1645) and Professor Francis Glisson (1650). During industrialisation and increasing air pollution in the English cities in the 19th century, rickets became very common. The Polish physician Sniadecky first associated rickets with lack of sun exposure in the 19th century. In the early 1920s several scientific experiments have led to a major breakthrough by the discovery that both lack of fat-soluble nutrients and lack of sunlight exposure could cause rickets. The chemical structure of vitamin D was discovered by Adolf Windaus, who was awarded for this with the Nobel Prize for Chemistry in 1928 (3).

Vitamin D – metabolism

Vitamin D is a fat-soluble vitamin with properties of both a vitamin and a hormone (4). It is mainly produced photochemically in the human skin from the precursor 7-dehydrocholesterol under influence of sunlight or ultraviolet light. A small amount of vitamin D is obtained from food, such as fatty fish, cod- liver oil, eggs, and fortified dairy products. The two major forms of vitamin D are vitamin D2 (ergocalciferol), industrially derived from UV irradiation of plants and yeast, and vitamin D3 (cholecalciferol), the more common form of vitamin D derived from animal food sources and produced in the skin (Figure 1).

Figure 1. Vitamin D metabolism



Vitamin D needs to be hydroxylated twice to become biologically active. First, vitamin D is transported to the liver where it is rapidly hydroxylated by 25-hydroxylase into 25-hydroxyvitamin D (25(OH)D) which has a strong affinity to vitamin D binding protein. The second hydroxylation occurs in the kidney by 1α-hydroxylase (1α-OHase), or CYP27B1, which results in the biologically active metabolite of vitamin D: 1,25-dihydroxyvitamin D (1,25(OH)2D). The elimination of vitamin D metabolites is initiated by CYP24A1 – the primary enzyme responsible for the catabolism of both 25(OH)D and 1,25(OH)2D.

Serum 25(OH)D is the major circulating form of vitamin D used as the main indicator of vitamin D status. Its half-life is 2-3 weeks compared to only 4-6 hours for 1,25(OH)2D (5).

Vitamin D – classical actions

Serum 1,25(OH)2D has a high affinity for the nuclear vitamin D receptor (VDR). The 1,25(OH)2D-VDR complex dimerises with the retinoid receptor (RXR), and this complex binds to vitamin D-responsive elements on target genes. This is known to selectively activate ~3% of the some 22,000 genes of the human genome, regulating the transcription of DNA to numerous proteins. The primary action of 1,25(OH)2D is to enhance intestinal calcium absorption and to promote osteoclast function, thereby maintaining calcium and phosphorus homeostasis and bone health (6). Serum 1,25(OH)2D is tightly regulated by the calcium-phosphorus-parathyroid hormone (PTH) axis, where an increase in PTH stimulates the production of 1,25(OH)2D. A negative feedback loop through serum calcium and directly through 1,25(OH)2D on the parathyroid cells, leads to a decrease in PTH with a direct negative effect on the production of 1,25(OH)2D.

Vitamin D – non classical actions

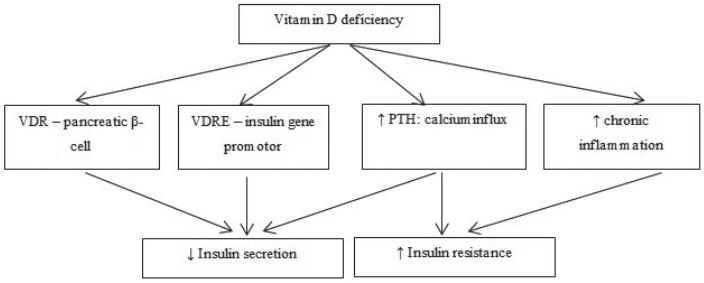
The VDR is known to occur in over 35 different cell types, including pancreatic β-cells, adipocytes, brain cells (neurons, astrocytes and microglia), lymphocytes, muscle cells, parathyroid cells and placenta, which means that the cells in these tissues have the potential to produce biological responses depending on the availability of appropriate amounts of 1,25(OH)2D (7,8). Until now it is known that at least ten tissues in addition to the proximal tubule of the kidney, which is the major source of 1,25(OH)2D, contain the enzyme 1α-OHase responsible for the extra-renal synthesis of 1,25(OH)2D for local activity as an intracrine or paracrine factor (8,9). These tissues include endothelial cells, human brain cells, pancreatic islet cells and the placenta. It is believed that the locally produced 1,25(OH)2D does not normally enter the circulation; thus, the plasma concentration of 1,25(OH)2D does not increase in a measurable way by the extrarenal synthesis. Extrarenal synthesis of 1,25(OH)2D was first documented more than a quarter of a century ago following studies of vitamin D metabolism in human pregnancy and the granulomatous disease sarcoidosis (10,11).
With these new discoveries mentioned above, it seems that 1,25(OH)2D functions as a hormone with actions on distance and having a negative feedback loop. Additionally, vitamin D does not mediates only the calcium metabolism, it also plays a role in the adaptive immune system, the innate immune system, insulin secretion by the pancreatic β -cell, cardiovascular system, blood pressure regulation, and brain and fetal development.

Vitamin D – insulin resistance

A growing body of research has identified several potential pathways to explain the role of vitamin D in insulin secretion and resistance. It appears to be mediated by direct and indirect pathways. A direct effect on insulin secretion may be mediated by activation of VDRs in the pancreatic β-cell with the addition of the presence of 1α-OHase to produce locally 1,25(OH)2D. Furthermore, the direct effect of vitamin D on insulin secretion is supported by the presence of the vitamin D-response element in the human insulin promotor gene (12). Vitamin D deficiency may also increase systemic inflammation, known to play an important role in the pathogenesis of type 2 diabetes (13). Finally, insulin secretion and insulin resistance are both calcium-dependent processes. Both could be influenced by vitamin D status through an alteration in calcium concentration and flux through cell membranes of pancreas and insulin-responsive tissues (Figure 2).

Despite accumulating evidence from several observational human and animal model studies emphasizing an inverse correlation of serum 25(OH)D with insulin resistance (14), intervention studies among patients with type 2 diabetes yielded conflicting results (15). Of importance is that most of these studies had a small sample size, used small amounts of vitamin D supplementation with a relatively short follow-up duration. Moreover, some of these studies were post-hoc analyses not using glycaemic control as primary outcome (15). In the general discussion the intervention studies will be extensively summarised.

Figure 2: Proposed mechanisms for the role of vitamin D in insulin secretion and resistance.



VDR, vitamin D receptor; VDRE, vitamin D-response element

Vitamin D deficiency

Vitamin D status is assessed through serum 25(OH)D, which is the sum of vitamin D3 produced in the skin and oral intake through foods and supplements which both are rapidly converted to 25(OH) D. Until now there is no consensus on the diagnosis of vitamin D deficiency and insufficiency , nor on the optimal serum level of 25(OH)D (16). Vitamin D deficiency is commonly defined by a serum 25(OH)D less than 30 nmol/l (12 ng/ml). This threshold level has been confirmed by the Institute of Medicine at the end of 2010 and the Endocrine Society Guideline (17). Optimal serum 25(OH) D is defined as a level above 50 nmol/l according to the Institute of Medicine and above 75 nmol/l according to the Endocrine Society. The Health Council of the Netherlands concluded that serum 25(OH)D is sufficient when it is above 30 nmol/l for adult men and women and above 50 nmol/l for people aged 70 years and over (18). They recommend a supplementation dose of 400 IU/day for children, women between 50 and 70 years old and people with a dark coloured skin, while 800 IU/ day is advised for elderly (> 70 years).

Vitamin D status is influenced by factors interfering with the production of vitamin D in the skin (i.e. skin pigmentation, dressing codes, season, aging, latitude, sun exposure, sunscreen use and air pollution) and by factors that affect its absorption or metabolism . Each of them can be a cause of an insufficient vitamin D level. People who are potentially at high risk for vitamin D deficiency are children, adolescents, pregnant women, obese people, elderly, non-western immigrants, and those living at higher latitudes (17).

Vitamin D and quality of life

Many observational studies found a link between vitamin D status and various factors which contributes to quality of life, as physical performance, chronic fatigue, chronic pain and depressive symptoms (19-22). Vitamin D has been related to quality of life in other study populations than patients with diabetes (23). It is known that patients with type 2 diabetes have a higher prevalence of depressive symptoms, less quality of life and suffer more often from chronic fatigue (24-26). This raises an important question whether vitamin D and quality of life in patients with type 2 diabetes are related, and if vitamin D supplementation has any effect on quality of life in patients with type 2 diabetes.

Vitamin D and advanced glycation end products

One of the chronic consequences of hyperglycaemia is the accelerated formation of advanced glycation end products (AGEs), which are suggested as one of the major pathogenic mechanisms causing end organ damage in diabetes (27). AGEs are formed nonenzymatically by the modification of proteins, lipids and nucleic acids by glucose and accumulate slowly over a persons' lifetime (28). AGEs can be measured non-invasively by skin autofluorescence which has a strong association with the severity of diabetes-related complications and mortality in patients with type 2 diabetes (29,30). Similar to the consequences of AGE accumulation, low vitamin D status has been linked to numerous biochemical and clinical disturbances, including the pathogenesis and progression of type 2 diabetes and cardiovascular disease (14). No observational studies have examined the association between vitamin D and skin auto fluorescence.

PCOS and vitamin D

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age, with a prevalence up to 12% depending on which diagnostic criteria are used (31). PCOS is diagnosed according the Rotterdam consensus criteria when at least two of the following criteria are present: 1) ovulatory dysfunction resulting in oligo- and/or anovulation, 2) hyperandrogenism (either biochemical abnormalities or clinical hirsutism) and/or 3) the presence of polycystic ovarian morphology (32). Metabolic disturbances are common in women suffering from PCOS: 30-40% have impaired glucose tolerance and insulin resistance with compensatory hyperinsulinemia, and as many as 10% will have type 2 diabetes mellitus by their fourth decade (33). Many studies have been conducted to clarify the mechanism of metabolic disturbances, in particular insulin resistance, in women affected by PCOS. In part, insulin resistance might be due to obesity. However, a substantial number of lean women affected by PCOS have insulin resistance as well, independent of obesity (34,35). Vitamin D deficiency has been proposed as the possible missing link between insulin resistance and PCOS. A review performed by Thomson et al. about the role of vitamin D in the aetiology and management of PCOS suggests that there is an association between vitamin D status and hormonal and metabolic dysfunctions in PCOS (36). Further research is necessary to answer the question whether vitamin D and metabolic disturbances are causally interrelated or that they constitute two independent features of women with PCOS.

Outline of this thesis

The purpose of this thesis is to gain more insight in the association between vitamin D and glycaemic control. For this we studied three different patient populations: patients with gestational diabetes, PCOS and type 2 diabetes. Second, this thesis investigates the associations between vitamin D status and first quality of life, and second AGEs in patients with type 2 diabetes.

Part I – Vitamin D in Gestational Diabetes

This part provides a systematic literature review and meta-analysis of vitamin D status and the onset of gestational diabetes.

Part II – Vitamin D in PCOS

This part includes two main topics. First, in chapter 2 a summary of the literature is provided in a systematic literature review about the association between vitamin D and metabolic disturbances in women suffering from PCOS. Second, in chapter 3 the association between vitamin D and metabolic disturbances was explored in women with PCOS (Rotterdam PCOS cohort) compared to controls.

Part III – Vitamin D in Type 2 Diabetes Mellitus

This part presents the data of our randomised placebo-controlled clinical trial performed among 275 patients with type 2 diabetes. Chapter 4 presents the study protocol. In chapter 5 we describe the effect of vitamin D supplementation on glycaemic control and other metabolic parameters. The secondary outcomes regarding the association between vitamin D status and quality of life, the association between vitamin D status and AGEs, and the effect of vitamin D supplementation on quality of life, are described in chapter 6, 7 and 8. Finally, chapter 9 provides a meta-analysis of the effect of vitamin D supplementation on glycaemic control in patients with type 2 diabetes.

In the general discussion the results of this thesis are summarised, placed in a broader context, and the implications for public health as well as the directions for further research will be discussed.

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PART I

GESTATIONAL DIABETES

CHAPTER 1

Vitamin D and Gestational Diabetes Mellitus: a systematic review and meta-analysis

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ABSTRACT

Background

Vitamin D metabolism is linked to glucose metabolism. The role of maternal vitamin D status in gestational diabetes mellitus (GDM) is uncertain. We sought to examine this role in women with GDM compared with normal glucose tolerance (NGT).

Methods

We performed a systematic review and meta-analysis by searching MEDLINE database, the Cochrane library and Uptodate® Online for English-language literature through September 2011. When necessary we contacted the authors of the reviewed articles for additional data. Summary odds ratios were calculated using a random-effects model meta-analysis.

Results

Randomised clinical trials have not yet been performed regarding the relationship between vitamin D and glucose metabolism in pregnancy. However, seven observational studies were eligible. Vitamin D status was assessed in 2146 participants of whom 433 were diagnosed with GDM. Four studies reported a high incidence of vitamin D deficiency in pregnant women (>50%). Overall vitamin D deficiency in pregnancy is significantly related to the incidence of GDM with an odds ratio of 1.61 (95% CI 1.19 – 2.17; p = 0.002). Serum 25-hydroxyvitamin D was significant lower in participants with GDM than in NGT (-5.33 nmol/l (95% CI -9.73 – -0.93; p = 0.018).

Conclusions

This meta-analysis indicates a significant inverse relation of serum 25-hydroxyvitamin D and the incidence of GDM. Clinical trials are needed to examine whether vitamin D supplementation will prevent GDM or decrease insulin resistance in GDM.

INTRODUCTION

Gestational diabetes mellitus (GDM), defined by glucose intolerance with onset or first recognition during pregnancy, is one of the most common complications of pregnancy. It affects 2-13% of all pregnancies depending on the population studied and the diagnostic cut-offs (1-3). GDM has serious adverse maternal outcomes, e.g. high Caesarean section rate, pre-eclampsia and long-term risk for developing metabolic syndrome and type 2 diabetes mellitus, and adverse fetal outcomes such as macrosomia which is related to shoulder dystocia and newborn asphyxia, infant respiratory distress syndrome and neonatal hypoglycaemia. Although after birth neonates are no longer exposed to a high glucose environment, they often have life-long increased risk of glucose intolerance and obesity (4-7). Early detection and intervention can greatly improve these outcomes. However, screening and diagnostic tests for GDM are not uniform worldwide (4,8). The International Association of Diabetes in Pregnancy Study Groups recently published a consensus derived from the Hyperglycaemia Adverse Pregnancy Outcome (HAPO) study data, suggesting that all pregnant women without known diabetes should have a 2 hour 75 g OGTT (4). Instead several guidelines report that an OGTT must be done if there are one or more predicting factors for GDM (3). The known risk factors for developing GDM include maternal age, obesity or being overweight, prior history of gestational diabetes, family history of type 2 diabetes, history of previous fetal death, being of a particular race/ethnicity and previous delivery of a macrosomic infant (5,7). Interest is growing in predicting which women will develop GDM given that early detection and intervention can greatly improve outcomes for both mother and child.

Vitamin D deficiency

A poor vitamin D status has been proposed as one of the factors being associated with the incidence of GDM. Vitamin D deficiency and insufficiency have been associated with impaired glucose metabolism and the metabolic syndrome (9-12). The number of vitamin D deficient or insufficient people worldwide is estimated at 1 billion (13). Although there is no consensus on optimal serum levels of 25-hydroxyvitamin D (25(OH)D), vitamin D deficiency is defined by a serum 25(OH)D level less than 25 nmol/l (10 ng/ml) (14). Serum 25(OH)D levels between 25 and 50 nmol/l (10-20 ng/ml) are considered as vitamin D insufficiency (15). Data from epidemiological studies such as the Longitudinal Aging Study Amsterdam show that threshold serum 25(OH)D for bone turnover markers is around 40 nmol/l and for bone mineral density at the hip is around 50 nmol/l (16). A round table conference could not reach a consensus, opinions ranging from 50 to 80 nmol/l (17). Recently, the Institute of Medicine, reviewing all the evidence, has set the required level of serum 25(OH)D at 50 nmol/l (14). The vitamin D status is influenced by factors regulating the production in the skin (i.e. skin pigmentation, skin covering by clothes, season, aging, latitude, sunscreen use and air pollution) and by factors that affects its absorption or metabolism (13). Each of them can be a cause of an insufficient or deficient vitamin D level. People who are potentially at high risk for vitamin D deficiency are elderly, children, pregnant women, obese people, people with increased skin pigmentation and non-western immigrants. It remains uncertain whether the optimal levels for non-pregnant adults are adequate for pregnant women, although the Institute of Medicine did not make a difference (14). The aim of this systematic review and meta-analysis is to evaluate the association between vitamin D deficiency and the incidence of GDM.

METHODS

Data sources and study selection

Two authors independently performed a formal computer-assisted search of the MEDLINE database, Embase, UpToDate® Online and The Cochrane Library for English-language literature through September 2011 using the search term 'gestational diabetes' combined with 'vitamin D', 'cholecalciferol' or '25-hydroxyvitamin D' and/or 'deficiency'. Additional publications were identified from citations from the recovered articles, review articles and reference lists. Studies that fulfilled the following criteria were included in this meta-analysis: 1) Studies identified a group with GDM and normal glucose tolerance (NGT) and 2) the outcome of interest was vitamin D status and/or deficiency. Details of studies included: country, design, publication year, participants (cases and cohort), variables controlled for in the analysis and odds ratios with CIs for the association between vitamin D deficiency and the diagnosis of gestational diabetes. When necessary the authors were contacted for additional data.

Statistical analysis

To obtain a summarised association between maternal serum 25(OH)D and the diagnosis of GDM, we used the mean differences and the adjusted p-value of each study. We performed a random-effect meta-analysis to combine the mean differences of each study. A p-value less than 0.05 was considered statistically significant. To identify potential associations of vitamin D deficiency and the incidence of gestational diabetes, the given ORs and 95% CIs from the reviewed articles were used. If the outcome measures were unsuitable for meta-analysis, the OR and 95% CIs were calculated by a 2 x 2 table using the data of the number of vitamin D deficient cases in the GDM and NGT group compared with the total number of participants in both groups. Study-specific ORs were pooled (Mantel-Haenszel) using a random-effect model meta-analysis to account for between-study heterogeneity that may have been introduced by the differences in study designs, methods for diagnosing GDM available in the trials and trial populations (18). Separate analyses were also performed for trials adjusting for confounders as maternal age, BMI and ethnicity.

We assessed statistical heterogeneity between studies with I2 statistic (with 95% CIs). The I2 is the proportion of total variation contributed by between-study variation. In general, I2values greater than 60-70% indicate the presence of substantial heterogeneity (19). In the presence of heterogeneity between studies, we assessed potential publication bias using formal tests, namely the funnel plot and Egger test (20).

RESULTS

Selected articles

Altogether 30 publications were identified through the literature search. Of those, 22 were excluded based on the inclusion criteria. In total, eight studies in which the association was studied between maternal vitamin D status and glucose homeostasis in pregnancy were included. Seven of these studies were observational studies (21-27) and one was a small intervention study with supplementation of 1.25-dihydroxyvitamin D (28), and was therefore excluded. Randomised controlled trials have not yet been published.

Table 1. Summary of the observational studies included in the review

Name, year[1], ref	Location	Study type	Cohort (n)	GDM (n)	GDM criteria	Mean 25(OH)D, nmol/l (SD) GDM	NGT	Prevalence 25(OH)D deficiency[2]	Significant difference[3]	Adjustments
Zhang (2008) (26)	US	Nested-case-control	171	57	ADA	59.5 (20.9)	74.0 (23.9)	19.8%	Yes	Age, BMI, ethnicity, family history of T2DM
Clifton-Bligh (2008) (21)	Australia	Cross-sectional	244	81	ADPS	48.6 (24.9)	55.3 (23.3)	48%	Yes	Age, BMI, ethnicity
Farrant (2009) (22)	India	Cross-sectional	559	39	Carpenter & Coustan	49.3 (31.2)	46.4 (30.9)	66%	No	Age, BMI, season, socio-economic status
Maghbooli (2007) (23)	Iran	Cross-sectional	579	52	Carpenter & Coustan	16.5 (10.4)	22.9 (18.3)	70.6%[3]	Yes	Age, BMI
Soheilykha h[4] (2010) (25)	Iran	Case-control	165	54	Carpenter & Coustan	23.7 (20.3)	31.7 (35.2)	78.4%	Yes	None
Makgoba (2011) (24)	UK	Case-control	248	90	WHO	47.2 (26.7)	47.6 (26.7)	58.8%	No	Age, BMI, season, ethnicity, previous GDM, family history of T2DM
Baker (2011) (27)	US	Nested-case-control	180	60	NDDG	97.0 (29.0)	86.0 (22.0)	7.2%	No	Age, BMI, season, gestational age

ADPS, Australasian Diabetes in Pregnancy Society criteria; NDDG, National Diabetes Data Group; T2DM, type 2 diabetes mellitus.
[1] Year of publication, [2] Defined by serum 25(OH)D < 50nmol/l, [3] Significant difference in serum 25(OH)D between GDM and NTG, [4] Defined by serum 25(OH)D < 25 nmol/l,
[5] The serum 25(OH)D is given in median and interquartile range

Description of the studies

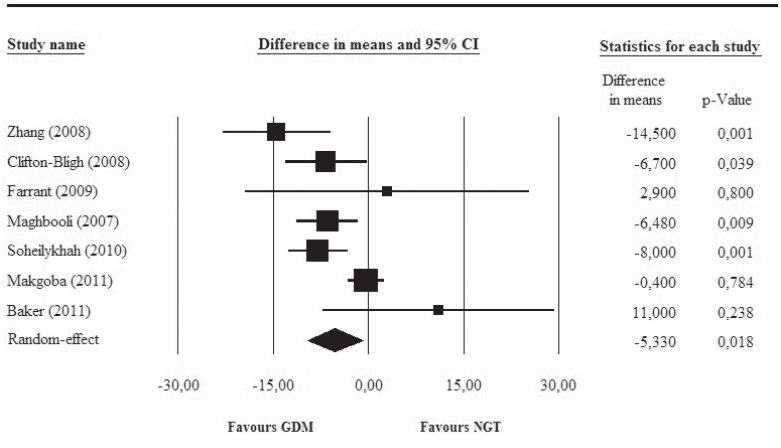
Seven observational studies representing a total of 2146 participants of whom 433 (20%) were diagnosed with GDM were included.

The studies were conducted in different countries and the participants consisted of various ethnicities. Three studies had a cross-sectional, two a case-control and two a nested case-control design. A summary of the studies is provided in table 1. Most studies collected the blood sample in the third trimester. Four studies found a high incidence (>50%) of maternal vitamin D deficiency during pregnancy, defined as 25(OH)D < 50 nmol/l. Five different criteria were used to diagnose GDM. Different assays were used measuring serum 25(OH)D.

Association of maternal vitamin D and GDM

Analysis of all studies demonstrates a wide variety between the mean serum 25(OH)D in women with GDM, ranging from 16.5 nmol/l in an Iranian study of Maghbooli et al. to 97.0 nmol/l found by Baker et al. in the United States. Four studies (21,23,25,26) found a significant difference in maternal serum 25(OH)D between women with GDM and NGT. Comparing the mean differences of all studies, using a random-effect meta-analysis model, a statistical significant difference in serum 25(OH)D of -5.33 nmol/l (95% CI -9.7 – -0.9; p=0.018) is demonstrated in detriment of gestational diabetes, meaning that woman with GDM has a significant lower vitamin D level. This difference shows statistical significant heterogeneity among studies (I2 = 69%, p=0.001) (Fig. 1). However, there was no evidence of publication bias (p=0.65).

Figure. 1. Meta-analysis of the association between maternal serum 25(OH)D and GDM.

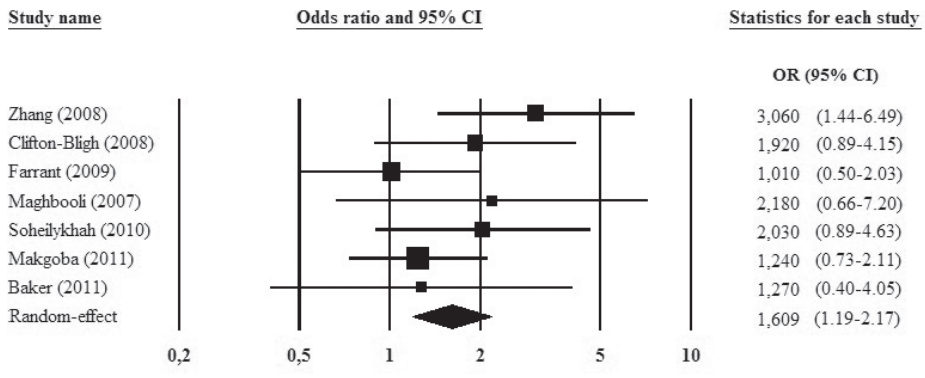


In the GDM-group 214 women (49%) had a serum 25(OH)D below 50 nmol/l and were defined as vitamin D deficient. Based on a random-effect model meta-analysis the combined OR for vitamin D deficiency associated with the diagnosis of GDM was 1.61 (95% CI 1.19 – 2.17; p=0.002) (Fig. 2.). No statistical significant heterogeneity was observed among the studies (I2 = 6%, p=0.38).

Obesity and high maternal age are risk factors for both vitamin D deficiency as GDM and thus potential confounders of the association between vitamin D deficiency and the incidence of GDM. When

we restricted the analysis to the six studies were adjustments for BMI and maternal age were made (21-24,26,27), the association remained significant: the combined OR was 1.57 (95% CI 1.11-2.22; test for heterogeneity I2 = 17%, p=0.011).
Three of these six studies adjusted the results, besides BMI and maternal age, also for ethnicity as a confounding factor (21,24,26). The combined OR of these studies, including 663 women (228 GDM), was 1.84 (95% CI 1.07-3.15; test for heterogeneity I2 = 47%, p=0.03).

Figure 2. Meta-analysis of the association between vitamin D deficiency and GDM



Two Iranian studies compared the maternal vitamin D status in women with an impaired glucose tolerance (IGT) and NGT (23,25). Both demonstrated a significant difference of serum 25(OH)D between IGT and NGT (median 25(OH)D: 16.2 versus 31.7 nmol/l, p=0.001 (25) and mean 25(OH)D: 18.9 versus 23.0 nmol/l p=0.013 (23), both measured in the 3rd trimester of pregnancy).

DISCUSSION

This systematic review and meta-analysis indicates that maternal vitamin D status is associated with GDM. A random-effect model meta-analysis demonstrates a significant association of vitamin D deficiency and the incidence of GDM. Women with GDM appear to have a significant lower serum 25(OH)D than women with NGT. However, it is unclear whether the association between vitamin D level and GDM is causal. In this respect, several important points should be considered.
First, due to the observational character of the reviewed studies, confounding cannot be excluded as a potential explanation for the observed association between vitamin D deficiency and GDM. As vitamin D status may be regarded as a marker of good health, possible confounders may be maternal age, BMI and physical activity. Indeed, older maternal age, high BMI and less physical activity were all inversely correlated with GDM (29,30). When the analysis was restricted to studies adjusted for maternal age, BMI and ethnicity the association between vitamin D status and GDM remained statistically significant (OR 1.84). However, these adjustments were only done in three studies (21,24,26).
Second, selection bias may also have contributed to the observed association between vitamin D status and GDM as the studies used different methods and criteria for the diagnosis of GDM which

could have influenced the final results (31,32). Diagnostic tests vary in the glucose load to be used, the timing and the type of blood sampling. The 100 g, 3 hour OGTT has been the gold standard for diagnosing GDM in the United States recommended by the ADA (3). The WHO recommends a 2 hour 75 g OGTT with a diagnostic cut off value of 7.8 mmol/l 2 hours after glucose load. The criteria in these guidelines are not uniform in screening for GDM. Recently the HAPO study, a large scale (25.000 pregnant women) multinational epidemiologic study, demonstrated that the risk of adverse maternal, fetal and neonatal outcomes continuously increased as a function of maternal glycaemia at 24-28 weeks, even within ranges previously considered normal for pregnancy. The HAPO study group recommended that all women without diabetes should have a 75 g OGTT at 24-28 weeks of gestation (4,7). Because of the different diagnostic criteria combined with the observational study design, it remains unclear whether the observed association is influenced by selection bias. Additional concerns are that serum 25(OH)D levels were measured in different trimesters of pregnancy and that different techniques are used. However, each study did use only one 25(OH)D assay, meaning that the relative differences between both groups remains in every study.

Inherent to any meta-analysis is the possibility of publication bias, which means that small studies with null results tend not to be published. However we found no evidence of publication bias in this meta-analysis.

Alarming is the high incidence of vitamin D deficiency in pregnancy. Four studies found an incidence of vitamin D deficiency in pregnancy above 50% (22-25). Two studies found a sufficient mean serum 25(OH)D (26,27), both conducted in the US whereas milk products are fortified with vitamin D which may explain the high vitamin D levels.

The mechanism behind the observed association between vitamin D and GDM is not well known. Alvarez et al. have summarised potential influences of vitamin D related to glucose metabolism (9): 1) the direct action of vitamin D on the pancreatic ß -cell function which occurs through the expression of vitamin D receptor as well as the enzyme 25-hydroxyvitamin D-1-α-hydroxylase in the pancreatic ß –cells; 2) the influence of vitamin D on insulin resistance through regulation of intracellular calcium which influences the glucose transport in target tissues and 3) the effect of vitamin D on systemic inflammation associated with insulin resistance in diabetes mellitus.

It remains unclear whether measurement of vitamin D levels during pregnancy should be recommended and vitamin D administered when deficiency is found. Even the definition of vitamin D deficiency is still not uniform worldwide. Further prospective clinical trials are required to evaluate the association between vitamin D deficiency and the diagnosis of GDM.

CONCLUSION

Maternal vitamin D appears to be associated with the incidence of GDM. We found a statistical significant association between maternal vitamin D deficiency and the incidence of gestational diabetes. A significant difference of 5.33 nmol/l in serum 25(OH)D was demonstrated in detriment of the participants with GDM. Due to the observational study design and the heterogeneity between the studies, the results remain difficult to interpret. Considering its safety, low costs and possible other beneficial effects, it could lead to structural measurement and use of vitamin D in pregnancy. However, clinical trials are necessary to resolve the uncertainties about the association between vitamin D deficiency and the glucose homeostasis in pregnant women.

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PART II

PCOS

CHAPTER 2

The role of vitamin D in metabolic disturbances in polycystic ovary syndrome (PCOS): a systematic review

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ABSTRACT

Background

Metabolic disturbances, in particular insulin resistance and dyslipidemia, is common in women suffering from PCOS. Evidence is accumulating that vitamin D status may contribute to the development of metabolic disturbances in PCOS.

Methods

The aim of this study was to provide a systematic review addressing the association between vitamin D status, vitamin D receptor polymorphisms and/or polymorphisms related to vitamin D metabolism, and metabolic disturbances in women with PCOS.

Design and Methods: A systematic search of electronic databases was performed up to January 2013 for observational studies and clinical trials in women suffering from PCOS with outcome measures that were related to vitamin D status. We conducted univariate and multivariate regression analyses of the weighted means to give insight in the association between vitamin D, BMI and insulin resistance based on existing literature.

Results

We found 29 eligible trials with inconsistence in their results. One well-designed randomised controlled trial has been performed until now. Univariate regression analyses of the weighted means revealed vitamin D as an significant and independent predictor for insulin resistance in both PCOS and control women. The significance disappeared after adjustment for BMI in PCOS women.

Conclusions

Current evidence suggests an inverse association between vitamin D status and metabolic disturbances in PCOS. Due to the heterogeneity of the studies it is hard to give a definite conclusion. The causal relationship between vitamin D status and metabolic disturbances in PCOS remains to be determined in well-designed placebo-controlled randomised clinical trials.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age, with a prevalence of 6-10% in the general population. PCOS is characterized by 1) ovulatory dysfunction resulting in oligo- and/or anovulation, 2) hyperandrogenism and/or hirsutism and 3) the presence of polycystic ovarian morphology (1). PCOS is by far the most common cause of anovulatory infertility, and has been associated with insulin resistance, hyperinsulinemia, dyslipidemia and central obesity (2-4), which are all risk factors for metabolic syndrome, type 2 diabetes mellitus and cardiovascular disease. Metabolic disturbances are common in women suffering from PCOS: 30-40% have impaired glucose tolerance and insulin resistance with compensatory hyperinsulinemia, and as many as 10% will have type 2 diabetes mellitus by their fourth decade (5-7). A recent meta-analysis revealed that dyslipidemia is more frequent in PCOS than in controls: women with PCOS had a higher LDL-cholesterol and non-HDL-cholesterol, regardless of BMI (4).
Current evidence suggests that insulin resistance has a central role in the pathogenesis of PCOS, contributing to both metabolic and reproductive disturbances (3).
Many studies have been conducted to clarify the mechanism of metabolic disturbances, in particular insulin resistance, in women affected by PCOS. In part, insulin resistance might be due to obesity. However, a substantial number of lean women affected by PCOS have insulin resistance as well, independent of obesity (8,9). Recently vitamin D deficiency has been proposed as the possible missing link between insulin resistance and PCOS. This assumption is supported by the finding that the active vitamin D-vitamin D receptor complex regulates over 300 genes, including genes that are important for glucose and lipid metabolism as well as blood pressure regulation (10). Moreover, poor vitamin D status and insulin resistance in patients with type 2 diabetes mellitus are associated (11-15). Still, it remains uncertain whether vitamin D and insulin resistance are causally interrelated or that they constitute two independent characteristics in women with PCOS. A recently published review performed by Thomson et al. about the role of vitamin D in the aetiology and management of PCOS suggests that there is an association between vitamin D status and hormonal and metabolic dysfunctions in PCOS (16). However literature about the association between vitamin D status and metabolic and hormonal disorders in women suffering from PCOS is scarce and has provided the scientific community with conflicting results. We therefore performed this systematic review to examine: 1) the association between vitamin D status and metabolic disturbances and/or endocrine parameters, in women with PCOS; 2) the effect of vitamin D supplementation on metabolic disturbances and/or endocrine parameters in PCOS, and 3) the influence of vitamin D receptor polymorphisms in women with PCOS.

MATERIALS AND METHODS

Search strategy

Two authors independently performed a formal computer-assisted search for observational studies and clinical trials that investigated the association between vitamin D status or vitamin D receptor polymorphisms and endocrine or metabolic parameters in PCOS women. This search was confined to the MEDLINE database, Embase and The Cochrane Library and included English-language literature

up to January 2013. The following search terms were used: polycystic ovary syndrome, polycystic ovaries, vitamin D, cholecalciferol, 25- hydroxyvitamin D (25(OH)D), vitamin D deficiency, vitamin D receptor, vitamin D receptor polymorphisms, and related terms. The detailed search strategy is displayed in Appendix 1.

Outcome of interest and criteria for inclusion

The primary endpoint for this systematic review was to examine the role of vitamin D in metabolic disturbances in women affected by PCOS. Studies that met the following criteria were included in this systematic review: 1) studies that included women with PCOS, with or without a control group, and 2) the outcome of interest included vitamin D status or vitamin D receptor polymorphisms or polymorphisms related to vitamin D metabolism, and their correlation with metabolic and/or endocrine parameters. We excluded letters, abstracts, and conference proceedings that were not published in fully peer reviewed journals. For study inclusion, PCOS was defined by the presence of a combination of oligo- or anovulation, PCO morphology, and hyperandrogenism, according to the National Institutes of Health (NIH) criteria or the Rotterdam-criteria (17).

Data extraction

The following data were extracted (in case they were available): author, study design, publication year, study location, sample size, criteria used for the diagnosis of PCOS, inclusion and exclusion criteria for cases and controls, characteristics of the study population, anthropometric parameters, metabolic parameters, endocrine parameters, serum 25(OH)D, method used for measurement of serum 25(OH)D, insulin resistance (defined as homeostasis model of assessment – insulin resistance (HOMA-IR)). To avoid multiple-publication bias, we excluded publications with overlapping patient populations in the regression models.

Data analysis

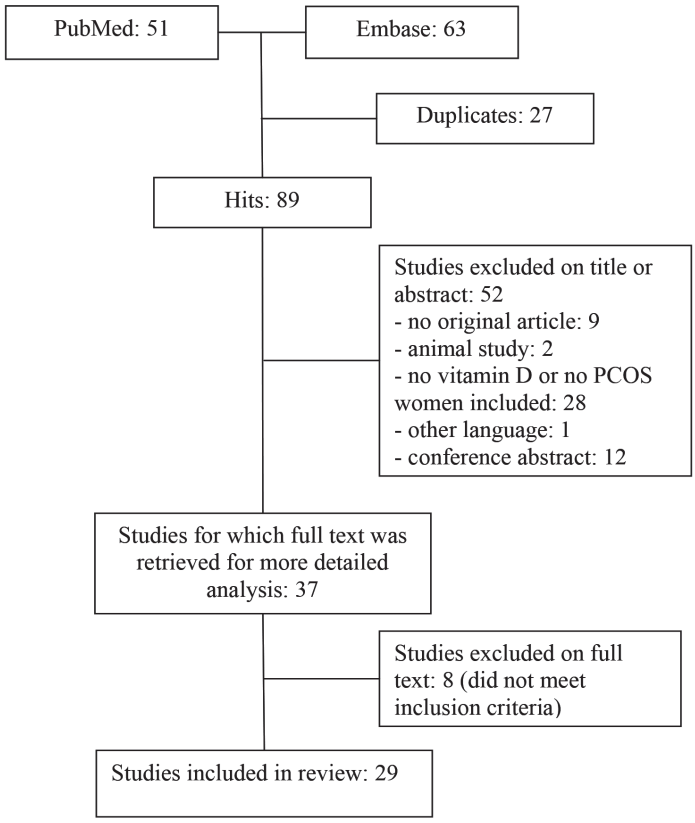
Statistical analyses were carried out using SPSS software (version 20.0, SPSS Inc, Chicago, IL). Mean values of serum 25(OH)D, HOMA-IR and BMI were employed if available in the reviewed articles. These data were weighted by the number of participants included in the study. In case the mean values were not reported, the median was used if available. Linear regression models of the weighted means were carried out to assess the independent relationships between serum vitamin D, BMI and insulin resistance in women affected by PCOS and controls. First univariate analyses were performed and subsequently multivariate analyses with HOMA-IR as independent and serum 25(OH)D and BMI as dependent variables. A p-value < 0.05 was considered statistically significant.

RESULTS

Identified studies

The initial literature search yielded 114 hits, 25 of which were duplicates, resulting in 89 non-duplicated publications. Of these, 52 were excluded based on the title or abstract and 8 studies were excluded based on the lack of inclusion criteria (Figure 1). In total 29 studies were included in this systematic review.

Figure 1. Flow chart for systematic review



The association between vitamin D status and metabolic disturbances in PCOS in observational studies

Twelve studies had an observational design (18-29), employing a cross-sectional or case-control design, with vitamin D status as one of the preliminary outcomes. A summary of these studies is provided in table 1. The studies included premenopausal women (14-50 years) of various ethnicities, with the number of participants ranging from 45 to 400. Different stratifications were used: seven studies compared PCOS women with controls (19,20,22-24,27,28), three studies compared lean versus obese PCOS women (18,21,26) one study compared PCOS women with and without the metabolic syndrome (25), and one study solely looked at PCOS women (29). Large differences were found in the prevalence

Table 1. Summary of the observational studies included in the review

Name, year[a], ref	Location	25(OH)D - assay	Study participants	Mean BMI kg/m2		Mean HOMA-IR		Prevalence 25(OH)D deficiency[b]	Mean serum 25(OH)D nmol/l		Main result regarding vitamin D and metabolic disturbances	Adjustments
Hahn et al. 2006 (18)	Germany	Immuno-assay	PCOS 98 Obese 32 Lean	37.1 (obese)	22.2 (lean)	5.7 (obese)	1.7 (lean)	67.5%	37.4 (obese)	53.2 (lean)	25(OH)D was inversely correlated with BMI, body fat, HOMA-IR and insulin. Positive association with QUICKI	Season
Hassan et al. 2012 (27)	Egypt	Immuno-assay	PCOS, 30 Control, 15	29.3 (PCOS)	25.8 (control)	7.9 (PCOS)	2.1 (control)		17.7 (PCOS)	79.2 (control)	25(OH)D was inversely correlated with BMI, HOMA-IR, QUICKI, fasting insulin	BMI
Li et al.[d] 2011 (19)	Scotland	LC-MS	PCOS, 25 Control, 27	30.8 (PCOS)	23.5 (control)	3.0 (PCOS)	1.4 (control)	PCOS 72%	27 (PCOS)	43 (control)	25(OH)D was inversely correlated with BMI and CRP, and positively correlated with QUICKI and HDL-C in PCOS	BMI, waist to hip ratio
Mahmoedi et al. 2010 (20)	Iran	Immuno-assay	PCOS, 85 Control, 115	27.6 (PCOS)	26.3 (control)	4.0 (PCOS)	2.9 (control)	nr	73.2 (PCOS)	48.4 (control)	PCOS had significant positive effects on serum 25(OH)D insulin and HOMA-IR	Age, BMI
Mazloomi et al. 2012 (28)	Iran	ELISA (DRG)	PCOS, 103 Control, 103	27.7 (PCOS)	26.2 (control)	2.3 (PCOS)	1.5 (control)	nr	30 (PCOS)	43.7 (control)	PCOS had a significant lower 25(OH)D and adiponectin. 25(OH)D is inversely correlated with BMI. Positive correlation with adiponectin. No association with HOMA-IR.	BMI, waist-circumference
Muscogiuri et al. 2012 (21)	Italy	Immuno-assay	PCOS 23 Obese 15 Lean	25.1 (all)		nr		37%	51.9 (obese)	75.4 (lean)	25(OH)D was inversely correlated with BMI waist-hip ratio and total fat mass. Positive association with glucose uptake during HEC.	BMI, season
Ngo et al. 2011 (22)	Australia	Immuno-assay	PCOS: 27 Control[c]: 20	26.9 (PCOS)	26.3 (control)	2.1 (PCOS)	1.4 (control)		79.3 (PCOS)	60.5 (control)	25(OH)D was positively correlated with QUICKI in PCOS group	
Panidis et al. 2005 (23)	Greece	Immuno-assay	PCOS, 291 Control, 109	25.5 (PCOS)	26.2 (control)	2.8 (PCOS)	2.6 (control)	nr	73.7 (PCOS)	53.5 (control)	25(OH)D was inversely correlated with BMI, insulin and HOMA-IR, however all were BMI-dependent	Age, BMI

Table 1. Continued

Name, year[a], ref	Location	25(OH)D - assay	Study participants	Mean BMI kg/m2		Mean HOMA-IR		Prevalence 25(OH)D deficiency[b]	Mean serum 25(OH)D nmol/l		Main result regarding vitamin D and metabolic disturbances	Adjust-ments
Patra et al. 2012 (29)	India	ELISA	PCOS, 60	27.1		5.5			52.3		25(OH)D was inversely correlated with HOMA-IR and fasting plasma glucose	BMI, waist to hip ratio
Savastano et al. 2011 (24)	Italy	Immuno-assay	PCOS, 90 Control[c]: 47	28.0 (PCOS)	28.5 (control)	3.1 (PCOS)	1.8 (control)	nr	32.4 (PCOS)	73.7 (control)	25(OH)D was inversely correlated with BMI, HOMA-IR, insulin, PED/PEA-15 and L/A ratio in women affected by PCOS	
Wehr et al. 2009 (25)	Austria	Immuno-assay	PCOS, 206	26.2 (all)		1.7 (all)		38.8%	43.2 (MSd)	64.4 (non-MS)	25(OH)D was inversely correlated with HOMA-IR, HOMA-β, QUICKI, and BMI, positive correlation with HDL	Age, BMI, season
Yildizhan et al.2009 (26)	Turkey	HPLC	PCOS 57 Obese 43 Lean	32.8 (obese)	22.1 (lean)	4.6	2.2	67%	31.9 (obese)	73.1 (lean)	25(OH)D was inversely correlated with BMI, WHR, HOMA-IR and TG	

[a] year of publication,
[b] vitamin D deficiency defined bij serum 25(OH)D < 50 nmol/l
[c] BMI matched control group, d median values
[d] MS defined by the National Cholesterol Education Program and the Adult Treatment Panel III

25(OH)D, 25-hydroxy vitamin D; FAI, free androgen index; HEC, hyperinsulinemic–euglycemic clamp; HOMA-IR, homeostasis model assessment–insulin resistance; L:A ratio, leptin:adiponectin ratio; LC–MS, liquid chromatography–tandem mass spectrometry; l, lean; MS, metabolic syndrome; NIH, National Institute of Health; NR, not reported; PCOS, polycystic ovary syndrome; PED/PEA15, phosphoprotein enriched in diabetes gene product; PTH, parathyroid hormone; QUICKI, quantitative insulin-sensitivity check index; SHBG, sex hormone-binding globulin.

of vitamin D deficiency (defined as: serum 25(OH)D < 50 nmol/l), varying from 37% in a study from Italy (21) to 72% in a study performed in Scotland (19).

The studies which compared serum 25(OH)D between PCOS and control women yielded conflicting results. In detail, three studies demonstrated a significantly lower serum 25(OH)D in PCOS women: 32.4 nmol/l versus 73.7 nmol/l in 90 PCOS and 47 control women (24), 30.0 nmol/l versus 43.7 nmol/l among 103 PCOS and their controls (28), and 17.7 nmol/l versus 79.2 nmol/l in 30 PCOS and 15 control women (27). In contrast, another study among 291 PCOS and 109 control women demonstrated a lower serum 25(OH)D in controls than in women affected by PCOS (53.5 nmol/l versus 73.7 nmol/l) (23).

Eleven of all included observational studies, investigated the correlation between vitamin D status and insulin resistance (18,19,21-29). Most of these studies used the HOMA-IR as an indicator of insulin resistance. Five studies reported the insulin sensitivity using the quantitative insulin-sensitivity check index (QUICKI) (18,19,22,25,27). One study by Muscoguiri et al. in 23 obese and 15 lean PCOS women, insulin resistance was evaluated by hyperinsulinemic euglycaemic clamp (HEC) method, the gold standard for the determination of insulin resistance. The authors found a positive correlation between serum 25(OH)D and glucose uptake during HEC ($r = 0.33$; $p = 0.03$). Serum 25(OH)D was inversely correlated with BMI ($r = -0.49$; $p = 0.04$), waist ($r = -0.41$; $p = 0.008$) and total fat mass ($r = -0.47$; $p = 0.02$). In a multivariate analysis the authors demonstrated that only the total fat mass was an independent predictor of serum 25(OH)D (21).

Nine studies found an inverse correlation between serum 25(OH)D and insulin resistance and/or insulin sensitivity in PCOS women (18,19,22-27,29). However, in one of these studies this correlation was BMI dependent (23) and four studies did not adjust for BMI or obesity (18,22,24,26). Two studies performed a multivariate analysis including serum 25(OH)D as explanatory variable regarding insulin resistance (22,25). Both demonstrated serum 25(OH)D as an independent predictor of insulin resistance ($p = 0.007$; $p = 0.036$). In this latter study 206 PCOS women were stratified by hypovitaminosis D (< 75 nmol/l, n = 150) and vitamin D sufficiency (≥ 75 nmol/l, n = 56). The authors demonstrated that the hypovitaminosis D group had a significantly higher HOMA-IR (1.96 versus 1.11, $p = 0.002$) than the vitamin D sufficient group. However, after a subsequent analysis in lean and obese PCOS women stratified by hypovitaminosis D and sufficient vitamin D, and no significant difference was found in HOMA-IR (25).

The studies which compared serum 25(OH)D between obese and lean women suffering from PCOS all observed a significantly lower serum 25(OH)D in obese PCOS women. (18,21,23,24,26).

The effect of vitamin D supplementation in women with PCOS

Ten intervention trials were performed and published until our search in January 2013 (30-40). Among those, four had a randomised controlled trial (RCT) design (30,35,36,40), five an open labeled single arm design (32,33,37-39), and one had a case-control study design (31). One RCT performed by Aradibili et al. had a randomised placebo-controlled double blind study design in which the effect of vitamin D3 supplementation on metabolic parameters and cardiovascular risk factors was evaluated in 50 PCOS women. This trial resulted in two articles included in this review (30,34). Sathyapalan et al. examined in their RCT the effect of atorvastatin versus placebo on serum vitamin D, and subsequently studied the effect of the rise in serum vitamin D on metabolic and endocrine parameters in PCOS women (36). The third RCT carried out by Rahsidi et al. was a pilot study in which 60 infertile PCOS women were randomised into a treatment with 1) calcium and vitamin D3, 2) calcium, vitamin D3 and metformin, or 3) metformin. They assessed effects on folliculogenesis and menstrual cycle (35). Bonakdaran et al. performed a RCT among 51 women in which active vitamin D (calcitriol) was administered, compared to metformin treatment and placebo (40).

The number of participants included in the intervention trials ranged from 11 to 100, all premenopausal PCOS women. Different treatment protocols of vitamin D supplementation were used in the trials varying from 400 IU a day in (35), to a single oral dose of 300.000 IU cholecalciferol (37). The follow-up time ranged from 3 weeks to 6 months. A summary of the studies is provided in Table 2. In detail, the first study performed in 1999 by Thys-Jacobs et al. among 13 PCOS women, who were treated with ergocalciferol 50,000 IU weekly or biweekly to achieve a target serum 25(OH)D of 75-100 nmol/l, demonstrated an improvement of menstrual regularity and acne, and two women became pregnant in a follow-up period of 6 months (38). In addition, a more recent study performed in Iran

Table 2. Summary of the intervention studies included in this review

Name, year[a], ref	Location	25(OH)D - assay	n =	Study design	Intervention	Duration (months)	Main outcome	Mean 25(OH)D nmol/l before treatment	Mean 25(OH)D nmol/l after treatment	Main results
Ardabili et al.[e] 2012 (30)	Iran	Immuno-assay	50[d]	RCT	Gr. 1: vitaminD3 50.000IU/ 20days Gr. 2: placebo	2	Metabolic parameters	Gr. 1: 17.2 Gr. 2: 19.5	58.4 nr	↑ HOMA-B = HOMA-IR, insulin, glucose, QUICKI, BMI
Bonakdaran et al. 2012 (40)	Iran	Immuno-assay	51	RCT	Gr. 1: metformin 1000mg/day Gr. 2: calcitriol 0.5ug/day Gr. 3: placebo	3	Endocrine and metabolic parameters	Gr. 1: 70.4 Gr. 2: 28.5 Gr. 3: 49.7	66.6 50.9 47.4	Gr 2: ↓ PTH = BMI, BP, HOMA-IR, insulin, glucose
Firouzabadi et al. 2012 (31)	Iran	Immuno-assay	100[b]	Case-control	Gr. 1: metformin 1500mg/d Gr. 2: metformin 1500mg/d, Ca 1000mg/d, vitaminD3 50.000IU/wk	6	Reproductive and anthropometric parameters	Gr. 1: 33.8 Gr. 2: 33.0	33.8 62.0	↑ menstrual regularity, follicle maturation, pregnancy (6 versus 9) ↓ BMI, hyperandrogenism NS difference between both groups
Kotsa et al. 2009 (32)	Greece	Immuno-assay	15[c]	Single arm	Alphacalcidol 1.0 ug/d	3	Glucose metabolism measured by IVGTT	37.8	71.4	↑ insulin secretion, HDL-cholesterol ↓TG = serum glucose and insulin
Pal et al. 2012 (33)	US	Immuno-assay	12[d]	Single arm	Vitamin D2 50.000IU/month, vitamin D3 2.000IU/d, Ca 530mg/d	3	Endocrine and metabolic parameters	43.9	71.8	↓ BP, total testosterone and androstenedione = insulin resistance, insulin, glucose,
Rahimi-Ardabili et al.[e] 2012 (34)	Iran	Immuno-assay	50[d]	RCT	Gr. 1: vitaminD3 50.000IU/ 20days Gr. 2: placebo	2	Cardiovascular risk factors	Gr. 1: 17.5 Gr. 2: 19.5	57.2 nr	↓ TC, TG and VLDL, PTH = HDL, LDL, hs-CRP, APO-A1, BMI
Rashidi et al. 2009 (35)	Iran	nr	60[b]	RCT, pilot	Gr. 1: Ca 1000 mg/d, Vit D3 400IU/d Gr. 2: Ca 1000 mg/d, Vit D3 400IU/d metformin 1500 mg/d Gr. 3: metformin 1500 mg/d	Treatment: 3 Follow-up: 6	Folliculogenesis and menstrual regularity	nr	nr	↑ menstrual regularity and folliculogenesis in metformin, calcium, vitamin D group

Table 2. Continued

Name, year[a], ref	Location	25(OH)D - assay	n =	Study design	Intervention	Duration (months)	Main outcome	Mean 25(OH)D nmol/l before treatment	Mean 25(OH)D nmol/l after treatment	Main results
Sathyapalan et al. 2010 (36)	UK	LC-MS	37	RCT	Gr. 1: atorvastatin 20 mg/d Gr. 2: placebo	6	Metabolic and endocrine parameters	Statin: 45.9 control: 44.8	61.9 42.1	↑ serum 25(OH)D = HOMA-IR and serum lipids
Selimoglu et al. 2010 (37)	Turkey	Immuno-assay	11[c]	Single arm	Vitamin D3 300.000 IU single dose orally	Follow-up: 3 weeks	Endocrine parameters and glucose parameters	42.2	92.6	↓ HOMA-IR = endocrine parameters, insulin, glucose
Thys-Jacobs et al. 1999 (38)	US	Radioligand-binding assay	13	Single arm	Ca 1500 mg/d and Ergocalciferol 50.000 IU weekly or biweekly, target: 25(OH)D 75-100 nmol/l	6	Reproductive and menstrual dysfunction	28.0	nr	↑ menstrual regularity and acne, 2 women became pregnant
Wehr et al. 2011a (39)	Austria	Immuno-assay	52	Single arm	Vitamin D3 20.000 IU/wk	24 weeks	Metabolic and endocrine parameters	69.9	130.8	↑ TC, LDL and menstrual frequency ↓ fasting and stimulated glucose, C-peptide, TG, PTH, estradiol and hip circumference = HOMA-IR

↑, increase; ↓, decrease; =, unchanged

[a] *Year of publication*

[b] *Infertile PCOS women*

[c] *Insulin resistant PCOS women*

[d] *Vitamin D deficient (< 50nmol/l) PCOS women*

[e] *Overlap in study population, only one study is used in the analysis*

Gr.1, Group 1; Gr.2, Group 2; Gr.3, Group 3; 25(OH)D, 25-hydroxy vitamin D; APO-A1, apolipoprotein A1; BP, blood pressure; HOMA-B, homeostasis model assessment–b-cell function; HOMA-IR, HOMA–insulin resistance; hs-CRP, high-sensitive C-reactive protein; IVGTT, intravenous glucose tolerance test; LC–MS, liquid chromatography–tandem mass spectrometry; NR, not reported; NS, no significance; OGTT, oral glucose tolerance test; PCOS, polycystic ovary syndrome; PTH, parathyroid hormone; TC, total cholesterol; TG, triglycerides, QUICKI, quantitative insulin-sensitivity check index

including 60 infertile PCOS women, observed an improvement in menstrual regularity after 3 months supplementation of calcium 1000 mg/day and vitamin D 400 IU/day (35).
Seven intervention trials examined the effect of vitamin D supplementation on insulin resistance and other metabolic characteristics (30,32,33,36,37,39,40). The only randomised placebo controlled trial who administered vitamin D3 and examined metabolic disturbances as primary prespecified outcome, did not demonstrate a significant effect on insulin resistance after three oral doses of vitamin D3 in 2 months. The rise in serum 25(OH)D was 40 nmol/l in the vitamin D group (30). The authors did find a significant increase in insulin secretion in the vitamin D group, but compared to the placebo group this was not significant. Total cholesterol, triglycerides and VLDL were decreased in the intervention group after vitamin D supplementation, but no effect was demonstrated on BMI, LDL and HDL. The RCT performed by Bonakdaran et al. in which calcitriol was administered, also failed to find an effect on insulin resistance (40). In the calcitriol group serum 25(OH)D raised from 29 to 51 nmol/l. The HOMA-IR decreased from 4.2 to 2.7, however this was not significant.
The other intervention trials yielded conflicting results: Selimoglu et al. observed a significant decrease in HOMA-IR after a single oral dose of 300.000 UI cholecalciferol (37), whereas the others failed to find a positive effect on insulin resistance after different regimes of vitamin D replacement (32,33,36,39).

The influence of vitamin D receptor polymorphisms and polymorphisms regarding vitamin D metabolism in women with PCOS

Literature assessing vitamin D receptor (VDR) polymorphisms and/or polymorphisms regarding vitamin D metabolism in women suffering from PCOS in relation with vitamin D status and metabolic disturbances is scarce. Six genetic linkage studies examined VDR and related polymorphisms in relation to PCOS (41-46). The number of participants ranged from 56 to 690 participants. None of the studies included a replication cohort. Besides single nucleotide polymorphisms (SNP's) regarding vitamin D receptor genotype, SNP's in the insulin receptor, calcium sensor receptor, parathyroid hormone receptor, adiponectin receptor, genes involved in cholesterol synthesis (DHCR7) and hydroxylation (CYP2R1), and genes involved in vitamin D transport (GC) were examined. A summary of the studies is provided in table 3.

Table 3. Summary of the genetic linkage studies

Name, year, ref	Location	Study participants	SNPs	Main results
Mahmoedi et al. 2009 (42)	Iran	PCOS, 162 Control, 162	VDR (Bsm-I, Fok-I, Apa-I, Taq-I) polymorphisms	VDR Apa-I was associated with PCOS VDR Fok-I'FF' independent increased risk for insulin level and insulin resistance in PCOS
Wehr et al. 2011 (45)	Austria	PCOS, 545 Control, 145	VDR (Cdx2, Bsm-I, Fok-I, Apa-I, Taq-I), GC[a], DHCR7[b], CYP2R1[c] polymorphisms	In PCOS women VDR Cdx2'AA' was associated with lower fasting insulin and HOMA-IR and higher QUICKI. GC'GG' and DHCR7'GG' had a higher risk for vitamin D deficiency
Ranzjad et al. 2012 (43)	Iran	PCOS, 181 Control, 181	INSR, ADIPOQ, PTH, VDR polymorphisms (SNPs[d])	No significant difference was observed in genotype and allele frequencies between women with PCOS and controls.
Ranzjad et al. 2011 (44)	Iran	PCOS, 56	VDR (Bsm-I, Fok-I, Apa-I, Taq-I, Tru9-I), PTH, CaSR, INSR, ADIPOQ polymorphisms[e]	CaSR'TG' was associated higher HOMA-IR VDR Taq-I was associated with elevated serum LH VDR Bsm-I and ADIPOQ (rs1501299) were associated with decreased SHBG
Zadeh-Vakili et al. 2012 (46)	Iran	PCOS, 260 Control, 221	VDR (Tru9-I)	No significant difference was observed in genotype and allele frequencies between women with PCOS and controls

[a] encodes vitamin D binding globulin
[b] encodes the enzyme 7-dehydrocholesterol reductase, interacting with the synthetic pathway of vitamin D3
[c] encodes a hepatic microsomal enzyme, hypothetically involved in the 25-hydroxylation of vitamin D in the liver
[d] INSR gene (rs2059806, rs1799817), ADIPOQ gene (rs2241766, rs1501299), PTH gene (rs6256), VDR gene (rs757343)
[e] PTH (rs6256), CaSR (rs1801725), INSR (rs2059806, rs1799817) , ADIPOQ (rs2241766, rs1501299)

ADIPOQ, adiponectin; CaSR, calcium-sensing receptor; HOMA-IR, homeostasis model assessment–insulin resistance; INSR, insulin receptor; NR, not reported; PCOS, polycystic ovary syndrome; PTH, parathyroid hormone; QUICKI, quantitative insulin-sensitivity check index; SHBG, sex hormone-binding globulin; SNPs, single-nucleotide polymorphisms; VDR, vitamin D receptor.

Five studies observed the association of VDR polymorphisms and metabolic disturbances in women with PCOS (41-43,45,46).

In a cohort of 162 PCOS and 162 control women variants of VDR were associated with an increase in insulin level and insulin resistance; The VDR Apa-I genotype was associated with the prevalence of PCOS (42). A study performed by Wehr et al. (45) among 545 PCOS and 145 control women found that the VDR Cdx2'AA' polymorphism was associated with lower fasting insulin (p=0.039) and HOMA-IR (p=0.041) compared to the other polymorphisms. These authors demonstrated that variants of the GC gene, which encodes for vitamin D binding globulin, and DHRC7 gene, which encodes for the enzyme 7-dehydrocholesterol reductase interacting with the synthetic pathway of vitamin D3, were both associated with a significant higher risk for vitamin D deficiency (OR 2.53 (1.27–5.06), p=0.009, and OR 2.66 (1.08–6.55), p=0.033). Ranzjad et al. observed that the CaSR'TG' polymorphism was associated with insulin resistance (43).

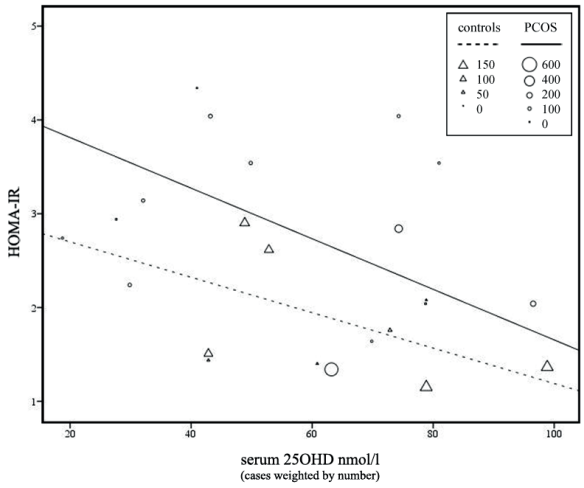
Another study of 181 Iranian PCOS women and 181 controls, examined whether different polymorphisms in VDR, adiponectin, parathyroid hormone, and insulin receptor genes were associated with PCOS. The authors observed no difference in vitamin D receptor gene polymorphisms between PCOS and control women (44). This result is in line with the study performed by Lin et al. (41) and Zadeh-Vakili et al. (46).

The correlation between vitamin D status, BMI and insulin resistance in women with PCOS

Vitamin D status and insulin resistance

In order to provide an overview of the relationship between vitamin D and insulin resistance in women affected by PCOS compared to healthy controls, we performed a regression analysis including serum 25(OH)D as independent and HOMA-IR as dependent variable. Eighteen of the 29 studies examined this correlation and reported the mean and/or median values and were therefore included in this analysis (18-20,22-24,26-30,33,37,39-41,43,45). Nine studies also reported these values in controls (19,20,22-24,27,28,41,45). The data were weighted by the number of participants in each study, resulting in a total of 1893 PCOS and 717 control women. The overall mean of serum 25(OH)D was 61.2 nmol/l in PCOS and 67.1 nmol/l in controls. The overall mean of the HOMA-IR was 2.71 in PCOS and 1.8 in controls. Univariate regression analysis revealed that an increase in serum 25(OH)D was significantly associated with a decrease in HOMA-IR in both PCOS (B1 = -0.027; CI -0.030 - -0.024; p= < 0.001) and in controls (B1 = -0.019; CI -0.021 - -0.017; p= < 0.001) (Figure 2). The results indicate that every 10 nmol/l increase in serum 25(OH)D, decreases the HOMA-IR with 0.27 in PCOS and 0.19 in controls. On multivariate regression analysis with serum 25(OH)D and BMI as independent variables, serum vitamin D was no longer an independent predictor of insulin resistance in PCOS. In controls the result remained significant.

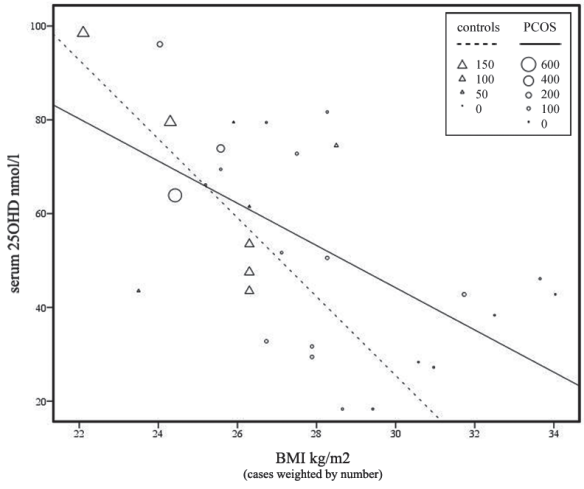
Figure 2. Association between vitamin D status and insulin resistance in PCOS and control women



Vitamin D status and BMI

Data on the association between anthropometric parameters and serum 25(OH)D were provided in 23 of the 29 reviewed studies (18-24,26-34,36-39,41,43,45), and nine studies reported these data also in control women (19,20,22-24,27,28,41,45). After regression analyses of the weighted means including a total of 2045 PCOS women and 717 controls, BMI appeared to be an independent predictor of serum 25(OH)D in both PCOS and controls (B1 = -4.5; $p < 0.001$; B1 = -8.4, $p < 0.001$) (Figure 3). The overall mean of the BMI in these studies was 26.6 kg/m2 in PCOS and 25.1 kg/m2 in controls.

Figure 3. Association between BMI and vitamin D status in PCOS and control women



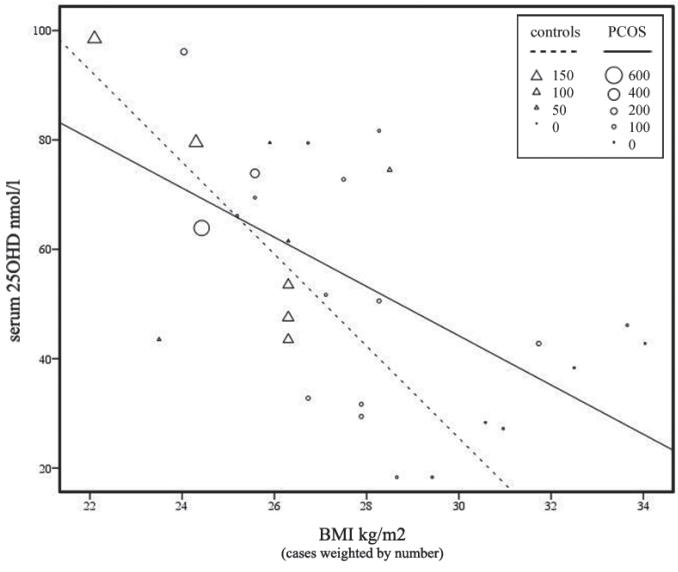
BMI and insulin resistance

Nineteen of the included studies investigated the correlation between BMI and HOMA-IR in PCOS women (18-20,22-30,33,37,39,41,43,45,46), and nine in controls (19,20,22-24,27,28,41,45). All studies revealed a significant association between BMI and HOMA-IR. A total of 2311 PCOS women and 717 controls participated in these studies. Multivariate regression analyses with serum 25(OH)D and BMI as independent variables and HOMA-IR as dependent variable, revealed that BMI was an independent predictor for HOMA-IR in PCOS and control women (B1 = 0.41; $p < 0.001$; B1 = 0.10; $p < 0.001$ respectively) (Figure 4).

DISCUSSION

The present systematic review was performed to examine the role of vitamin D status and polymorphisms on metabolic disturbances, in particular insulin resistance, in women suffering from PCOS. Inconsistent results were observed in the studies analyzing the relation of vitamin D status and metabolic disturbances in PCOS women. These conflicting findings might be due to the small sample sizes, the lack of adjustments for confounders, different definitions for PCOS, different assays used for serum 25(OH)D measurement, the time of intervention, different amounts of vitamin D supplementation used in the intervention trials, and the lack of an optimal serum 25(OH)D level in the general population.

Figure 4. Association between BMI and insulin resistance in PCOS and control women



Only one well-designed randomised placebo controlled trial has been performed until now, demonstrating no effect of vitamin D3 supplementation on insulin resistance (30). Maybe the small sample size and the relatively short follow-up time could account for the lack of an effect. Another RCT among 51 PCOS women performed by (40) did show a decrease in HOMA-IR after 3 months of calcitriol supplementation. However, likely due to the small sample size this decrease was not significant.

Currently, the relation between obesity, vitamin D status and insulin resistance has not been clarified. There is an ongoing discussion on whether vitamin D deficiency and insulin resistance are causally interrelated or that they are both BMI-dependent. In this review we provided an overview of the correlation between these variables by performing a linear regression analysis. Univariate linear regression analysis revealed both serum vitamin D and BMI as independent predictors for insulin resistance. However, in the multivariate analysis serum 25(OH)D was no longer an independent predictor for insulin resistance in PCOS women. A strong independent relationship was seen between BMI as explanatory and serum 25(OH)D as outcome variable. A limitation of the data analyses conducted in our review is that we used the weighted means of the reviewed studies instead of the original data. Another important note is the different methodology used for vitamin D measurement in the reviewed studies. At the moment, many discussion is going on which test is the most reliable for vitamin D measurement. Due to the increasing recognition of the high prevalence and diverse consequences of vitamin D deficiency, a massive rise in vitamin D testing is observed worldwide. A recent trial demonstrated the limitation of different assays (47). Both could have influenced the results.

The demonstrated inverse relation between BMI and vitamin D status has been established in earlier reports (48,49). It has been shown that in obese individuals a higher proportion of vitamin D, which is fat soluble, is sequestered in adipose tissue and thereby lowers the bioavailability of vitamin D. Alternatively, obese individuals tend to spend less time outdoors exposed to sunlight, leading to insufficient biosynthesis of vitamin D generated through the skin. Moreover, obesity is highly prevalent in women affected by PCOS, with the highest prevalence reported in studies conducted in the United States and Australia, with 61% to 76% of women with PCOS considered obese, and 85 % considered overweight or obese (1). In addition, obesity has a strong correlation with insulin resistance and type 2 diabetes mellitus (50). This association is in line with our results. Despite the strong association between obesity and insulin resistance, multiple studies demonstrate that women affected by PCOS have more severe insulin resistance than expected on the basis of their body weight (51-53). In this regard vitamin D status has more than once been designated as a possible contributing factor for insulin resistance in women affected by PCOS. This hypothesis is supported by animal studies with vitamin D receptor null mice and in human models (10). The largest cross-sectional study (NHANES data) demonstrated an inverse correlation between serum 25(OH) D concentrations and diabetes prevalence. After multivariate adjustment, serum 25(OH)D concentrations were negatively correlated with insulin resistance (15). Pittas et al. confirmed these results in a three year follow-up study while participants with impaired fasting glucose who used cholecalciferol plus calcium supplementation, had a smaller increase in insulin resistance compared to controls who received a placebo (54).

The potential mechanisms by which vitamin D can affect glucose metabolism could be the result of a direct and indirect action of serum 25(OH)D: 1) direct stimulation of insulin release through the expression of VDR as well as the enzyme 1α-OHase in the pancreatic ß-cells; 2) through the 1,25(OH)2D - VDR complex binding to the vitamin D response element of the insulin receptor at tissue level and thereby enhancement of insulin responsiveness for glucose transport; 3) suppression of the release of proinflammatory cytokines that are believed to mediate insulin resistance (11). The latter mechanism is supported by studies showing an association between low serum 25(OH)D and increased CRP levels (25,55). Indirectly vitamin D may play a role through its influence on the extracellular and intracellular calcium regulation which is essential in mediating glucose transport in target tissues.

Nine of the reviewed studies compared PCOS women with controls. An important observation is that vitamin D status did not markedly differ between both groups (overall mean 61.2 nmol/l in PCOS versus 67.1 nmol/l in controls). It is remarkable that despite a slight difference in mean BMI in both groups, the HOMA-IR differed considerably, suggesting that PCOS per se is a risk factor for the development of insulin resistance. This is in line with some, but not all earlier results (8,9,53). In contrast, PCOS per se does not seem to be a risk factor for vitamin D defiency. The studies performed by Ngo et al, Panadis et al. and Mahmoedi et al. even found a higher serum 25(OH)D in PCOS women than in controls. However, these data were not adjusted for season of measurement and diet.

Another important note is that the inconsistent results in the reviewed articles may be explained by the hypothesis that vitamin D supplementation may only be effective on insulin resistance in case a significant insulin resistance exists. As the majority of the reviewed studies did not include diabetic PCOS women, this could be a possible explanation why an effect of vitamin D supplementation on insulin resistance was not evident in all studies. However, the RCT performed by Aradibli et al. did not show an effect of vitamin D supplementation despite a HOMA-IR of 3.17 in PCOS women.

Another hypothesis for the lack of effect may be when baseline serum 25(OH)D is already sufficient. It seems plausible that in case of an adequate vitamin D status, no effect can be seen after vitamin D supplementation. In this respect, it should be noted that until now, the optimal serum 25(OH)D level is not clear. Although there is no consensus on optimal serum levels of 25(OH)D, frequently vitamin D deficiency is defined by a serum 25(OH)D level less than 25 nmol/l (10 ng/ml). Serum 25(OH)D levels between 25 and 50 nmol/l (10-20 ng/ml) are considered as vitamin D insufficiency, and serum 25(OH)D > 50 nmol/l are considered as sufficient. Recently, the Institute of Medicine, reviewing all the evidence, has set the required level of serum 25(OH)D at 50 nmol/l (56), but expert opinion remain divided. In addition, no optimal treatment regime is established yet for the treatment of vitamin D deficiency in different populations. In this aspect, this may influenced the study results whereas all included intervention studies used different amounts and duration of vitamin D supplementation.

In conclusion, the relation between vitamin D status and metabolic disturbances in women with PCOS remains uncertain. Current literature suggests an inverse correlation between vitamin D status and insulin resistance in women with PCOS. However, due to the heterogeneity of the studies, it is hard to give an informed conclusion. Future research with adequately powered randomised placebo-controlled double-blind studies of vitamin D supplementation in women affected by PCOS is warranted. Until that time screening women who are at risk for vitamin D deficiency and supplementation with vitamin D could be considered.

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CHAPTER 3

Vitamin D and metabolic disturbances in polycystic ovary syndrome (PCOS): a cross-sectional controlled study

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ABSTRACT

Background

Women with polycystic ovary syndrome (PCOS) commonly have metabolic disturbances, in particular, insulin resistance and dyslipidemia, independent from body mass index (BMI). Evidence is accumulating that the vitamin D status plays a role in the development of these metabolic disturbances.

Methods

Serum 25-hydroxyvitamin D (25(OH)D) concentrations were measured using the LC-MS/MS method in 639 women with PCOS and 449 control women. Metabolic profile measurements were performed in PCOS women. Serum 25(OH)D was stratified into a severe deficient <25 nmol/l, insufficient 25-50 nmol/l, moderate 50-75 nmol/l and adequate >75 nmol/l status. Multivariable linear regression analysis was used to study the associations between serum 25(OH)D and metabolic parameters.

Results

Serum 25(OH)D was significantly lower in PCOS women compared to controls (mean 25(OH)D of 49.0 nmol/l versus 64.5 nmol/l). Multivariable linear regression analysis adjusted for BMI, ethnicity, waist to hip ratio, cholesterol/HDL ratio, and season resulted in a significant difference in homeostasis model assessment (HOMA-IR) between the highest and lowest vitamin D groups (β= 0.76; 95% CI: 0.63 – 0.91; $p = 0.003$). A significant adjusted association was seen between serum 25(OH)D and HDL-cholesterol (β= 0.20; 95% CI: 0.05 – 0.60, $p < 0.01$) and apolipoprotein A1 (β= 26.2; 95% CI: 7.5 – 45.0, $p < 0.01$) with higher levels in the highest compared to the lowest vitamin D group.

Conclusions

Women with PCOS had a significantly lower serum 25(OH)D level compared to controls. A compromised vitamin D status in PCOS women is associated with a higher HOMA-IR and an unfavourable lipid profile. Large randomised controlled trials are necessary to explore the causality of this linkage.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common endocrinopathy in women of reproductive age, with a prevalence up to 10% depending on which diagnostic criteria are used (1). It is characterized by ovulatory dysfunction, hyperandrogenism and/or polycystic ovarian morphology (2). Metabolic disturbances are present in a majority of the women suffering from PCOS, i.e. 30-40% have impaired glucose tolerance and insulin resistance with compensatory hyperinsulinemia, and as many as 10% will develop type 2 diabetes mellitus during their fourth decade (3). Adipose tissue dysfunction has been implicated as a contributor to insulin resistance in women with PCOS. However, a substantial number of lean women affected by PCOS have insulin resistance as well, independent of obesity (4,5).

Vitamin D deficiency has been proposed as the possible missing link between insulin resistance and PCOS (6). Vitamin D is a fat-soluble vitamin that is synthesized endogenously through sunlight-induced photochemical conversion of cholesterol to 7-dehydrocholesterol in the skin or obtained from the diet. Subsequently vitamin D undergoes a hydroxylation twice, first vitamin D is transported to the liver where it is rapidly hydroxylated by 25-hydroxylase into 25-hydroxyvitamin D (25(OH) D). The second hydroxylation occurs in the kidney and is catalyzed by 1 alpha-hydroxylase to form 1,25-dihydroxyvitamin D (1,25(OH)2D), the active metabolite of vitamin D. Circulating 1,25(OH)2D binds to vitamin D receptors (VDR) to initiate its effect. Serum 25(OH)D is the major circulating form of vitamin D used as the main indicator of vitamin D status. Its half-life is 2-3 weeks compared to only 4-6 hours for 1,25(OH)2D (7). Since many years, a role for vitamin D has been suggested outside the calcium and bone homeostasis, due to the identification of the VDR, and the enzyme 1 alpha-hydroxylase in many more tissues, including the pancreatic beta-cells, immune cells, (8) and reproductive organs in both genders (6). Moreover, this assumption is supported by the finding that the active vitamin D-vitamin D receptor complex regulates over 300 genes, including genes that are important for glucose and lipid metabolism as well gonadal function (9).

Women with PCOS seem to have a higher risk for vitamin D deficiency (10). Associations of vitamin D status and metabolic disturbances has been investigated in a large number of studies, which are summarised in a systematic review. However, due to the heterogeneity of the studies, small sample sizes, and small number of studies no firm conclusion could be drawn (11). In addition, the association between vitamin D and insulin resistance has been studied thoroughly in patients with diabetes, resulting in convincing evidence from observational studies that vitamin D deficiency is inversely related to the degree of insulin resistance (12).

So far, it is not clear whether vitamin D deficiency per se is a risk factor PCOS, and whether vitamin D status is associated with metabolic disturbances in PCOS women. Therefore, we performed a case-control study to examine vitamin D status in PCOS women and controls. We also did an analysis in this large PCOS cohort to extend the current knowledge about the association between vitamin D and metabolic disturbances in PCOS women.

METHODS

Subjects

This retrospective comparison study included 639 PCOS women from the Rotterdam PCOS cohort and 449 control women from the HAVEN cohort.

PCOS women who were screened for anovulatory infertility at the outpatient clinic of the Erasmus Medical Centre Rotterdam, and subsequently diagnosed with PCOS according to the Rotterdam criteria were eligible for inclusion in this study. Trained professionals performed the screening procedure according to a standardized protocol which has been previously described in greater detail (13). First, a thorough general medical, reproductive and family history was taken, including self-reported ethnicity. Second, anthropometric measurements were performed, including height, weight, body mass index (BMI), waist circumference measured midway between the arcus costae and anterior superior iliac spine, hip circumference measured at the level of the anterior superior iliac spine, systolic and diastolic blood pressure, and the level of hirsutism measured with the use of the Ferriman-Gallwey (FG) score. Subsequently, a systematic transvaginal ultrasonography was performed to assess ovarian volume, endometrial thickness, and the total number of antral follicles measuring 2–10 mm. Finally, an extensive metabolic and endocrine profile was assessed.

PCOS was diagnosed according to the Rotterdam criteria; i.e., requiring the presence of at least two out of the three following criteria: ovulatory dysfunction resulting in oligomenorrhea (mean bleeding interval 35–182 days in last six menstrual bleeds) and/or amenorrhea (absence of menstrual bleeding for >182 days), hyperandrogenism and/or hirsutism, and the presence of polycystic ovarian morphology (PCOM) (14). Clinical and biochemical hyperandrogenism was defined as an FG score >8, and/or a free androgen index (FAI: [(T/SHBG) x 100] >4.5) (16). We defined PCOM as ≥12 follicles measuring 2–10 mm in diameter in at least one ovary, or an increased ovarian volume (>10 cm3) (15). In all women, basal levels of luteinizing hormone (LH), follicle stimulating hormone (FSH), and estradiol (E2) were assessed to rule out hypo- and hypergonadotropic hypogonadism. In addition, to screen for hyperprolactinemia and thyroid dysfunction prolactin level and thyroid stimulating hormone (TSH) were measured. Women were excluded from inclusion if the blood withdrawal was not in a fasting state.

Controls were recruited at child health centers in the same geographic area as the study population. The control group consisted of a random group of mothers who had a spontaneous and uneventful pregnancy and delivered a healthy child without congenital malformations at the age of 15 months. They were all assessed, at 15 – 17 months after delivery at the hospital using a standardized study protocol. Controls have been described previously (16,17). Information on general health and cycle history was gathered by questionnaire. This study was approved by the Central Committee on Research Involving Human Subjects (the Hague, The Netherlands) and the institutional review board at the Erasmus Medical Center. Informed consent was obtained from all participants.

Assessment of metabolic profile and vitamin D

Regarding the cardiovascular profile, information was collected on BMI and systolic and diastolic blood pressure in both PCOS and control women. In PCOS women additionally waist circumference, fasting glucose and insulin, and lipid profile (i.e. total cholesterol, triglycerides, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol) was measured. Insulin resistance was as

sessed using the homeostasis model assessment (HOMA-IR: [fasting glucose (mmol/L) x fasting insulin [mU/L]]/22.5). Metabolic syndrome was defined according the National Cholesterol Education Program (NCEP) ADP III criteria (18).

Venous blood samples were drawn at examination and stored at –80°C after centrifugation at 3000 rpm for 10 min at 20 °C. Serum 25(OH)D was measured using the liquid chromatography tandem-mass spectrometry (LC-MS/MS) in November 2013 (PCOS women) and September 2015 (control women). Liquid-liquid extraction (hexane) of the samples and analysis, carried out with a Waters ACQUITY UPLC system couple to a Waters Xevo TQ mass spectrometer, were performed as described elsewhere (19). Intra-assay CV was <6% and intra-assay CV was < 9% at the concentration of 16 and 80 nmol/L. The measurement of serum 25(OH)D was carried out by the central chemical laboratory of the Medical Centre Alkmaar, the Netherlands. This laboratory is a (ISO-15189) certified laboratory.

Endocrine evaluation included serum levels of gonadotropic hormones (LH, FSH) and estradiol (E2), androgens, testosterone, androstenedione (AD), dehydroepiandrosterone (DHEA), and dehydroepiandrosterone sulfate (DHEAS), progesterone and 17-hydroxyprogesterone (17-OH-Pg), sex hormone binding globulin (SHBG), fasting glucose and insulin, TSH, and prolactin. LH, FSH, TSH, prolactin, and insulin were measured by immunoradiometric assay. Testosterone, AD, E2, SHBG, progesterone, 17-OH-Pg, DHEA, and DHEAS were determined by RIAs. Intra-assay and inter-assay coefficients of variation were less than 5% and less than 15% for LH, less than 3% and less than 8% for FSH, less than 3% and less than 5% for T, less than 8% and less than 11% for AD, less than 5% and less than 7% for E2, and less than 4% and less than 5% for SHBG, respectively.

Statistical analyses

Data are presented as means ± standard deviation if normally distributed, and otherwise as median and interquartile range in case of skewed distribution. For the main analyses we compared PCOS women with controls regarding vitamin D status. For this, we used a linear regression analysis with serum 25(OH)D as dependent variable and PCOS versus control as independent variable. We adjusted for season in which blood was collected, BMI, age, blood pressure, and ethnicity as potential confounders or effect modifiers.

Subanalyses were performed to assess the association between vitamin D status and metabolic outcome parameters in women with PCOS, by applying multivariable linear regression analyses. As the relationship between serum 25(OH)D and the outcome measures was not linear, serum 25(OH)D was divided in four groups by the widely used cut-off values of vitamin D: 1) serum 25(OH)D: < 25 nmol/l, 2) serum 25(OH)D: 25-50 nmol/l, 3) serum 25(OH)D: 51-75 nmol/l, and 4) serum 25(OH)D: > 75 nmol/l (20). The vitamin D group with a serum 25(OH)D > 75 nmol/l was used as reference value. Regarding the lipid profile the highest vitamin D group (> 75 nmol/l) was compared to the lowest vitamin D group (< 25 nmol/l). We adjusted for confounding variables which are known to influence the association between vitamin D level and cardiovascular outcomes (e.g. BMI, lipid profile, ethnicity etc.). A p-value < 0.05 was considered as statistically significant. All data were analysed using the Statistical Package of the Social Sciences (SPSS software, version 22.0; SPSS Inc., Chicago, IL).

RESULTS

Vitamin D status in PCOS and control women (main analyses)

A total of 639 PCOS women and 449 control women were included in the analyses. Demographic, anthropometric and clinical characteristics are presented in Table 1. The mean age was 34 ± 5 and 32 ± 5 years, with a median BMI of 25.2 (22.0 – 30.4 kg/m2) and 24.0 (22.0 – 27.0 kg/m2) in PCOS and control women, respectively. Overall median serum 25(OH)D was 49.0 (27.1 – 74.1 nmol/l) in PCOS versus 64.5 (39.2 – 85.7) in the control group. A severe vitamin D deficiency was present in 136 (21%) women out of 639 PCOS women, 190 (30%) women had a serum 25(OH)D between 25.1 and 50.0 nmol/l, 165 (26%) women had a serum 25(OH)D between 50.1 and 75.0 nmol/l, and 148 (23%) women had a serum 25(OH)D > 75 nmol/l. In the control group 49 (11%) women had a serum 25(OH)D ≤ 25 nmol/l, 106 (24%) women between 25.1 and 50.0 nmol/l, 131 (29%) women between 50.1 and 75.0 nmol/l, and 163 (36%) women had a serum 25(OH)D > 75 nmol/l. PCOS women had a significantly higher risk to have a lower serum 25(OH)D than control women (β = 0.78; 95% CI: 0.72 – 0.84, p < 0.001) which was found in the crude analysis, meaning PCOS women have a 22% higher risk of a lower serum 25(OH)D than control women (Table 2). Correcting this analysis for age, BMI, blood pressure, ethnicity and season the difference remains significant (β = 0.93; 95% CI: 0.87 – 0.99, p = 0.03).

Table 1. Baseline characteristics of PCOS and control women

	PCOS	Control	p-value
N	639	449	
Age (y)	34 ± 5	32 ± 5	< 0.001
Body Mass Index (kg/m2)	25.2 (22.0 – 30.4)	24.0 (22.0 – 27.0)	< 0.001
Ethnicity (%)			< 0.001
Caucasian	385 (60)	382 (85)	
Non-Caucasian	254 (40)	67 (15)	
Season of blood collection			
Winter	145 (23)	114 (25)	0.304
Spring	114 (18)	142 (32)	< 0.001
Summer	207 (32)	93 (21)	< 0.001
Autumn	173 (27)	200 (22)	0.072
BP systolic (mmHg)	118 ± 13	114 ± 11	< 0.001
BP diastolic (mmHg)	77 ± 11	73 ± 9	< 0.001
Serum 25(OH)D (nmol/l)	49.0 (27.1 – 74.1)	64.5 (39.2 – 85.7)	< 0.001
Storage of samples (years)	6	10	< 0.001

25(OH)D, 25-hydroxy vitamin D; BP, blood pressure

Table 3. Baseline demographic and clinical characteristics

		Vitamin D groups (serum 25(OH)D)			
	All	*≤ 25.0 nmol/l*	*25.1 – 50.0 nmol/l*	*50.1 – 75.0 nmol/l*	*> 75.0 nmol/l*
N	639	136	190	165	148
Age (y)	34 ± 5	32 ± 5	33 ± 6	34 ± 6	35 ± 5
Ethnicity (%)					
Caucasian	385 (60)	19 (14)	101 (53)	129 (78)	136 (92)
Non-Caucasian	254 (40)	117 (86)	89 (47)	36 (22)	12 (8)
Season of blood collection					
Winter	145 (23)	40 (29)	52 (27)	33 (20)	20 (14)
Spring	114 (18)	31 (23)	35 (19)	31 (19)	17 (11)
Summer	207 (32)	25 (19)	52 (27)	59 (36)	71 (48)
Autumn	173 (27)	40 (29)	51 (27)	42 (25)	40 (27)
Storage (y)	6 ± 1	6 ± 1	6 ± 1	6 ± 1	6 ± 1
Body Mass Index (kg/m2)	26.6 ± 6.0	29.1 ± 6	26.7 ± 6.1	26.4 ± 5.9	24.3 ± 5.3
Waist/hip ratio	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
FG score	5 (1 – 8)	9 (2 – 13)	5 (1 – 7)	4 (0 – 5)	4 (0 – 6)
BP systolic (mmHg)	118 ± 13	119 ± 12	118 ± 13	118 ± 13	117 ± 13
BP diastolic (mmHg)	77 ± 11	78 ± 11	77 ± 10	77 ± 11	76 ± 9
Metabolic syndrome yes (%)	90 (15)	33 (24)	26 (14)	23 (14)	12 (8)
Serum 25(OH)D (nmol/l)	49.0 (27.1 – 74.1)	16.8 (12.9 – 21.4)	37.0 (30.0 – 43.4)	62.9 (56.3 – 69.9)	94.2 (81.7 – 102.4)
Fasting glucose (mmol/L)	4.33 ± 0.68	4.49 ± 0.73	4.37 ± 0.78	4.28 ± 0.57	4.20 ± 0.55
Fasting insulin (mU/L)	10.0 (4.6 – 12.4)	14.2 (6.2 – 18.2)	10.6 (5.3 – 12.8)	8.5 (4.6 – 10.9)	7.2 (3.5 – 9.4)
HOMA-IR	1.99 (0.85 – 2.46)	2.94 (1.13 – 3.66)	2.09 (0.95 – 2.48)	1.64 (0.85 – 2.13)	1.36 (0.66 – 1.72)
Total cholesterol (mmol/L)	5.1 ± 1.2	5.0 ± 1.3	5.1 ± 1.2	5.2 ± 1.3	5.2 ± 1.1
LDL-cholesterol (mmol/L)	3.6 ± 1.0	3.6 ± 1.0	3.6 ± 1.0	3.7 ± 1.1	3.7 ± 1.0
HDL-cholesterol (mmol/L)	1.4 ± 0.5	1.3 ± 0.4	1.4 ± 0.5	1.5 ± 0.5	1.6 ± 0.5
Triglycerides (mmol/L)	1.1 ± 0.7	1.2 ± 0.8	1.1 ± 0.6	1.2 ± 0.7	0.9 ± 0.4
TC/HDL ratio (mmol/L)	3.9 ± 1.4	4.3 ± 1.6	3.9 ± 1.3	3.8 ± 1.4	3.5 ± 1.3
Apo A1	213.3 ± 59.4	198.1 ± 47.6	215.4 ± 59.2	215.6 ± 67.8	221.9 ± 57.3
Apo B	121.9 ± 37.9	126.5 ± 42.1	123.1 ± 37.0	120.8 ± 37.8	117.1 ± 34.6
TSH	1.87 (1.08 – 2.34)	2.21 (1.12 – 2.68)	1.90 (1.09 – 2.41)	1.77 (0.99 – 2.16)	1.63 (1.09 – 2.01)

Table 3. Continued

		Vitamin D groups (serum 25(OH)D)			
	All	*≤ 25.0 nmol/l*	*25.1 – 50.0 nmol/l*	*50.1 – 75.0 nmol/l*	*> 75.0 nmol/l*
Cortisol	321 ± 118	321 ± 116	311 ± 118	312 ± 121	342 ± 115
Testosterone	2.01 (1.40 – 2.50)	2.18 (1.50 – 2.70)	2.02 (1.30 – 2.60)	1.96 (1.40 – 2.40)	1.89 (1.20 – 2.35)
Free androgen index	6.5 (3.0 – 8.6)	9.3 (4.9 – 12.6)	6.7 (3.1 – 8.9)	5.6 (2.8 – 7.5)	4.6 (2.1 – 6.3)
Estradiol	301 (176 – 361)	285 (181 – 361)	301 (179 – 356)	313 (175 – 361)	302 (168 – 394)
DHEAS	5.36 ± 2.47	5.51 ± 2.89	5.36 ± 2.34	5.18 ± 2.35	5.42 ± 2.37
LH	10.9 (4.9 – 13.2)	9.9 (4.9 – 13.4)	10.9 (4.7 – 13.7)	12.1 (5.6 – 13.7)	10.3 (4.4 – 12.5)
FSH	6.2 ± 2.6	5.8 ± 2.1	6.3 ± 2.9	6.5 ± 2.8	6.3 ± 2.4
AMH	11.0 (6.0 – 14.2)	10.2 (5.8 – 12.7)	10.1 (5.6 – 13.6)	11.0 (6.0 – 14.9)	12.7 (6.8 – 15.1)

25(OH) D, 25-hydroxy vitamin D; AMH, anti mullerian hormone; Apo, apolipoprotein; DHEAS, Dehydroepiandrosterone sulfate; FSH, follicle stimulation hormone; HOMA-IR, homeostatic model assessment – insulin resistance; TC, total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; LH, Luteinizing hormone; TG, triglyceride; TSH, thyroid stimulating hormone

Table 2. Linear regression analysis of serum 25(OH)D* in PCOS versus controls.

	B (95% CI)	p	
Model 1:	*crude analysis*	0.78 (0.72 – 0.84)	< 0.001
Model 2:	*Model 1 + age, BMI and systolic blood pressure*	0.79 (0.73 – 0.86)	< 0.001
Model 3:	*Model 2 + season and ethnicity*	0.93 (0.87 – 0.99)	0.030

** serum 25(OH)D was natural log transformed*

PCOS women (subanalyses)

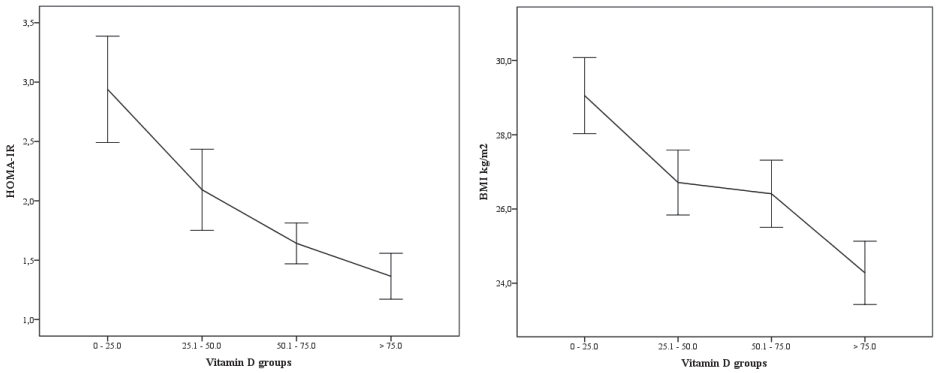
Demographic, anthropometric and clinical characteristics of all PCOS women and stratified by vitamin D group are presented in Table 3. The majority (60%) of included women were of Caucasian ethnicity. The distribution of the various ethnicities differed significantly between women with a severe vitamin D defiency compared to women with a sufficient vitamin D status, with 90% Caucasian women in the highest vitamin D group versus 14% of the women in the lowest vitamin D group. An expected difference was seen in season of

measurement between the vitamin D groups, with the highest percentage of blood samples taken in the summer presenting in the highest vitamin D group and the lowest percentage of blood samples taken in the summer in the lowest vitamin D group (48% versus 19%, respectively).

Anthropometric parameters

Median BMI of all PCOS women included was 25.2 (22.0 – 30.4 kg/m2). Overweight/obese women (BMI > 25 kg/m2) had a mean serum 25(OH)D 46.3 ± 27.9 nmol/l, which was significantly lower compared to women with a BMI < 25: serum 25(OH)D 59.7 ± 30.0 nmol/l (p < 0.001). A total of 94 (15%) women fulfilled the NCEP APT III criteria for metabolic syndrome, who had a significant lower serum 25(OH)D than women without metabolic syndrome (mean serum 25(OH)D 44.3 ± 27.4 nmol/l and 54.2 ± 29.9 nmol/l (p < 0.001), respectively).

Figure 1. Association between serum 25(OH)D (nmol/l) groups and HOMA-IR and BMI



Glycaemic parameters

Linear regression analysis correcting for BMI, ethnicity, waist to hip ratio, cholesterol/HDL ratio, and season resulted in a significant difference in HOMA-IR between the highest and lowest vitamin D groups. In the women with the lowest vitamin D level, HOMA-IR was on average 24% higher compared to the women with the highest vitamin D level (β= 0.76; 95% CI: 0.63 – 0.91; p = 0.003, Table 4) (Figure 1). Also, a significant difference was found between the middle vitamin D group with a serum 25(OH)D between 25.1 and 50.0 nmol/l and the highest vitamin D group (β=0.83; 95% CI: 0.72 – 0.96, p = 0.01). No significant difference was found between the middle-high vitamin D group (serum 25(OH)D: 50.1 – 75.0 nmol/l) compared to the women with a serum 25(OH)D > 75 nmol/l regarding HOMA-IR. No significant difference between the vitamin D groups was seen in fasting glucose after correction for BMI, age, ethnicity and season (β = -0.18; 95% CI: -0.39 – 0.03, p = 0.09 (data not shown).

Table 4. Linear regression analysis of vitamin D groups* and HOMA-IR**

	B (95% CI)	p-value
Model 1: *crude analysis*		
1.Serum 25(OH)D ≤ 25.0 nmol/l	0.49 (0.41 – 0.58)	< 0.001
2.Serum 25(OH)D 25.1 – 50.0 nmol/l	0.68 (0.57 – 0.80)	< 0.001
3.Serum 25(OH)D 50.1- 75.0 nmol/l	0.80 (0.67 – 0.94)	0.007
Model 2: *Model 1 + BMI*		
1.Serum 25(OH)D ≤ 25.0 nmol/l	0.70 (0.60 – 0.81)	< 0.001
2.Serum 25(OH)D 25.1 – 50.0 nmol/l	0.81 (0.71 – 0.93)	0.003
3.Serum 25(OH)D 50.1- 75.0 nmol/l	0.90 (0.79 – 1.04)	0.149
Model 3: *Model 2 + ethnicity*		
1.Serum 25(OH)D ≤ 25.0 nmol/l	0.75 (0.62 – 0.89)	0.001
2.Serum 25(OH)D 25.1 – 50.0 nmol/l	0.84 (0.73 – 0.97)	0.019
3.Serum 25(OH)D 50.1- 75.0 nmol/l	0.91 (0.79 – 1.05)	0.200
Model 4: *Model 3 + W/H ratio, Chol/HDL ratio*		
1.Serum 25(OH)D ≤ 25.0 nmol/l	0.76 (0.65 – 0.91)	0.003
2.Serum 25(OH)D 25.1 – 50.0 nmol/l	0.84 (0.73 – 0.96)	0.012
3.Serum 25(OH)D 50.1- 75.0 nmol/l	0.91 (0.80 – 1.04)	0.186
Model 5: *Model 4 + season*		
1.Serum 25(OH)D ≤ 25.0 nmol/l	0.76 (0.63 – 0.91)	0.003
2.Serum 25(OH)D 25.1 – 50.0 nmol/l	0.83 (0.72 – 0.96)	0.011
3.Serum 25(OH)D 50.1- 75.0 nmol/l	0.91 (0.79 – 1.04)	0.160

BMI, body mass index; Chol/HDL ratio, cholesterol – high-density lipoprotein ratio; W/H ratio, waist to hip ratio

** Vitamin D group with serum 25(OH)D > 75 nmol/l is the reference group*

***HOMA-IR natural logarithm (0.76 means 1- 0.76 = 24% higher HOMA-IR in vitamin D group ≤ 25 nmol/l than > 75 nmol/l)*

Lipid profile

Mean total cholesterol (TC) /HDL ratio was 3.9 ± 1.4 mmol/L in our study population. The ratio differed significantly between the vitamin D groups with a difference of -0.79 mmol/L between the lowest and highest vitamin D group ($p < 0.01$). After correcting for confounders, the association did not remain significant between the lowest and highest vitamin D groups (B= -0.31; 95% CI: -0.73 – 0.12, $p = 0.16$). A significant difference was seen in HDL-cholesterol between the lowest and highest vitamin D group after correction for confounders (B= 0.20; 95% CI: 0.05 – 0.60, $p < 0.01$). Apolipoprotein A1 was significantly higher in the highest vitamin D group compared to the lowest group after correction for confounders (B= 26.2; 95% CI: 7.5 – 45.0, $p < 0.01$). A small significant difference was seen in serum total cholesterol demonstrating higher serum total cholesterol in the highest vitamin D group compared to the lowest vitamin D group (B= 0.41; 95% CI: 0.02 – 0.70, $p = 0.05$). No difference between the vitamin D groups was found in serum triglycerides, LDL-cholesterol, and apolipoprotein B (Table 5).

Table 5. Regression analysis of serum 25(OH)D > 75 nmol/l versus < 25 nmol/l and parameters of lipid profile.

	Model 1		Model 2		Model 3	
	B (95% CI)	**p**	**B (95% CI)**	**p**	**B (95% CI)**	**p**
TC	0.16 (-0.13 – 0.46)	0.27	0.34 (0.04 – 0.65)	0.03	0.41 (0.02 – 0.70)	0.05
TG	-0.28 (-0.44 – -0.12)	< 0.01	-0.12 (-0.28 – 0.04)	0.13	-0.14 (-0.35 – 0.06)	0.18
HDL	0.34 (0.23 – 0.45)	< 0.01	0.23 (0.12 – 0.34)	< 0.01	0.20 (0.05 – 0.34)	< 0.01
LDL	0.05 (-0.20 – 0.29)	0.70	0.24 (0.00 – 0.49)	0.06	0.28 (-0.04 – 0.60)	0.09
TC/HDL ratio	-0.79 (-1.12 – -0.46)	< 0.01	-0.33 (-0.65 – -0.01)	0.048	-0.31 (-0.73 – 0.12)	0.16
Apo A1	23.1 (9.2 – 37.0)	< 0.01	18.6 (4.1 – 33.1)	0.01	26.2 (7.5 – 45.0)	< 0.01
Apo B	-9.8 (-18.9 – -0.8)	0.03	-0.03 (-9.1 – 9.0)	0.99	7.9 (-3.6 – 19.5)	0.18

25(OH) D, 25-hydroxy vitamin D; TC, total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; TG, triglycerides
Model 1: crude.
Model 2: adjusted for BMI, fasting glucose
Model 3: adjusted for BMI, fasting glucose, season, ethnicity and age

DISCUSSION

The aim of this study was to explore vitamin D status in women with PCOS compared to controls. Second, associations between vitamin D status and metabolic disturbances in PCOS women were evaluated. This is the largest study in its kind in a cohort of women with PCOS which confirmed the previously reported lower vitamin D status in women with PCOS as compared to controls (11). Low serum 25(OH)D status is significantly associated with a higher insulin resistance in women with PCOS independent of major confounders as BMI, season and ethnicity. The HOMA-IR differed

substantially (24%) between the highest and severe vitamin D deficient group. Finally, women with PCOS and a severe vitamin D deficiency had the lowest levels of HDL cholesterol and apolipoprotein A1.

The strength of our study is that we have studied the largest cohort of PCOS women and compared our findings with a control group without PCOS, which allows a well-powered comparison between cases and controls. Second, all samples were measured using the LC-MS/MS method, which is the golden standard for measurement of serum 25(OH)D (19).
A disadvantage of our study is that we did not have metabolic measurements in the control women, so a comparison of the association between vitamin D status and metabolic disturbances in PCOS versus controls was not possible. In this context we were not able to explore if this association is different in PCOS women than in controls. Despite, the control women were enrolled from the general population and not selected on fertility, which could result in PCOS women included in the control group, it is not likely that a substantial part of the control women had PCOS due to the fact that they all had a spontaneous pregnancy and regular menstrual cycles. It is known that vitamin D metabolism is altered in pregnancy however, this was not of influence in the present results as the blood collection of the control women was carried out 15 to 17 months after pregnancy.
Although serum 25(OH)D is stable over time and multiple freeze-thaw cycles, theoretically long time storage of serum samples at -80 ^{0}C could have influenced the vitamin D concentration (21). However, any influence of the storage time does not seem very likely as the mean storage time in control women was significantly higher than in controls (10 versus 6 years), in which you would expect a lower 25(OH)D level in the group with a higher storage time, which was not the case .
Another important point is the possibility of residual confounding which cannot be ruled out, despite correction for several confounders we applied in the regression analyses. Last point of importance, is we have used a surrogate marker of insulin resistance (HOMA-IR) and not the golden standard to measure insulin resistance, i.e. insulin clamp technique.

Interpretation of study findings

Several epidemiologic studies have explored the effect between vitamin D status and metabolic disturbances in PCOS women and controls. These studies are summarised in an earlier systematic review (11), in which the authors concluded serum 25(OH)D to be a significant predictor for insulin resistance in PCOS women. However, this effect disappeared after adjustment for BMI. Important limitations of the earlier performed observational studies were the small sample sizes and poor analyses with lack of correcting for confounding factors as ethnicity, season of blood collection and BMI.
The finding of a higher prevalence of vitamin D defiency in PCOS women compared to controls is in line with earlier reports, which observed a percentage of vitamin D deficiency (defined as serum 25(OH)D < 50nmol/l) of 67 – 85% among women with PCOS (10).
The mean vitamin D level in control women of our study was comparable with the overall mean vitamin D level that was reported in control women in the earlier performed systematic review (11). However, the mean vitamin D level in women with PCOS is much lower than what was reported in the same review. This discrepancy could be explained by the finding of some of the reviewed studies that the vitamin D level was even higher in women with PCOS than in control women (22-24). Probably, this inconsistent finding is caused by the lack of adjustment for the major confounders, i.e.

the season of measurement of vitamin D level and ethnicity.
To date, the vitamin D experts worldwide are still discussing about the optimal serum 25(OH)D level and the required supplementation dose. The Institute of Medicine (20) has determined that serum 25(OH)D levels greater than 50 nmol/l are sufficient based on the current studies available, although other experts corresponding to The Endocrine Society consider that optimal levels should be higher (> 75 nmol/l) (25). In our study population only 138/639 (22%) had a serum 25(OH)D concentration > 75 nmol/l. In the lower groups a more disturbed metabolic profile was already seen with higher levels of insulin resistance and a worse lipid profile, thereby supporting the recommendation from the Endocrine Society to reach a serum 25(OH)D concentration > 75 nmol/l.

Vitamin D is thought to influence the development of PCOS through gene transcription and influences metabolic metabolism (26). The effect of vitamin D status on glucose metabolism appears to be mediated by direct and indirect pathways. A direct effect on insulin secretion may be mediated by activation of VDRs in the pancreatic beta-cell with the addition of the presence of 1α-hydroxylase to produce locally 1,25(OH)2D. Furthermore, the direct effect of vitamin D on insulin secretion is supported by the presence of the vitamin D-response element in the human insulin promoter gene (27). Vitamin D deficiency may also increase systemic inflammation, known to play an important role in the pathogenesis of insulin resistance (28). Finally, insulin secretion and insulin resistance are both calcium-dependent processes. Both could be influenced by vitamin D status through an alteration in calcium concentration and flux through cell membranes of pancreas and insulin-responsive tissues.

The association found between vitamin D and dyslipidemia (i.e. HDL-cholesterol and apolipoprotein A1) had previously been described (29). Low HDL-cholesterol is one of the central features of metabolic syndrome. As dyslipidemia should be considered as an additional therapeutic target in women suffering from PCOS (30), vitamin D might be useful in the complex treatment of these women.

Apart from these cross-sectional findings, there are several small intervention studies among vitamin D supplementation in PCOS women. Until now, seven intervention studies examining the effect of vitamin D supplementation on metabolic disturbances in PCOS have been performed, which are summarised in a recently published systematic review (31). A significant inverse association was found between serum 25(OH)D and insulin resistance in the included observational studies, which is in line with our results. However, no significant improvement in metabolic functions was found among PCOS women supplemented with vitamin D. An important drawback is that most of the intervention studies were not randomised, and were conducted with relatively small samples sizes with a short follow-up. The largest study with a RCT design among 104 obese, vitamin D deficient PCOS women did reveal a positive effect of weekly 50.000 IU vitamin D plus calcium 1000mg/day on insulin resistance (32).

In conclusion, this is the largest epidemiologic study showing a significant association between vitamin D status and metabolic disturbances in patients with PCOS. Moreover, PCOS women had a significant lower serum 25(OH)D compared to controls. For future research large randomised controlled clinical trials are necessary to explore whether this association has a causal linkage.

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PART III

TYPE 2 DIABETES

CHAPTER 4

Study protocol:

A randomised placebo-controlled clinical trial to study the effect of vitamin D supplementation on glycaemic control in type 2 Diabetes Mellitus (SUNNY trial)

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ABSTRACT

Background

Besides the classical role of vitamin D on calcium and bone homeostasis, vitamin D deficiency has recently been identified as a contributing factor in the onset of insulin resistance in type 2 diabetes mellitus. However, it is uncertain whether vitamin D deficiency and poor glycaemic control are causally interrelated or that they constitute two independent features of type 2 diabetes mellitus. There are limited clinical trials carried out which measured the effect of vitamin D supplementation on glycaemic control.

The objective of this study is to investigate the effect of vitamin D supplementation on glycaemic control and quality of life in patients with type 2 diabetes mellitus.

Methods

In a randomised double-blind placebo-controlled trial conducted in five general practices in the Netherlands three hundred patients with type 2 diabetes mellitus treated with lifestyle advises or metformin or sulphonylurea-derivatives are randomised to receive either placebo or 50,000 IU Vitamin D3 at monthly intervals. The primary outcome measure is the change in glycated haemoglobin level between baseline and six months. Secondary outcome measures include blood pressure, anthropometric parameters, lipid profile, insulin resistance, quality of life, advanced glycation end products and safety profiles. Quality of life will be measured by The Short Form (SF-36) Health Survey questionnaire. Advanced glycation end products are measured by an AGE-reader.

Conclusions

This trial will be the first study exploring the effect of vitamin D supplementation on both glycaemic control and quality of life in patients with type 2 diabetes mellitus. Our findings will contribute to the knowledge of the relationship between vitamin D status and insulin resistance in patients with type 2 diabetes mellitus.

BACKGROUND

Type 2 diabetes mellitus, characterized by peripheral insulin resistance and pancreatic beta-cell dysfunction, represents a worldwide epidemic with significant co-morbidity and mortality due to microvascular and macrovascular complications (1). Although therapies for type 2 diabetes and its co-morbidity have improved over the last few decades, the need for new insights for the prevention and management of type 2 diabetes remains needed due to the increased impact of the disease. There is accumulating evidence suggesting that vitamin D status plays a role in many non-skeletal functions including diabetes mellitus (2,3). The prevalence of vitamin D deficiency is increasing with an estimating number of one billion people worldwide (3). A recent study in the Netherlands among older people revealed a prevalence of vitamin D deficiency of 47.8% (defined as 25 (OH) D < 50 nmol/l) (4).

Vitamin D is a secosteroid that is obtained from dietary sources, either food or supplements, and exposure to sunlight. It needs to be hydroxylated twice to become biologically active. Vitamin D is transported to the liver where it is first hydroxylated by 25-hydroxylase into 25-hydroxyvitamin D (25 (OH) D). This is the major circulating form used as an indicator of vitamin D status. The second hydroxylation occurs in the kidney by 1α-hydroxylase (1α-OHase), a product of the CYP27B1. Here the largest amount of the biologically active form of vitamin D: 1,25 dihydroxyvitamin D (1,25 (OH) 2D) is formed. Besides its classical role in calcium and bone homeostasis, vitamin D deficiency has recently been identified as contributing factor in the onset of insulin resistance in type 2 diabetes (5,6). In a meta-analysis of observational studies a relatively consistent association between low vitamin D status and the prevalence of type 2 diabetes or metabolic syndrome was reported (6). However, due to confounding and selection bias in epidemiological studies, a causal link cannot be established. To determine whether the relation between vitamin D deficiency and glycaemic control is causal in nature, randomised controlled trials with vitamin D supplementation are needed. To date, only few clinical trials examining this relation, with glycaemic control as primary outcome in type 2 diabetes, have been performed (7-13). The results of these clinical trials are inconsistent mostly due to the small sample size, low dose of vitamin D supplementation and short duration of the trials. Recently, a meta-analysis done by George et al. (14) demonstrated a small effect of vitamin D supplementation on fasting glucose and insulin resistance, with no effect on HbA1c. However most of these reviewed studies did not include diabetic patients nor had insulin resistance as primary outcome (15-22). Therefore, adequately powered, randomised placebo-controlled clinical trials with vitamin D supplementation are needed.

The potential mechanisms by which vitamin D can affect glucose metabolism could be the result of a rapid non-genomic effect or slower genomic effect of serum 25 (OH) D: 1) stimulation of insulin release by the increased expression of vitamin D receptor (VDR) as well as the enzyme 1α-OHase in the pancreatic beta-cells; 2) by binding of the 1,25 (OH) 2D - VDR complex to the vitamin D response element of the insulin receptor at tissue level enhancing insulin responsiveness for glucose transport; 3) suppression of the release of pro-inflammatory cytokines that are believed to mediate insulin resistance (2,5,23). The latter hypothesis is supported by studies showing an association between low serum 25 (OH) D and increased C-reactive protein levels (24). Indirectly, vitamin D may influence the extracellular and intracellular calcium regulation which is essential in mediating glucose transport in target tissues.

Another hypothesis may be the influence of serum vitamin D on oxidative stress and thereby reducing the formation of advanced glycaemic end products (AGEs). AGEs are a heterogeneous group of compounds formed nonenzymatically by glycation and oxidation of proteins. In patients with type 2 diabetes AGE formation is enhanced as a consequence of a hyperglycaemic and free radical rich environment (25). The AGE accumulation in the skin, as measured by skin autofluorescence, has found to be an independent strong predictor of microvascular and macrovascular complications in both type 1 and type 2 diabetes (26-28). Until now, solely a study conducted in diabetic rats is available which demonstrated a relation between AGEs and vitamin D (29).

We proposed to start a RCT with the following objectives:

i. To investigate the effect of vitamin D supplementation on glycaemic control and insulin resistance in patients with type 2 diabetes.
ii. To investigate the effect of vitamin D supplementation on health related quality of life measured by SF-36 in patients with type 2 diabetes.
iii. To investigate the association between skin autofluorescence and serum vitamin D

METHODS/DESIGN

Study design

We designed a double-blind, randomised placebo-controlled clinical trial among patients with type 2 diabetes. We randomly assigned three hundred patients into 1:1 ratio to receive a monthly dose of cholecalciferol 50,000 IU or placebo. The follow-up duration is six months. The study will be conducted in five general practices in and around Alkmaar, a city in north-western Netherlands at latitude 52°.

Participants

All patients with type 2 diabetes at the general practices fulfilling the inclusion criteria at their last visit by the general practitioner will be invited for screening for their eligibility.

Inclusion/exclusion criteria

Adults (≥18 years) with type 2 diabetes, diagnosed according to the World Health Organisation, who are treated with lifestyle advises, metformin, or sulfonylurea-derivatives (SU-derivatives), whether or not in combination, were invited for participation. Serum glycated hemoglobin (HbA1c) had to be stable and below or equal to 64 mmol/mol (8.0%) for the last three months without recent changes in hypoglycaemic agents.

The exclusion criteria are: an impaired renal function (estimated creatinine clearance < 30 ml/min measured by the MDRD formula: $186 \times (\text{serum creatinine})^{-1.154} \times (\text{age})^{-0.203} \times 0.742$ (if the subject is female) or $\times 1.212$ (if the subject is black)), any granuloma forming disorder, known history of renal stones or hypercalcaemia (serum calcium > 2.65 mmol/l), hypo- or hyperphosphatemia, serum 25 (OH) D < 15 nmol/l or > 150 nmol/l, known intolerance for cholecalciferol, using other antidiabetic treatment as mentioned above, insufficient knowledge of the Dutch language, mental retardation or psychiatric treatment for schizophrenia or bipolar disorder, participation in any other trial, being pregnant or lactating, or no signed informed consent.

Outcomes

The primary outcome of the study is HbA1c. Secondary outcomes are insulin resistance and beta-cell function measured by the homeostasis model of assessments (HOMA) and quantitative insulin sensitivity check index (QUICKI). Further secondary outcomes include the alteration of serum 25 (OH) D over time, blood pressure, lipid profile, parathyroid hormone, calcium, thyroid function, AGE accumulation in the skin, safety profile of vitamin D supplementation, urine analysis for micro-albuminuria, and quality of life assessed by The Short Form (SF-36) Health Survey questionnaire.

Sample size estimation

It was calculated that 126 patients with type 2 diabetes would be required in this trial to demonstrate a significant difference at 80% power and 5% significance. Power calculations were based on the literature and aimed at a difference of 0.5% in HbA1c value in the treated group as compared to the placebo group with a standard deviation of 1.0% (30). With an expected rate of 50% vitamin D deficient participants, 252 subjects would be required. According to an expected drop out rate of 20%, 300 subjects will be recruited.

Randomisation

A double-blind randomised placebo-controlled trial design has been chosen. Randomisation for the parallel treatment phase will be carried out by the pharmaceutical department after checking the inclusion- and exclusion criteria. The patient will be assigned to the treatment order as defined by the code. The patients will be randomised 1:1 according to the method of block randomisation with a block size of 10. No stratification is used. The study medication will be delivered in similar flagons and labels with its own sequence number. The allocation sequence number will be kept by the pharmacist in a secure place during the course of the study.

Intervention

In this trial the dose of vitamin D (cholecalciferol) being supplemented is 50,000 IU. The consumption frequency will be once a month (equivalent to ~1667 IU/day in the treatment group) for six consecutive months. We chose the oral route of administration. Cholecalciferol is supplied in a vial of 8 ml containing 50,000 IU per ml, meaning the patients will use 1 ml per month. The placebo will be identical in taste, texture and appearance as the active supplement. The vitamin D supplement and placebo are manufactured and packed by the pharmacy of the Meander Medical Centre, Amersfoort, the Netherlands. Both the active supplements and the placebo will be given to participants in a translucent flagon with only their sequence number on the label. Labeling of the flagons will be done by the same person doing the randomisation and allocation sequence. A pipette of 1 ml will be delivered by the vial to make sure all patients use the right dose of the study medication. During their first visit patients will be instructed how to take in the study medication correctly with the pipette. To ensure compliance during the study, reminders about the intervention appointments will be sent regularly via email of phone calls.

The safety of vitamin D supplementation has well been established. The recommended dose for adults is 600–800 IU/day, depending on age, with an upper limit of 4,000 IU/day (31). Toxicity of vitamin D supplementation did not occur with a dose of 10,000 IU/day (32). Side effects (hypercalcaemia, hypercalcuria, renal stones, gastro-intestinal symptoms or hypersensitivity symptoms) of

vitamin D supplementation are rare. For the safety of all patients serum creatinine and calcium are measured at three months. Patients with known hypercalcaemia are excluded for this reason. A 24 hour telephone number is available in the case hypersensitivity symptoms occur.

Handling and storage of data and documents

For each participant a Case Record Form (CRF) is provided by the sponsor of the trial. All personal data are stored on a coded drive which is only available to the study coordination team. These documents will be stored in the hospital for a minimum of ten years. The Subject Identification Codes must be kept for at least 15 years. All these requirements are in accordance with the 'Wet Medisch Onderzoek met Mensen'.

Statistical analysis

The primary efficacy analysis to explore the intervention effect on glycaemic control will be based on the intention-to-treat (ITT) method, in which all randomised patients for whom outcome data are available, will be recorded. In the ITT analysis the patients will be analysed according to their original group assignment, whether or not they accepted and/or adhered to the intervention. This analysis avoids the possibility of any bias associated with loss, misallocation or non-adherence of participants. If there will be a substantial difference between those allocated to receive an intervention and those who actually received it (and adequately adhere to it), we will perform an additional analysis adjusting for actual treatment received ('per protocol' analysis). The results of the 'per protocol' analysis will be compared with the ITT analysis and the numbers involved will be precisely described.

All raw data will be entered into SPSS software (version 20.0, SPSS Inc, Chicago, IL). Ten percent of all data will be double-entered to check for duplication and outliers before starting statistical analyses. Baseline characteristics of the study patients will be summarised separately for each randomised group. Dichotomous and categorical data will be presented as number and percentages of patients within both groups. Continuous variables will be checked for normality and will be presented in terms of means and standard deviation if normally distributed, or when a variable has a skewed distribution as median, 25th and 75th percentiles. Nominal variables including subgroups will be analysed using the Chi-squared test, ordinal variables by the Mann-Witney test or Kruskall-Wallis test and continuous variables by the t-test or analysis of variance model (ANOVA).

The primary efficacy endpoint, HbA1c, will be compared between placebo and treatment group using a repeated measures analysis of variance with adjustment for baseline characteristics. For secondary outcomes a paired t-test, Wilcoxon's test or repeated measures analysis of variance will be performed. Delta values will be calculated to compare the differences in means over time between the treatment and placebo group.

Correlation coefficients will be measured using Pearson or Spearman, depending on the level of measurement. A logistic and/or linear regression model will be used for multivariate analysis to relate an outcome variable to causal variables. The multivariate analysis will be used to rule out the possible role of confounders for vitamin D deficiency as season of measurement, maternal age, ethnicity, diet, physical activity and BMI.

Subgroup analyses will be performed by stratifying vitamin D into three groups: deficient (15–49 nmol/l), insufficient (50–74 nmol/l), and sufficient (75–150 nmol/l). Furthermore, subgroup analyses

for vitamin D deficient patients (serum 25 (OH) D 15–35 nmol/l), and poor glycaemic control (HbA1c ≥ 53 mmol/mol) will be performed using the same method as described for the primary analysis. A p-value < 0.05 will be considered as statistical significant.

Funding/ethics

This trial was approved by the Medical Ethics Committee of North-Holland, the Netherlands. No funding is given for the trial.

RESULTS

Study procedure

The study will be divided into two phases: Phase I and Phase II.

Phase I

Patients with type 2 diabetes who met the inclusion criteria at their last visit at the general practice will be invited through a brief introduction letter for participation. All patients will be approached by phone in three weeks after receiving the letter whether or not they are interested for participation. If interested, the extensive patient information form will be given and an appointment will be scheduled at the general practice for informed consent and start of the trial.

Phase II

The trial has a total of three visits at the patient's own general practice (Figure 1). The initial visit includes informed consent, assessment of eligibility, questionnaires regarding demographics, sunlight exposure, physical activity, dietary intake (fish and dairy products), diabetes history and medication, co-morbidity and health related quality of life (SF-36). Anthropometric parameters and skin AGE accumulation will be measured. Blood collection will be collect at this stage. The study medication will be given for six months with a prior instruction. The second visit at three months is for safety assessment and observation of consumption. This includes: discussion of any adverse events, any change in medication, control of the ingestion of the study medication, blood collection (including renal function, serum 25 (OH) D and HbA1c). The final visit is at the end of six months, when questionnaires and anthropometric measurements are repeated and the last set of blood samples are collected (Table 1). The inclusion of all patients will be spread over one year to prevent large seasonal influences, as well as the results will be adjusted for seasonal influences. All participants will be informed of their anthropometry and blood pressure measurement at the start and during the course of the trial. The blood test results during the course of the trial will only be discussed when these are abnormal and lead to premature termination of the trial. The following criteria are drawn for premature termination of the trial: hypercalcaemia (calcium > 2.65 mmol/l), onset of any granuloma forming disease, serum 25 (OH) D < 15 nmol/l or > 250 nmol/l, impaired renal function (eGFR < 30 ml/min), hypersensitivity for cholecalciferol, onset of urolithiasis, change in hypoglycaemic agents or HbA1c > 69 mmol/mol (8.5%). After completion of the trial, all participants will be informed of their blood results. In case of vitamin D deficiency (serum 25 (OH) D < 50 nmol/l) at the end of the trial, supplementation with cholecalciferol will be started. The patients will be informed by letter in which group they participated after every patient completed the trial.

Table 1 Outcome variables and time of measurement (n = 300)

Variables	Months		
	0	3	6
Informed Consent	x		
Sociodemographics	x		
Medical and family history	x		
Diet, sun exposure, physical activity	x		x
Advanced Glycation End products (AGEs)	x		x
Questionnaire SF-36	x		x
Medication alterations		x	x
Adverse events		x	x
Anthropometry			
Height, weight, BMI	x	x	x
Blood pressure	x	x	x
Waist/hip circumference	x		x
Blood analysis			
Serum 25 (OH) D	x	x	x
HbA1c	x	x	x
Fasting blood glucose	x	x	x
Fasting blood insulin	x	x	x
C-peptide	x	x	x
Serum creatinine	x	x	x
Lipid profile	x		x
Serum calcium	x		x
Serum phosphate	x		x
Serum albumin	x	x	x
Serum PTH	x		x
Serum TSH	x		x
AST	x		x
ALT	x		x
Alkaline phosphatase	x		x
γ-GT	x		x
Hb, Platelets, Leukocytes	x		x
C-reactive protein	x		x
Urine analysis	x	x	x

25(OH)D, 25-hydroxyvitamin D; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; Hb, haemoglobin; PTH, parathyroid hormone; TSH, thyroid stimulating hormone, γ-GT, gamma glutamyltransferase.

Anthropometric parameters, AGE measurement and health related questionnaire

Height and weight will be measured using a stadiometer (up to 2 meters) and a weighing scale (200 kilograms). Weight will be measured without shoes and with light clothes in the morning. Blood pressure will be measured using a sphygmomanometer. The blood pressure will be measured at the right arm whilst the patient is sitting for at least 5 minutes. Tape-measurements will be performed for waist and hip circumferences. Body Mass Index (BMI) will be calculated by dividing weight through the square of height. Waist to hip ratio will be calculated by the formula: waist circumference/hip circumference. The AGE accumulation in the skin will be measured using an AGE reader (Diagnoptics Technologies B.V., Groningen, The Netherlands). This is a non-invasively device that can assess the tissue accumulation of AGEs using fluorescence of ultraviolet light in human tissue. The forearm is put on the machine and the concentration is assessed by ultraviolet light. This procedure takes 3 minutes and will be done at baseline and at the final visit.

SF-36 health survey will be used to assess health-related quality of life on emotional and physical wellbeing. The patients will receive the questionnaire at baseline and at the end of the trial with a self-addressed envelope.

Blood collection

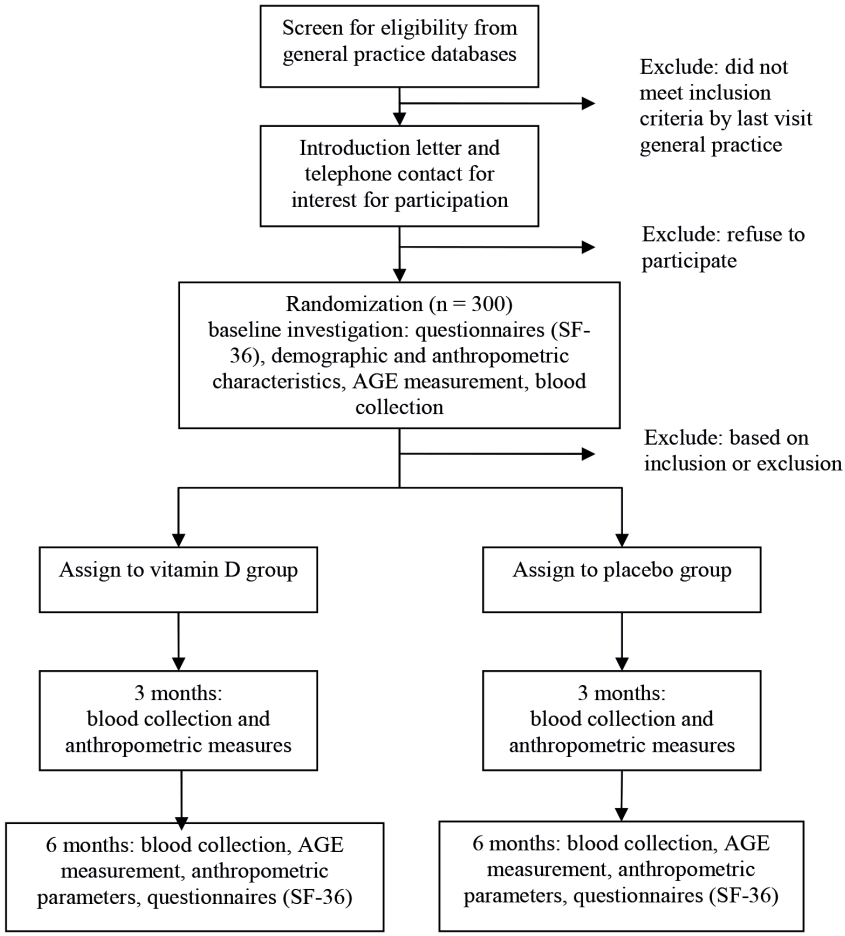
Venous blood sampling will be drawn at 0, 3 and 6 months by registered staff nurses using a sterile vacutainer needle between 8.00 and 9.30 am. All participants will be asked to fast overnight for at least 8 hours before blood collection. Blood serum will be used for the analysis of HbA1c, C-peptide, fasting insulin, calcium, phosphate, albumin, PTH, liver enzymes, lipid profile, C-reactive protein, TSH, platelets count, leukocytes and hemoglobin and 25 (OH) D ((iSYS automated immunoanalyser (IDS GmbH, Frankfurt, Germany)). The total 25-OH vitamin D assay detects 25-OH VD3 and 25-OH VD2, both with a specificity of 100%. The quality of the test is controlled by applying Westgard QC-rules on 3 different QC-samples (33). The accepted interassay coefficients of variation are <10% for all 3 QC-samples (low, medium and high level). TSH and intact-PTH will be determined on a Beckman Coulter UniCel DxI 600 Synchron Access immunoanalyser (Beckman Coulter Nederland B.V., Mijdrecht, the Netherlands). Calcium, phosphate, glucose, albumin, liver enzymes, C-reactive protein and lipid profiles will be determined on a Beckman Coulter UniCel DxC 860i Synchron Clinical system chemistry analyzer. C-peptide and insulin will be determined on Siemens Immulite XPi automated immunoanalyser (Siemens Medical Solutions Diagnostics B.V., Breda, the Netherlands). HbA1c will be measured on a HA-8180 automated HPLC system Menarini (Florence, Italy). The total white blood cell count, platelets count and haemoglobin content will be performed with an automated Sysmex XE-2100 blood cell counter (Sysmex Coorperation, Kobe, Japan).

Fasting blood glucose will be measured in blood plasma. Homeostasis model assessment of insulin resistance (HOMA-IR), calculated by fasting glucose (mmol/l) x fasting insulin (IU/ml)/22,5 and QUICKI, calculated by 1/log (fasting insulin (IU/ml)) + log (fasting glucose (mg/dl)), will be used to evaluate insulin resistance. Pancreatic beta-cell function will be assessed through the HOMA-B, calculated by (20 x fasting insulin (IU/ml))/(fasting glucose (mmol/l) – 3,5). All assays will be performed according to the manufacturer's instructions and carried out by the central chemical laboratory of the Medical Centre Alkmaar, the Netherlands. This laboratory is a certified CCK laboratory. Aliquots of serum and plasma will be stored at −70/80'C for future research questions.

Adverse events

All serious adverse events (SAEs) will be reported through the web portal ToetsingOnline to the accredited Medical Ethics Committee that approved the protocol, within 15 days after the sponsor has first knowledge of the serious adverse reaction. SAEs that result in death or are life threatening episodes should be reported expedited. The expedited reporting will occur not later than 7 days after the responsible investigator has first knowledge of the adverse reaction. This is for a preliminary report with another 8 days for completion of the report.

Figure 1. Recruitment flow chart



DISCUSSION

The main purpose of this study is to measure the effect of vitamin D supplementation on glycaemic control and health related quality of life in patients with type 2 diabetes. There is widespread interest in the potential causal role of vitamin D on the pathogenesis and progression of type 2 diabetes. We hope this study will give new insight into this causality. Until now conflicting results are seen in observational studies and the few clinical trials performed, which could not confirm a causal association (7-14). Factors for the lack of effect found in these studies includes: short trial duration, relatively low doses of vitamin D supplementation whether or not combined with calcium supplements, and heterogeneous study populations.

This trial has several strengths: the study sample size allows us to perform a subgroup analysis of vitamin D deficient patients. Taken into account that only in vitamin D deficient patients an effect of vitamin D supplementation on glycaemic control will be found, we chose to recruit double the number of participants as calculated with the power estimation, hypothesizing that 50% of the included patients will be vitamin D deficient (serum 25 (OH) D < 50 nmol/l). Secondary, the relatively high dose chosen for intervention and the duration of the trial are strengths of our study. The dose of vitamin D supplementation of 50,000 IU of vitamin D per month (daily equivalent ~1667 IU) is based on the recommendations of the Institute of Medicine and on the study done by van Groningen et al. (31,34). In this latter study, performed in the Netherlands, it was demonstrated that a cumulative dose of 100,000 IU given in 2 months increases the serum 25 (OH) D meanly with 29 nmol/l. Assuming that the mean serum 25 (OH) D in our study population will be around 50 nmol/l at baseline, the serum 25 (OH) D should be raising to a sufficient status (≥75 nmol/l) in two months. To measure a difference in HbA1c level we hypothesise that the maximal effect will be seen at least at six months, regarding the fact that the red blood cells circulate about 100 days in the blood and HbA1c levels takes around six weeks to change. Furthermore, this is the first study examining the association between vitamin D status and skin autofluorescence value as well as the effect on skin AGEs after vitamin D supplementation. For this secondary outcome the duration of our trial will be a limitation, regarding the stability and long half-life of skin AGEs with could be the cause to find no effect of vitamin D supplementation on skin AGEs.

The results of this trial should provide more insight into the potential causal association between vitamin D status and glycaemic control in patients with type 2 diabetes.

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CHAPTER 5

Effect of vitamin D supplementation on glycaemic control in patients with Type 2 Diabetes (SUNNY trial): A Randomised Placebo-Controlled Trial

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ABSTRACT

Background

Low vitamin D status has been associated with impaired glycaemic control in patients with type 2 diabetes. The purpose of our study was to evaluate the effect of vitamin D supplementation on glycaemic control in patients with type 2 diabetes.

Methods

This randomised, double-blind, placebo-controlled trial was conducted in 275 adult patients with type 2 diabetes without insulin treatment. Patients were randomly assigned to receive either vitamin D3 (50,000 IU per month) or placebo for six months. To assess the primary outcome of the study, change in HbA1c, we performed a linear regression analysis.

Results

Mean baseline serum 25-hydroxyvitamin D [25(OH)D] increased from 60.6 ± 23.3 to 101.4 ± 27.6 nmol/l and 59.1 ± 23.2 to 59.8 ± 23.2 nmol/l in the vitamin D and placebo group, respectively. Mean baseline HbA1c was 6.8 ± 0.5% (51 ± 6 mmol/mol) in both groups. After six months no effect was seen on HbA1c (mean difference: $\beta = 0.4$, 95% CI: -0.6 to 1.5; $p = 0.42$), and other indicators of glycaemic control (homeostasis model assessment of insulin resistance, fasting insulin and glucose) in the entire study population. Subgroup analysis in patients with a serum 25(OH)D < 50 nmol/l or an HbA1c level > 7% (53 mmol/mol) did not differ the results.

Conclusions

In a well controlled group of patients with type 2 diabetes, intermittent high-dose vitamin D supplementation did not improve glycaemic control.

INTRODUCTION

Type 2 diabetes, characterised by peripheral insulin resistance and pancreatic beta-cell dysfunction, represents a worldwide epidemic with significant co-morbidity and mortality due to microvascular and macrovascular complications (1). Although therapies for type 2 diabetes have improved over the last few decades, new insights for the prevention and management of type 2 diabetes remain necessary due to the increased prevalence of the disease.

Over the past decade, vitamin D has attracted substantial interest towards extra-skeletal outcomes in various disease conditions, including diabetes mellitus (2,3). Vitamin D deficiency (defined as serum 25-hydroxyvitamin D [25(OH)D] < 50 nmol/l) is highly prevalent in patients with type 2 diabetes (4,5). Several potential mechanisms involving vitamin D might affect glycaemic control in patients with type 2 diabetes. Most cells, including the pancreatic beta-cells, contain the vitamin D receptor (VDR), and most of them also have the capability to produce the biologically active 1,25-dihydroxyvitamin D [1,25(OH)2D] which allows intracrine and paracrine functions. In vitro studies have shown that the active vitamin D metabolite 1,25(OH)2D stimulated insulin release by the pancreatic beta-cells (6,7). In addition, vitamin D is known to have immuno-modulatory and anti-inflammatory effects and might reduce peripheral insulin resistance by altering low-grade chronic inflammation (8,9). Furthermore, insulin secretion and insulin sensitivity are both calcium-dependent processes.

A large number of cross-sectional studies generally demonstrated an inverse association between vitamin D status and prevalence of hyperglycemia (4,10). Longitudinal studies have reported that low vitamin D status is a predictor for incident type 2 diabetes (11,12). Still, it remains unclear whether vitamin D deficiency and insulin resistance are causally related or whether they constitute two independent features of patients with type 2 diabetes. Results from previous intervention studies with vitamin D supplementation have been conflicting. A systematic review and meta-analysis of 15 studies examining the effect of vitamin D supplementation concluded that there is currently insufficient evidence of beneficial effect to recommend vitamin D supplementation as a means of improving glycemia or insulin resistance in patients with diabetes, impaired glucose tolerance or normal fasting glucose (13). A weak positive effect of vitamin D supplementation was seen on fasting glucose and insulin resistance in patients with type 2 diabetes. Inconsistency in these results may be due to the fact that many of the included reviewed studies used a different supplementation regime, had a lack of power, did not have glycaemic control as primary outcome, or did not include patients with type 2 diabetes.

Taken together, the causality of the association between vitamin D status and glycaemic control in patients with type 2 diabetes has not yet been proven. We therefore designed this double-blind randomised placebo-controlled trial to determine the effect of vitamin D supplementation on glycaemic control in patients with type 2 diabetes.

METHODS

Study design and participants

The SUNNY trial, acronym for study on the effect of vitamin D supplementation on glycaemic control in type 2 diabetes, is a double-blind, randomised placebo-controlled clinical trial in which the effect of vitamin D supplementation on glycaemic control was examined in patients with type 2 diabetes. A detailed description of the protocol can be found elsewhere (14).

In brief, the trial was conducted in five general practices in and around Alkmaar, the Netherlands at latitude 52º. Adult patients (≥ 18 years) with type 2 diabetes treated with lifestyle advice, metformin, or sulfonylurea-derivatives (SU-derivatives), whether or not in combination, were invited by letter for participation in the study. Serum HbA1c had to be stable and below or equal to 8.0% (64 mmol/mol) for the last three months without recent changes in hypoglycaemic agents. All patients were included between July 2012 and April 2013. This trial was approved by the Medical Ethics Committee of North-Holland, the Netherlands and was conducted according to the principles of the Declaration of Helsinki.

The main exclusion criteria were: an impaired renal function (estimated glomerular filtration rate [eGFR] < 30 ml/min/1.73m2, calculated from serum creatinine using the MDRD formula), any granuloma forming disorder, hypercalcemia (serum calcium > 2.65 nmol/l) of any reason, serum 25(OH)D < 15 nmol/l or > 150 nmol/l and urolithiasis. The patients were not allowed to take vitamin D supplements during the study. Throughout the study drug alterations regarding hypoglycaemic agents and statins were not allowed. All patients gave written informed consent. Withdrawal criteria for premature termination of the trial were: onset of hypercalcemia, serum 25(OH)D < 15 nmol/l or > 250 nmol/l, hypersensitivity to cholecalciferol or placebo, onset of urolithiasis, any change in hypoglycaemic agents, or HbA1c > 8.5% (69 mmol/mol).

Intervention

All participants were randomised according to either an oral dose of cholecalciferol 50,000 IU once a month or a identically looking placebo 50,000 IU once a month for 6 months (Meander Medical Center, Amersfoort, the Netherlands). The patients were randomised 1:1 according to the method of block randomisation with a block size of 10. No stratification was used. The randomisation procedure was carried out by the pharmacist. The participants and the research team remained blinded till the end of the study.

Outcome measures

The primary outcome of the study was the change in serum HbA1c between vitamin D and placebo group after six months of intervention . Secondary outcomes were insulin resistance and beta-cell function, measured through the homeostasis model assessment of insulin resistance (HOMA-IR) and beta-cell function (HOMA-B), quantitative insulin sensitivity index (QUICKI), lipid profile, blood pressure, and safety profiles. Furthermore pre-specified subgroup analysis in patients with serum 25(OH)D < 50 nmol/l or HbA1c between 7 and 8% (53 - 64 mmol/mol) at baseline were performed. An exploratory subgroup analysis was performed in patients with severe vitamin D defiency (serum 25(OH)D < 30 nmol/l). Outcome measurements were obtained at baseline (immediately prior to dosing), at three and six months. Venous blood samples for serum 25(OH)D, HbA1c, fasting blood

glucose and insulin, lipid profile, serum calcium, albumin, creatinine and parathyroid hormone (PTH) were collected after an overnight fast at 8.00 – 9.30 am. Serum 25(OH)D was measured on an iSYS automated immunoanalyser (IDS GmbH, Frankfurt, Germany). PTH was determined using an Access intact PTH-assay on a Beckman Coulter UniCel DxI immunoanalyser (Beckman Coulter Nederland B.V., Mijdrecht, the Netherlands). All assays were performed according to the manufacturer's instructions and carried out by the clinical chemistry laboratory of the Medical Center Alkmaar, the Netherlands. This laboratory is CCKL certified.

Statistical analysis

We calculated that 126 patients with type 2 diabetes would be required in this trial to demonstrate a significant difference at 80% power and 5% significance. Power calculations were based on the literature and aimed at a difference of 0.5% in HbA1c value in the treated group as compared to the placebo group with a standard deviation of 1.0 % (15). With an expected rate of 50% of the subjects having a serum 25(OH)D < 50 nmol/l, 252 subjects would be required to draw conclusions in vitamin D subgroups (deficient versus sufficient). According to an expected drop out rate of 20%, 300 subjects were recruited.

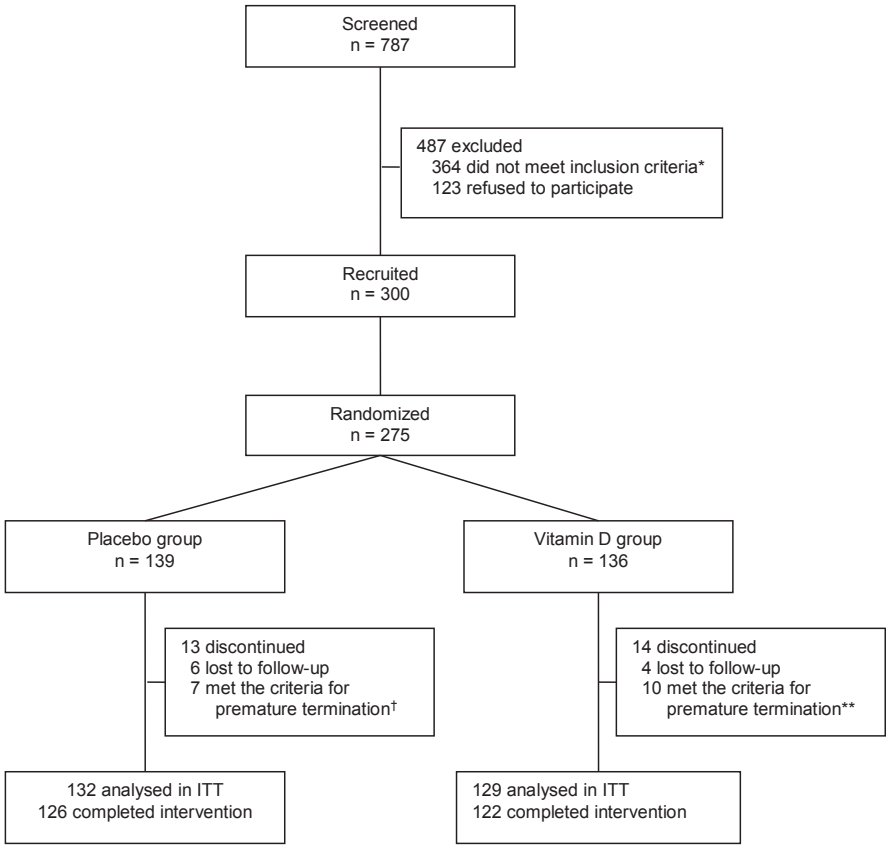
All data were analyzed using the statistical package Statistical Package of the Social Sciences (SPSS software, version 20.0, SPSS Inc, Chicago, IL). Baseline characteristics were presented as means ± standard deviation (SD), frequencies (%), or as median (IQR) in case of a skewed distribution. According to the guidelines of the institute of medicine (IOM) vitamin D deficiency was defined by a serum 25(OH)D < 50 nmol/l (16). Severe vitamin D deficiency is defined by a serum 25(OH)D < 30 nmol/l according to the Dutch Health Council. The efficacy analyses to explore the intervention effect on glycaemic control, were based on a modified intention-to-treat (ITT) protocol, in which all randomised patients with at least one available post-baseline HbA1c value were included. For patients with missing data at six months the last measurement was carried forward. Per-protocol analysis was also performed including all patients who completed the trial. Linear regression analysis was used to assess the mean difference between intervention and placebo groups after six months (mean difference is reported as beta). Change in HbA1c was analysed as primary outcome with randomisation group and baseline values as explanatory measures. All effects were adjusted for baseline value, sex, season of measurement, baseline age and BMI, and ethnicity in line with earlier literature. For the pre-specified subgroup analysis among patients with vitamin D deficiency and an HbA1c level > 7% (53 mmol/mol) at baseline, the same analyses were used. For the exploratory analysis among patients with a severe vitamin D defiency, the results were solely adjusted for baseline value, age and season of measurement, due to the small sample size with a lack of statistical power. Skewed continuous variables were natural log transformed before analysis. A two-sided p-value < 0.05 was considered as statistically significant.

RESULTS

Of the 787 patients who were screened for eligibility, 423 were invited for participation. Of these, 300 potential participants were recruited for the study of whom 275 showed up at the first visit and were randomised (139 and 136 in the placebo and vitamin D group, respectively). In total 261 (95%), 132

in the placebo group and 129 in the vitamin D group accomplished the three months measurement and were included in the intention to treat analysis (Figure 1).

Figure 1. Paraticpant flow chart



**Most patients did not meet the inclusion criteria because of insulin therapy;*
†criteria for premature termination of the trial: increase of HbA1c (n = 5), change in antidiabetic medication (n = 10), or change in serum 25(OH) < 15 or > 150 nmol/l (n = 2).
ITT, intention to treat.

The main reasons for premature termination between start and completion of the trial were: any alteration in oral hypoglycaemic agents (n =10), serum 25(OH)D < 15 or > 150 nmol/l (n = 2), lost to follow-up (n = 10), and HbA1c level > 69 mmol/mol (8.5%) (n = 5).

Baseline demographic, anthropometric and biochemical characteristics of both groups are presented in Table 1. The mean age of all patients was 67 ± 8 years and 65% were male. The mean diabetes duration was 6 ± 5 years with a mean baseline HbA1c value of 6.8 ± 0.5% (51 ± 6 mmol/mmol). The anti-diabetic treatment regimen did not differ between both groups. Overall mean serum 25(OH)D was 59.8 ± 23.2 nmol/l. Vitamin D deficiency was present in 98 out of 261 patients (38%), 102 patients (39%) had a serum 25(OH)D level between 50 - 74 nmol/l, and 61 patients (23%) had a 25(OH)D level between 75 and 150 nmol/l at baseline. No differences were reported in diet (dairy products and fish intake) at baseline between both groups. Statin use was high in both groups (84%).

Serum 25(OH)D increased significantly in patients who received vitamin D supplementation: 60.6 ± 23.3 nmol/l to 101.4 ± 27.6 nmol/l at six months, compared to no change in the placebo group: serum 25(OH)D: 59.1 ± 23.2 nmol/l to 59.8 ± 27.4 nmol/l. 75% of the patients in the intervention group achieved a serum 25(OH)D level ≥ 75 nmol/l at three months, and 85% after six months of vitamin D supplementation. A significant inverse association was found between baseline serum 25(OH)D and the increase in serum 25(OH)D at six months in both groups ($r = -0.42$, $p = <0.001$; and $r = -0.38$, $p = <0.001$, in vitamin D and placebo group respectively). No significant association was found between baseline BMI and the change in serum 25(OH)D.

Serum 25(OH)D and glycaemic control

Concerning the primary outcome, the change in HbA1c from baseline to six months including all patients, did not differ significantly between both groups ($\beta = 0.4$, 95% CI: -0.6 to 1.5; $p = 0.42$) (Table 2a). Regarding mean change of the secondary outcomes, no significant differences between both groups were seen in other indicators of glycaemic control (HOMA-IR, HOMA-B, fasting glucose, fasting insulin and QUICKI) and anthropometric variables (Table 2a). A significant difference, to detriment of the vitamin D group, was observed in total cholesterol/HDL ratio. This result however, remained no longer significant after adjustment for the change in BMI over six months and baseline total cholesterol/HDL ratio (data not shown). Serum 25(OH)D and PTH significantly differed between both groups. Systolic blood pressure fell significantly in both groups (-6.4 ± 17.7 mmHg, $p = < 0.001$ in the vitamin D group; -6.8 ± 17.2 mmHg, $p = < 0.001$ in the placebo group), but the difference between the intervention and control group was not significant. Per protocol analysis did not change the results (data not shown).

Pre-specified subgroup analysis in patients with a serum 25(OH)D < 50 nmol/l (n = 98) did neither reveal any change in HbA1c between both groups ($\beta = 0.07$, 95% CI: -2.0 to 1.9; $p = 0.95$), nor in the other indicators of glycaemic control (Table 2b). In addition, no effect was seen in subgroups with reduced glycaemic control (baseline HbA1c > 7.0% [53 mmol/mol]) (data not shown).

Table 1 Patient demographics and baseline characteristics

	Vitamin D group	Placebo group
	n = 129	**n = 132**
Demographic parameters		
Male, n (%)	88 (68)	82 (62)
Age (years)	67 ± 8	67 ± 9
Diabetes duration (years)	6 ± 4	6 ± 5
White skin colour, n (%)	122 (95)	122 (93)
Antidiabetic treatment, n (%)		
Lifestyle adjustments	4 (3)	7 (5)
Metformin	91 (71)	75 (57)
SU-derivatives	3 (2)	5 (4)
Metformin + SU-derivatives	31 (24)	45 (34)
Microvascular complications ≥ 1, n (%)*	35 (27)	16 (12)
Cardiovascular disease, n (%)	35 (27)	48 (36)
Current smoker, n (%)	18 (14)	19 (14)
Alcohol use ≤ 2 EH/day, n (%)	114 (88)	114 (86)
Dairy intake ≥ 2 units/day, n (%)	85 (66)	100 (76)
Fish intake > 1 servings/week, n (%)	45 (35)	56 (42)
Vitamin D supplements, n (%)†	18 (14)	12 (9)
Exposure to sun, n (%)		
< 5 hours/week	50 (39)	52 (39)
5-10 hours/week	57 (44)	59 (45)
>10 hours/week	22 (17)	21 (16)
Physical activity, n (%)		
< 2 hours/week	40 (31)	38 (29)
2-5 hours/week	56 (43)	67 (51)
>5 hours/week	33 (26)	27 (20)
Season of blood collection, n (%)		
Spring	15 (12)	12 (9)
Summer	31 (24)	32 (24)
Autumn	63 (49)	64 (45)
Winter	20 (15)	24 (18)
Clinical characteristics		
BMI (kg/m2)	28.7 ± 4.6	28.5 ± 4.5
Systolic BP (mmHg)	146 ± 18	146 ± 18
Diastolic BP (mmHg)	81 ± 10	81 ± 9
Waist circumference (cm), median (IQR)	105 (97 - 111)	103 (95 - 109)
Fasting glucose (mmol/l)	7.7 ± 1.1	7.6 ± 1.1
Fasting insulin (mU/l)	15.6 ± 9.9	16.6 ± 10.1
HbA1c (% / mmol/mol)	6.8 ± 0.5 / 51 ± 6	6.8 ± 0.5 / 51 ± 5
HOMA-IR, median (IQR)	4.63 (2.61 - 6.78)	4.59 (3.1 - 7.0)

Table 1. Continued

	Vitamin D group	Placebo group
	n = 129	**n = 132**
QUICKI	0.31 ± 0.03	0.31 ± 0.03
HOMA-B, median (IQR)	64.8 (43.3 - 102.6)	66.7 (46.9 - 122.9)
Cholesterol (mmol/l)	4.4 ± 1.0	4.4 ± 1.0
HDL-cholesterol (mmol/l), median (IQR)	1.1 (1.0 - 1.3)	1.1 (1.0 - 1.3)
Triglycerides (mmol/l), median (IQR)	1.5 (1.1 - 2.0)	1.4 (1.0 - 2.1)
LDL-cholesterol (mmol/l)	2.5 ± 0.9	2.5 ± 0.9
Chol/HDL-ratio (mmol/l)	3.88 ± 1.06	3.92 ± 1.28
Serum 25(OH)D (nmol/l)	60.6 ± 23.3	59.1 ± 23.2
Serum creatinine (µmol/l)	83 ± 18	81 ± 18
Serum calcium (mmol/l)	2.32 (2.28 – 2.38)	2.33 (2.28 – 2.40)
Serum PTH (pmol/l)	5.4 ± 2.2	5.6 ± 2.1

Data are presented as mean ± SD, unless indicated otherwise.
** including nephropathy, neuropathy and retinopathy*
† maximum dose of 400IU vitamin D supplement daily before start of the trial

25(OH)D, 25-hydroxyvitamin D; BMI, body mass index; BP, blood pressure; HDL, high density lipoprotein; HOMA-IR, homeostasis model assessment – insulin resistance; LDL; low density lipoprotein; PTH, parathyroid hormone; QUICKI, quantitative insulin-sensitivity check index.

Performing an exploratory subgroup analysis in severe vitamin D deficient patients (n = 19) demonstrated a significant mean difference of 3.1 mmol/mol in HbA1c after six months of vitamin D supplementation between both groups (β = -3.1, 95% CI: -6.0 to -0.1; p = 0.04). This result remained significant after adjustments for baseline HbA1c, season of measurement and age (β = -3.5, 95% CI: -6.6 to -0.4; p = 0.02) (Table 2c). In the safety profiles, one patient in the treatment group experienced new onset urolithiasis who was excluded after three months. No other side effects were seen in the vitamin D group, in particular, none of the patients developed hypercalcemia during the study.

DISCUSSION

In this double-blind placebo-controlled randomised clinical trial the effect of six months oral vitamin D3 supplementation on glycaemic control was investigated in patients with well-controlled type 2 diabetes. We did not find a significant effect of vitamin D supplementation on glycaemic control and metabolic profile in the entire study population, despite an significant increase in serum 25(OH) D in patients who received vitamin D. A significant effect of vitamin D supplementation on HbA1c was seen after six months in severe vitamin D deficient patients. However, despite we adjusted for baseline HbA1c value, this significant result may be explained by the imbalance in baseline HbA1c value between the group receiving vitamin D compared to placebo.

The current study adds to an increasing body of evidence that vitamin D supplementation in vitamin D sufficient patients with type 2 diabetes does not improve glycaemic control. Unfortunately, studies or subgroup analyses in patients with a vitamin D deficient status regarding glycaemic indices in patients with type 2 diabetes are limited (17-19). Three studies included solely vitamin D deficient (serum 25(OH)D < 50 nmol/l) patients with type 2 diabetes, of which one reported endothelial function as primary outcome (19). In a study performed among 129 Korean patients with a mean serum 25(OH)D level of 26.1 ± 11.2 nmol/l, the patients were randomised into either vitamin D3 2000 IU/day combined with calcium 200 mg or placebo. After a follow-up of 24 weeks no difference was seen in the intervention group on HbA1c or insulin resistance, despite a significant rise in serum 25(OH)D (25.2 ± 9.7 nmol/l to 75.4 ± 25.2 nmol/l) (18).

Our findings are consistent with a recent meta-analysis, which reported that there is insufficient evidence of a beneficial effect to recommend vitamin D supplementation as a means of improving glycaemic control in patients with type 2 diabetes (13). Most studies which investigated the effect of vitamin D supplementation in patients with type 2 diabetes did not find a significant effect on glycaemic control (20-24). However, in one study performed by Nikooyeh et al., in which patients were randomised into plain yoghurt drink or vitamin D3 fortified yoghurt drink (~1000 IU a day), after 12 weeks, there was a significant reduction in HbA1c, HOMA-IR, fasting insulin and glucose (25). Intervention studies among other study populations than type 2 diabetes yielded conflicting results (26-29). Possible reasons for the lack of an effect found in intervention studies might be related to the patient characteristics (e.g. baseline serum 25(OH)D and HbA1c), duration of the study, sample size, dosage or formulation of vitamin D supplement and primary outcome of the studies.
The discrepancy between epidemiologic studies and intervention studies regarding vitamin D status is remarkable; almost all epidemiologic studies reported a significant association between vitamin D status and glycaemic control or incident diabetes, whereas almost all intervention studies did not show an effect of vitamin D supplementation on glycaemic indices. This observation suggests that vitamin D status could be more an expression of ill-health than a cause of poor glycaemic control.
Strengths of our study are the randomised double-blind placebo-controlled study design, the use of a large sample size with similar characteristics at baseline in intervention and control group, the relatively high dose of vitamin D supplementation leading to adequate target levels of serum 25(OH)D. Our study also has important limitations, which may partly be responsible for the lack of an effect found on glycaemic control in the whole study population.

First, our study population consisted of patients who were relatively well controlled in their diabetes regulation, treated with lifestyle advice whether or not in combination with oral hypoglycaemic agents, with the majority of the patients having no cardiovascular complications. Moreover, only 37% of our included patients had a serum 25(OH)D < 50 nmol/l, instead of the estimated 50% to have enough power for a subgroup analysis of patients with a serum 25(OH)D < 50 nmol/l. It is imaginable that vitamin D supplementation is only effective in patients with vitamin D deficiency and that due to a power problem we did not find an effect on glycaemic control in the subgroup with a serum 25(OH)D < 50 nmol/l (type II error).

Second, an important limitation is the use of a large intermittent dose of vitamin D supplementation, which is currently not promoted. Debate is ongoing whether vitamin D supplementation has to be given in a lower oral dosage once daily instead of a higher dose once a month (30). We believe that another supplementation regimen would not have altered our results, because the intervention group achieved a rise in serum 25(OH)D from 60.6 to 101.4 nmol/l, indicating an optimal increase. Another limitation of our study is the use of HOMA-IR to measure insulin resistance instead of hyperinsulinemic-euglycaemic clamp which is the gold standard method to assess insulin sensitivity.
In conclusion, a large intermittent dose of vitamin D supplementation at a level optimising serum 25(OH)D, did not improve glycaemic control in patients with well-controlled type 2 diabetes. Further research among vitamin D deficient patients with poorly regulated type 2 diabetes will be necessary to elucidate the question whether vitamin D supplementation is effective on glycaemic control or if it appears to be a marker of ill health.

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CHAPTER 6

Vitamin D status and health-related quality of life in patients with Type 2 Diabetes

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ABSTRACT

Background

Evidence from longitudinal studies suggest that low levels of vitamin D increase the risk for many adverse health outcomes, including depression, cardiovascular and metabolic diseases. The objective of the present study was to test whether vitamin D status was associated with health-related quality of life in patients with type 2 diabetes.

Methods

Demographic and clinical characteristics including health-related quality of life were obtained from 241 adult patients with type 2 diabetes managed with oral hypoglycaemic agents. Health-related quality of life was assessed by the Short Form 36 (SF-36) Health Survey. Multiple logistic regression analysis was used to investigate the association between vitamin D status and health related quality of life with adjustment for confounders.

Results

Mean age of all patients included was 67 ± 8 years with a mean HbA1c of 52 ± 8 mmol/mol (6.9 ± 0.7%) and mean serum 25-hydroxyvitamin D of 59 ± 23 nmol/l. Vitamin D deficiency (serum 25-hydroxyvitamin D < 50 nmol/l) was present in 38%. No significant associations were found between vitamin D status and health-related quality of life.

Conclusions

Vitamin D status was not associated with health-related quality of life in patients with type 2 diabetes. This could be explained by the relatively high serum 25-hydroxyvitamin D, well-controlled glycaemic control and relatively good health-related quality of life of all patients. A prospective study among vitamin D deficient patients with a poor glycaemic control would be interesting for future research.

INTRODUCTION

Diabetes mellitus is a chronic disease affecting approximately 382 million persons worldwide in 2013 (1). Patients with type 2 diabetes mellitus are at increased risk of developing micro- and macrovascular complications and cardiovascular disease, which subsequently compromises health-related quality of life (HRQOL) (2,3). Previous research has demonstrated that patients with Type 2 diabetes had a poorer (health-related) quality of life, a higher prevalence of generalized anxiety disorder and elevated symptoms of anxiety compared to the general population (2-5). Moreover, depression is common in patients with Type 2 diabetes, with a prevalence up to 24% in women and 13% in men. Depression in diabetes is associated with a considerably lower quality of life, higher HbA1c levels, increased risk of developing diabetes specific micro- and macrovascular complications, an increased mortality risk, and higher health-care costs (6-10).

Much research has been conducted examining the factors that contribute to a decreased quality of life in patients suffering from type 2 diabetes (2,3). A potential factor could be low vitamin D status, which is highly prevalent in patients with type 2 diabetes, and has been linked to quality of life in several other populations with conflicting results (11-16).

Vitamin D is a secosteroid that is obtained from dietary sources, either food or supplements, and exposure to sunlight. It needs to be hydroxylated twice to become biologically active. Besides its classical action in calcium and bone homeostasis, vitamin D deficiency has recently been linked to numerous non-skeletal conditions, including type 2 diabetes. This is the result of the discovery that most tissues and cells, including the brain, immune system and pancreatic β-cells contain the vitamin D receptor and the enzyme 1-alpha hydroxylase to convert serum 25-hydroxyvitamin D (25(OH)D) to its biologically active form which allows intracrine and paracrine functions (17). Vitamin D deficiency is a growing worldwide problem. In a cross-sectional study from a population-based cohort among 538 white Dutch patients with type 2 diabetes aged 60–87 years, the prevalence of serum 25(OH)D < 50 nmol/l was 34% in the summer and up to 51% during winter (11). Low serum 25(OH)D has been linked to quality of life in several observational studies including study populations other than type 2 diabetes, e.g. patients with osteoporosis, Crohn's disease, end-stage renal disease (new on dialysis) and patients suffering from chronic pain (11-15). The mechanism how vitamin D status may affects quality of life in patients with type 2 diabetes is not known. Hypothetically vitamin D may indirectly improve quality of life by influencing glycaemic control which has been correlated to vitamin D status in many observational studies (18). A recent meta-analysis performed by George et al. found a small, non-significant, effect on glycaemic control (i.e. fasting glucose and insulin resistance) after vitamin D supplementation in patients with type 2 diabetes or impaired glucose tolerance compared to controls (19). In addition, vitamin D might influence systemic inflammation, which is linked to insulin resistance in type 2 diabetes, by modulating immune responses and oxidative stress (20). Moreover, vitamin D may have cardio-protective and anti-depressant effects that could help to maintain a good HRQOL (21,22).

The aim of this study is to investigate whether vitamin D status is associated with HRQOL in patients with stable type 2 diabetes managed with oral hypoglycaemic therapy.

PATIENTS AND METHODS

Study design and participants

We conducted a cross-sectional study among 241 patients with type 2 diabetes derived from five general practices in and around Alkmaar, the Netherlands at latitude 52º. The participants were included between July 2012 and April 2013 in a randomised placebo-controlled trial ("the SUNNY trial"), in which the effect of 50,000 IU vitamin D3 once a month during six months versus placebo was examined on glycaemic control in patients with type 2 diabetes. The trial was approved by the Medical Ethics Committee of North-Holland, the Netherlands. The trial protocol is described in greater detail elsewhere (23). In brief, adult patients (≥ 18 years) with type 2 diabetes treated with lifestyle advice, metformin, or sulphonylurea-derivatives, whether or not in combination, were invited for participation in the study. Serum HbA1c had to be stable and below or equal to 64 mmol/mol (8.0%) for the last three months without recent changes in hypoglycaemic agents. The main exclusion criteria were: an impaired renal function (estimated glomerular filtration rate (eGFR) < 30 ml/min calculated from serum creatinine using the MDRD formula), any granuloma forming disorder, hypercalcaemia (serum calcium > 2.65 nmol/l) of any reason, serum 25(OH)D < 15 nmol/l or > 150 nmol/l, urolithiasis, psychiatric treatment for schizophrenia, organic mental disorder or bipolar disorder currently or in the past, insufficient knowledge of the Dutch language and substance abuse (other than nicotine) or no signed informed consent. The patients were allowed to take vitamin D supplements with a maximum dose of 400 IU a day prior to inclusion.

Study variables

The following date were collected: age, gender, ethnicity, marital status, education level, employment status, diabetes duration, diabetic specific complications, medication use and diabetic therapy, co-morbidities, smoking status, alcohol use, dietary fish and dairy intake, physical activity, sun exposure and season of blood collection. Also standard anthropometric data (height, weight) and venous blood collection were obtained from each patient. Serum 25(OH)D was measured on an iSYS automated immunoanalyser (IDS GmbH, Frankfurt, Germany). The total 25(OH) D assay detects 25(OH)D2 and 25(OH)D3, both with a specificity of 100%. The quality of the test is controlled by applying Westgard QC-rules on 3 different QC-samples.

Health-related quality of life (HRQOL)

HRQOL was assessed at baseline using the Short Form 36 (SF-36) Health Survey in Dutch language. The SF-36 consists of 36 questions and set response choices on an ordinal scale. There are eight domains and two summary measures: physical functioning, role limitations due to physical problems, bodily pain, general health perceptions (together presenting the physical component summary), and mental health, vitality, social functioning and role limitations due to emotional problems (together presenting the mental component summary measure) (24). For each domain, the HRQOL scores are converted to a 0 to 100 scale, with higher scores indicating a better HRQOL. The SF-36 has adequate internal consistency (Cronbach's α from 0,65 to 0,94, diabetes specific from 0,76 to 0,93) and test-retest reliability (r = 0.63 - 0.81) (25,26). In 1994 Aaronson et al. translated and validated the Dutch Language version of the SF-36 (Cronbach's α from 0,66 to 0,93, mean 0.84) (27).

Statistical analyses

For the purpose of the present cross-sectional study participants were stratified into two groups according to their vitamin D status: 1) serum 25(OH)D < 50 nmol/l, defined as vitamin D deficiency, and 2) serum 25(OH)D ≥ 50 nmol/l, indicating a sufficient vitamin D status. Patient demographic and clinical characteristics were compared using a Pearson's chi-squared test for categorical variables and an independent sample t-test or mann-whitney test for continuous variables depending on normality. Multiple logistic regression analyses were performed to explore the association between vitamin D status (serum 25(OH)D below and above 50 nmol/l) and each domain of HRQOL. All analyses were adjusted for age, gender, season of measurement, pre-existing cardiovascular disease and BMI, based on earlier literature and in case of a regression correlation coefficient difference > 10%. Subgroup analyses regarding glycaemic control (HbA1c ≥ 53 mmol/mol [≥ 7.0%]) and poor level of HRQOL for each domain (using national Dutch means stratified by gender found by Aaronson et al. to distinguish reduced HRQOL) (27), were performed using the same method as the primary analysis. Subgroup analyses regarding patients with a reduced HRQOL however, were solely adjusted for season of measurement and BMI due to the small number of patients included in this analysis resulting in insufficient power to correct for all confounders. All analyses were also performed stratified by gender due to differences in HRQOL between men and women. All data were analysed using the statistical package SPSS software (IBM version 20.0; SPSS Inc., Chicago, IL, USA). Data are presented as numbers (%), median (interquartile range) or means ± standard deviation. A p-value of < 0.05 was considered as statistically significant.

RESULTS

A total of 300 patients were recruited in 'the SUNNY trial' of whom 275 appeared at the first visit and were randomised. Of the 275 patients included, 241 (88%) returned their SF-36 questionnaire which they received at the first visit. Mean age was 67 ± 8 years and 65% were men. The median diabetes duration was 6 (3 - 8) years with a mean HbA1c of 52 ± 8 mmol/mol (6.9 ± 0.7%). Overall mean serum 25(OH)D was 59 ± 23 nmol/l. The prevalence of vitamin D deficiency, serum 25(OH)D < 50 nmol/l, was 38% and 150 patients (68%) had a serum 25(OH)D ≥ 50 nmol/l. . Demographic and clinical characteristics, and HRQOL scores of all participants and stratified by serum 25(OH)D below and above 50 nmol/l are presented in Table 1.

Table 1. Demographic, clinical characteristics and health-related quality of life of all patients and stratified by vitamin D level.

		Serum 25(OH)D		
	All patients	**<50 nmol/l**	**≥ 50 nmol/l**	**p-value**
	n = 241	**n = 91**	**n = 150**	
Demographic characteristics				
Male (%)*	157 (65)	52 (57)	105 (70)	0.04
Age (years)	67 ± 8	67 ± 9	67 ± 8	0.13
Diabetes duration (years)	6 (3 - 8)	5 (4 - 8)	6 (3 - 8)	0.38
White skin colour (%)*	224 (93)	77 (85)	147 (98)	<0.01
Anti diabetic treatment (%)				0.32
Lifestyle adjustments	11 (5)	5 (6)	6 (4)	
Metformin	143 (59)	48 (53)	95 (63)	
SU-derivates	10 (4)	3 (3)	7 (5)	
Metformin and SU-derivates	77 (32)	35 (39)	42 (28)	
Microvascular complications† ≥1 (%)	52 (22)	19 (21)	33 (22)	0.84
Cardiovascular disease (yes, %)	78 (32)	27 (30)	51 (34)	0.49
Single (%)*	45 (19)	26 (29)	19 (13)	<0.01
Education level (%)				0.73
Low	91 (38)	33 (36)	58 (39)	
Middle	83 (34)	30 (33)	53 (35)	
High	67 (28)	28 (31)	39 (26)	
Employment status (%)				0.34
Paid employment	68 (28)	29 (32)	39 (26)	
Unemployed or incapacitated	19 (8)	9 (10)	10 (7)	
Retired	154 (64)	53 (58)	101 (67)	
Current smoker (%)*	35 (15)	19 (21)	16 (11)	0.03
Alcohol use >2 units/day (%)	30 (12)	14 (15)	16 (11)	0.28
Physical activity (%)*				0.04
<2 hours/week	78 (32)	37 (41)	41 (27)	
2-5 hours/week	109 (45)	40 (44)	69 (46)	
>5 hours/week	54 (22)	14 (15)	40 (27)	
Sun exposure (%)*				<0.01
<5 hours/week	99 (41)	52 (57)	47 (31)	
5-10 hours/week	109 (45)	35 (39)	74 (49)	
>10 hours/week	33 (14)	4 (4)	29 (19)	
Season of blood collection (%)				0.29
Spring	27 (11)	13 (14)	14 (9)	
Summer	57 (24)	16 (18)	41 (27)	
Autumn	121 (50)	48 (53)	73 (49)	
Winter	36 (15)	14 (15)	22 (15)	

Table 1. Continued

		Serum 25(OH)D		
	All patients	**<50 nmol/l**	**≥ 50 nmol/l**	**p-value**
	n = 241	**n = 91**	**n = 150**	
Clinical characteristics				
BMI (kg/m^2)*	28.6 ± 4.5	29.5 ± 4.7	28.0 ± 4.3	0.01
HbA1c (mmol/mol)	52 ± 8	52 ± 10	52 ± 6	0.50
HbA1c (%)	6.9 ± 0.7	6.9 ± 0.9	6.9 ± 0.6	0.50
e-GFR	83 ± 18	84 ± 20	82 ± 17	0.42
Serum 25(OH)D (nmol/l)*	59 ± 23	37 ± 8	73 ± 17	<0.01
Serum PTH (pmol/l)	5.5 ± 2.2	5.7 ± 1.9	5.4 ± 2.4	0.25
Health-related quality of life				
Physical component summary:	76 (60 - 88)	77 (60 - 88)	76 (60 - 88)	0.87
Physical functioning	85 (65 - 95)	80 (60 - 90)	85 (70 - 95)	0.06
Role limitations physical	100 (50 - 100)	100 (50 - 100)	100 (50 - 100)	0.28
Bodily pain	74 (52 - 100)	72 (51 - 100)	74 (52 - 100)	0.84
General health perceptions	62 (47 - 77)	62 (47 - 72)	67 (52 - 77)	0.36
Mental component summary:	87 (72 - 93)	89 (68 - 94)	86 (74 - 92)	0.63
Mental health	84 (68 - 92)	84 (64 - 92)	84 (72 - 92)	0.67
Role limitations emotional	100 (100 - 100)	100 (100 - 100)	100 (100 - 100)	0.75
Vitality	70 (55 - 85)	70 (50 - 85)	70 (55 - 80)	0.95
Social functioning	100 (75 - 100)	100 (88 - 100)	100 (75 -100)	0.31

Data are presented as numbers (%), mean ± SD or median (IQR)
** p-value <0.05*
† Including retinopathy, nephropathy and neuropathy
25(OH)D, 25-hydroxyvitamin D; BMI, body mass index; e-GFR, estimated glomerular filtration rate; PTH, parathyroid hormone

Vitamin D deficient patients consisted of more women, had more often a dark coloured skin, had a higher incidence of smoking, less physical activity and sun exposure, had a higher BMI, and were more often single for marital status compared to the group with a serum 25(OH)D ≥ 50 nmol/l (all $p < 0.05$). No differences were observed in the eight HRQOL domains, nor in the physical or mental component summary, between both groups. HRQOL scores were higher in men compared to women, particularly the domains physical functioning, bodily pain, social functioning, mental health, vitality, and general health perceptions (data not shown).
Concerning the main outcome, the association between HRQOL and serum 25(OH)D in patients with type 2 diabetes, no significant associations were observed after correction for the following confounders: age, gender, season of measurement, pre-existent cardiovascular disease and BMI

(Table 2). In addition, no effect was seen in the summary components as well. A significant association was observed between physical functioning and serum 25(OH)D ≥ 50 nmol/l (OR: 1.01, 95% CI: 1.00 - 1.03, p = 0.04), however this remained no longer significant after adjustment for confounders. Pre-specified subgroup analysis in patients with a reduced glycaemic control (HbA1c ≥ 53 mmol/mol, [≥ 7.0%], n = 71) demonstrated a small association between serum 25(OH)D ≥ 50 nmol/l and role limitations due to emotional problems before and after adjustment for confounders (OR: 1.02, 95% CI: 1.00 - 1.04, p = 0.02) (data not shown). The results did not differ significantly including only patients with a relatively poor HRQOL for one of the SF-36 domains.

Table 2. Association between HRQOL and vitamin D status.

	Serum 25(OH)D ≥ 50 nmol/l					
	Model 1			Model 2		
	OR	95% CI	p	OR	95% CI	p
Physical component summary:	1.00	0.99 - 1.02	0.57	1.00	0.99 - 1.02	0.99
Physical functioning	1.01	1.00 - 1.03	0.04*	1.01	1.00 - 1.03	0.14
Role limitations physical	0.99	0.99 - 1.00	0.43	1.00	1.00 - 1.00	0.39
Bodily pain	1.00	0.99 - 1.01	0.66	0.99	0.99 - 1.01	0.65
General health perceptions	1.01	0.99 - 1.02	0.32	1.01	0.99 - 1.02	0.46
Mental component summary:	1.00	0.99 - 1.02	0.60	1.00	0.99 - 1.02	0.63
Mental health	1.00	0.99 - 1.02	0.77	1.00	0.98 - 1.02	0.91
Role limitations emotional	1.00	1.00 - 1.01	0.42	1.00	1.00 - 1.01	0.36
Vitality	1.00	0.99 - 1.02	0.61	1.00	0.99 - 1.02	0.86
Social functioning	0.99	0.98 - 1.01	0.66	0.99	0.98 - 1.01	0.59

Model 1: unadjusted
Model 2: adjusted for age, gender, season of measurement, pre-existing cardiovascular disease and BMI
**p-value <0.05*

DISCUSSION

In this cross-sectional study among Dutch patients with type 2 diabetes managed with oral hypoglycaemic therapy, no association between serum 25(OH)D and HRQOL was observed. The question whether vitamin D status and HRQOL are associated has not been studied before in patients with type 2 diabetes. However, studies examining the association between vitamin D status and (health-related) quality of life in other populations are available. In a large population-based cohort study among 15.954 post-menopausal women a small difference on mental HRQOL was seen between

women with a low (< 400 IU/day) and high vitamin D3 intake (≥ 800 IU/day). However, this result was attenuated after controlling for the confounders checked in this study: age, energy intake, BMI, education, smoking, living arrangement, antidepressant usage, comorbidity history, and physical activity (16). In contrast with our study results, a recent population-based observational study among Dutch patients aged > 70 years, found a significant lower score on the physical component of HRQOL in patients with a severe vitamin D deficient status (serum 25(OH)D < 25 nmol/l). However, physical performance, depressive symptoms and the number of chronic diseases explained this association largely (28).

Despite our study population was at risk for vitamin D deficiency, mean serum 25(OH)D was 59 ± 23 nmol/l and only nine patients had a serum 25(OH)D < 25 nmol/l, defined as severe deficient vitamin D status. This relatively high vitamin D status could be an explanation for the lack of an association found between serum 25(OH)D and HRQOL in our study population. Our results regarding physical activity and sun exposure, with less physical activity and sun exposure reported among vitamin D deficient patients, is equivalent with today's literature (29). It can be hypothesised that active patients go outside more often, resulting in higher exposure to sunlight and subsequently in higher serum 25(OH)D levels. To explore the influence of season of measurement, which could play an important role in HRQOL, we stratified all patients by season of inclusion. These results showed no better HRQOL of patients included in summer or spring season (Appendix 1). Also notable is the significant difference between both vitamin D groups in number of single participants. Hypothetically single persons go less outside into the sunlight compared to persons who have a partner. HRQOL scores were higher in men compared to women in all domains and significantly higher in six domains. These findings are consistent with the results of a Dutch population-based study investigating HRQOL using the SF-36 questionnaire (27). In addition, Wexler et al. found a correlation between female sex and a decreased HRQOL in patients with type 2 diabetes using the Health Utilities Index-III to evaluate HRQOL (3).

Several limitations in our study need to be discussed. First, our study population, selected from general practices, consisted of patients with type 2 diabetes with well-controlled glycaemic control (mean HbA1c: 52 ± 8 mmol/mol [6.9 ± 0.7%]). This could implicate relative low disease burden with less physical and mental limitations. In addition, men in our study population had HRQOL scores mainly comparable with the general population (27). In women, a lower level of HRQOL was seen in five domains compared to the general population, however stratifying the study population by gender did not change the results significantly. Given the low prevalence of diabetes-specific complications and the fact that the participants were treated with lifestyle advice and/or oral hypoglycaemic agents, we assume that our study population consisted of patients with a relatively good HRQOL, almost comparable with the general population. This could be another possible reason for not finding an association between serum 25(OH)D and HRQOL in our study. An important limitation in our study, which could have altered the results regarding HRQOL, is that we had no detailed information regarding previous falls and fractures of all patients included. It is known that patients with a low vitamin D status have a higher risk for falls and fractures and this could have influenced HRQOL. Second, in several HRQOL domains, high percentages of men and women with a maximum score on the SF-36 questionnaires were seen. Because of the inability of the SF-36 questionnaire to record HRQOL above the maximum, it is impossible to distinct any differences between patients or vitamin

D groups, if there are any. This prompted us to question the applicability of the SF-36 questionnaire in patients with type 2 diabetes derived from general practices. In line with this thought, Woodcock et al. compared the SF-36 questionnaire with a diabetes-specific questionnaire (Audit of Diabetes Dependent Quality of Life) in examining HRQOL among patients with type 2 diabetes in general practices, resulting in a recommendation for adding a diabetes-specific questionnaire to the SF-36 questionnaire due to the more accurate measurement of diabetes-specific components of type 2 diabetes (30).

Selection bias in this study might exist as patients with a poor quality of life and/or a mental disorder may have less energy and motivation to participate in the study, and if included, respond to the questionnaire. To partly resolve this point, we compared the demographic and clinical characteristics between all patients who did return their questionnaire (n = 241) and who did not (n = 34). This demonstrated no significant differences.

Furthermore this cross-sectional analysis demonstrates secondary outcomes of a randomised placebo-controlled trial (SUNNY trial) with the effect of vitamin D supplementation on glycaemic control as primary outcome for which the sample size was calculated. For this secondary outcome we could question if the sample size was large enough to observe differences in HRQOL anyway. By performing a logistic regression analysis with a binary outcome this limitation taken into account as much as possible.

In conclusion, no significant association was found between vitamin D status and HRQOL among patients with type 2 diabetes managed with oral hypoglycaemic therapy. This could be explained by the relatively good glycaemic control, high mean serum 25(OH)D, and high HRQOL scores of the study participants. Consequently, a randomised placebo-controlled trial in patients with type 2 diabetes with a poor glycaemic control and/or insulin treatment will be of high interest.

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CHAPTER 7

Effect of vitamin D supplementation on health-related quality of life in patients with type 2 diabetes mellitus: a randomised double-blind placebo-controlled trial

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ABSTRACT

Background

Increased levels of depressive symptoms, fatigue or pain (all dimensions of reduced health-related quality of life, [HRQOL]) are common in patients with type 2 diabetes. Earlier studies have reported associations between low vitamin D status and fatigue and depressive symptoms. The aim of the present study was to examine the effects of vitamin D supplementation on dimensions of HRQOL in patients with type 2 diabetes.

Methods

In a randomised, double-blind, placebo-controlled trial in which the effect of monthly cholecalciferol 50,000 IU versus placebo was assessed in 275 adults with type 2 diabetes derived from general practices, we also collected data on HRQOL at baseline and after six months using the Short Form 36 Health Survey (SF-36). Linear regression analyses were used to compare the change in HRQOL over time between the vitamin D and placebo group.

Results

187/275 (68%) completed baseline and follow-up SF-36 and were included in the analysis. Median serum 25-hydroxyvitamin D almost doubled in the intervention group compared to the placebo group (58.5 to 106.0 nmol/l versus 60.0 to 61.5 nmol/l, respectively). A small significant difference (adjusted B: -8.90; 95% CI: -17.16 to -0.65) between both groups was seen concerning the SF-36 domain role limitations due to physical problems in disadvantage of the vitamin D group.

Conclusions

Six months of vitamin D supplementation did not improve HRQOL in patients with type 2 diabetes managed on oral antidiabetic therapy.

INTRODUCTION

With a total number of 415 million patients in 2015, expecting to increase to a number of 642 million patients in 2040, diabetes mellitus is a growing worldwide epidemic. It is common knowledge that patients with diabetes mellitus are at increased risk for micro- and macrovascular complications, including neuropathy, nephropathy, retinopathy, peripheral artery disease and cardiovascular disease (1). Furthermore, in patients with type 2 diabetes, relatively high prevalence of depression, fatigue and (neuropathic) pain were found resulting in a decreased quality of life (2-6). Depressive symptoms and fatigue in patients with diabetes are related to an increased risk of developing diabetes-specific complications (2,3). Moreover, patients with depressive symptoms and diabetes had an almost 50% increased all-cause mortality rate, probably due to non-optimal self-care (2,7).

Low vitamin D status is common in patients with type 2 diabetes (8) and previous observational studies demonstrated an association between low vitamin D status and a reduced health-related quality of life (HRQOL), fatigue and depressive symptoms (9-16). Two recent meta-analyses (17,18) based on the results of randomised controlled trials which investigated the effect of vitamin D supplementation on depressive symptoms, suggest an improvement of depressive symptoms after vitamin D supplementation (17,18). Intervention studies concerning the effect of vitamin D supplementation on fatigue are scarce and the studies that have been executed are difficult to compare as their research designs are very different. Lima et al. (19) performed a randomised placebo-controlled trial in adolescents and young adults with juvenile systemic lupus erythematosus and found a significant reduction of "fatigue related to social life" score (when using the Kids Fatigue Severity Scale) in the vitamin D group compared to placebo after 24 weeks of oral cholecalciferol 50,000 IU per week (19). In addition, a significantly improve in fatigue score in all five scales (general, physical, emotional, mental and vigor) of the Multidimensional Fatigue Symptom Inventory Short Form was seen in primary care patients with a low vitamin D status and fatigue as their main problem, after five weeks of vitamin D supplementation (oral ergocalciferol 50,000 IU three times per week) (20). However, this study was not blinded or placebo-controlled. It should be noted that the majority of the patients included in these studies did not have type 2 diabetes.

The biological mechanisms linking vitamin D status to HRQOL, depressive symptoms, and fatigue in patients with type 2 diabetes are not clear. Hypothetically, vitamin D deficiency may contribute to poor glycaemic control (21), which in turn leads to a higher risk to develop micro- and macrovascular complications in the long-term (21). Furthermore, due to immuno-modulatory properties of vitamin D and its association with oxidative stress, vitamin D may influence low-grade systemic inflammation, which is linked to both depressive symptoms and insulin resistance (22,23). Another possible link between vitamin D status and depressive symptoms is an elevated parathyroid hormone (PTH) level that has been linked to depressive symptoms and insulin resistance and is increased in state of vitamin D deficiency (24,25). Moreover, vitamin D itself seems to have cardio-protective effects as well (26). Based on these findings, we hypothesise a positive effect of vitamin D supplementation on fatigue and depressive symptoms in patients with type 2 diabetes. The aim of this study was to test whether six months of vitamin D supplementation improves the Short Form 36 (SF-36) Health Survey domain scores, especially the domains physical functioning, role limitations due to physical problems, social functioning, role limitations due to emotional problems, mental health and vitality, in patients with type 2 diabetes, using a randomised double-blind placebo-controlled trial design.

RESEARCH DESIGN AND METHODS

Study design and patients

The SUNNY trial (acronym for study the effect of vitamin D supplementation on glycaemic control in type 2 diabetes) is a double-blind randomised placebo-controlled clinical trial, with the primary aim to determine the effect of vitamin D supplementation on glycaemic control in patients with type 2 diabetes (27,28). Secondary aim was to investigate whether vitamin D supplementation improved dimensions of HRQOL (28). The trial was conducted in five general practices in and around the city of Alkmaar, the Netherlands, latitude 52º, between July 2012 and April 2013. Adult persons (≥ 18 years) with type 2 diabetes treated with lifestyle advice, metformin, and/or sulfonylurea-derivatives (SU-derivatives) were invited for participation in the study. Serum HbA1c had to be stable and below or equal to 8.0% (64 mmol/mol) for the last three months without recent changes in hypoglycaemic agents. Main exclusion criteria were: an impaired renal function (estimated glomerular filtration rate (eGFR) < 30 ml/min calculated from serum creatinine using the MDRD formula), any granuloma forming disorder, hypercalcemia (serum calcium > 2.65 nmol/l) of any reason, serum 25-hydroxyvitamin D (25(OH)D) < 15 nmol/l or > 150 nmol/l, urolithiasis, psychiatric treatment for schizophrenia, organic mental disorder or bipolar disorder currently or in the past, insufficient knowledge of the Dutch language and substance abuse (other than nicotine) or no signed informed consent. Withdrawal criteria for premature termination of the trial were: increase of HbA1c > 8.5% (69 mmol/mol), hypersensitivity to cholecalciferol or placebo, onset of urolithiasis, any change in antidiabetic medication or serum 25(OH)D < 15 or > 250 nmol/l.

This trial protocol was approved by the Medical Ethics Committee of Noord-Holland, the Netherlands and was conducted according to the principles of the Declaration of Helsinki [NTR3154]. A detailed description of the protocol can be found elsewhere (28).

Intervention

All participants were randomised according to either an oral dose of cholecalciferol
50,000 IU once a month or an identically looking placebo 50,000 IU once a month for 6 months (Meander Medical Center, Amersfoort, the Netherlands).

Outcome measures

Change in HRQOL after six months of vitamin D supplementation was one of the secondary outcomes described in the study protocol of the SUNNY trial (28). HRQOL was assessed at baseline and six months after baseline, using the Dutch version of the Short Form 36 (SF-36) Health Survey, which was translated and validated by Aaronson et al. in 1994 (29). The SF-36 questionnaire is composed of 36 questions and represents eight domains and two summary measures: physical functioning, role limitations due to physical problems, bodily pain, general health perceptions (together presenting the physical component summary), mental health, vitality, social functioning and role limitations due to emotional problems (together presenting the mental component summary). For each domain, scores are summed and converted to a scale from 0 to 100, with lower scores indicating a poorer HRQOL (30).

Demographic data, medical history, medication use and diabetes specific elements (treatment, complications, duration) were collected from medical records and during interviews. Lifestyle

information including smoking status (yes/no), alcohol use (units per week), sun exposure (hours per week), and physical activity (hours per week) were self-reported and gathered through interviews. Standard anthropometric data (height, weight) and venous blood collection were obtained from each patient. Serum 25(OH)D was measured using an iSYS automated immunoanalyzer (IDS GmbH, Frankfurt, Germany). Data were collected at baseline and after six months.

Randomisation

The patients were randomised 1:1 according to the method of block randomisation with a block size of 10. No stratification was used. The randomisation procedure was performed by the pharmacist. The participants and the research team remained blinded until the end of the study.

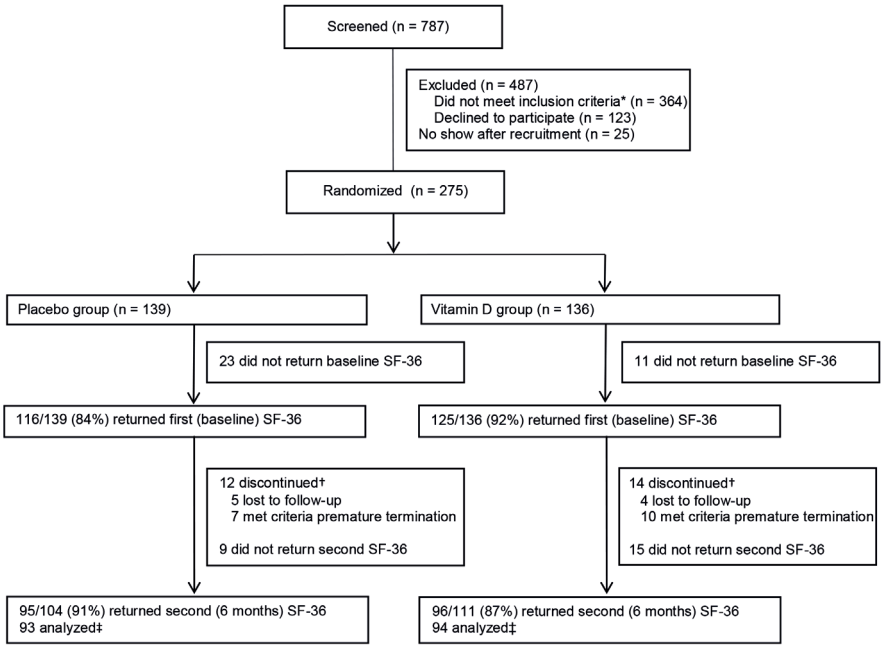
Statistical Analysis

Patients who completed the study (returned questionnaires at baseline and 6 months) were included in the statistical analyses. In case of one or two missing SF-36 domains, linear imputation was used, when more SF-36 domains were missing the patients were excluded. All data were analyzed using the Statistical Package of the Social Sciences (SPSS software, version 20.0; SPSS Inc., Chicago, IL). Baseline characteristics were presented as means ± SD, frequencies (%), or as median (interquartile range [IQR]) in case of a skewed distribution. Linear regression analysis was used to assess the mean difference between intervention and placebo group after six months (mean difference reported as B and β). Change in SF-36 domain score was analyzed as dependent outcome with randomisation group as explanatory variable. To correct for regression to the mean, all analyses were adjusted for its baseline value. In case of skewed distribution, the separate SF-36 domains were log transformed. As we know that men and women provide different outcome on the SF-36 questionnaire and estrogen use may increase the concentration of the vitamin D binding protein and improve hydroxylation of vitamin D in the liver, the models will be tested for effect modification by gender (29,31). Furthermore, all analyses were corrected for confounding variables, which were selected based on literature, including age, gender (if no effect modification), BMI, and season of blood collection. Subgroup analyses among patients with low vitamin D status, defined as 25(OH)D < 50 nmol/l according to the practical guidelines of the Endocrine Society, were performed (32).
A two sided p-value of 0.05 or smaller was considered as significant.

RESULTS

A total number of 787 patients were screened for eligibility of which 300 persons were recruited and finally 275 persons (no show: n = 25) were randomised to either vitamin D supplementation (n = 136) or placebo (n = 139) (Figure 1). 487 (62%) patients were excluded from the study because they did not met the inclusion criteria (75%, mostly because they used insulin) or refused to participate (25%).

Figure 1. Participant flow chart



**Most patients did not meet the inclusion criteria because of insulin therapy*

† Did not received second SF-36

‡ 2 patients excluded from analyses because ≥ 2 SF-36 domains were missing at baseline or follow-up

Table 1. Baseline demographic and clinical characteristics in the vitamin D group and the placebo group (n = 187)

	Vitamin D group n = 94	Placebo group n = 93
Demographic parameters		
Age (years)	67 ± 8	68 ± 9
Male (%)	68 (72)	57 (61)
Diabetes duration (years)	6 (3 - 8)	6 (4 - 8)
White skin colour (%)	91 (95)	90 (95)
Antidiabetic treatment (%)		
Lifestyle adjustments	3 (3)	6 (7)
Metformin	66 (70)	48 (52)
SU-derivatives	2 (2)	5 (5)
Metformin and SU-derivatives	23 (25)	34 (37)
Microvascular complications* ≥1 (%)	25 (27)	13 (14)
Cardiovascular disease (yes, %)	28 (30)	33 (36)
Single (%)	8 (9)	20 (22)

Table 1. Continued

	Vitamin D group n = 94	Placebo group n = 93
Education level (%)		
Low	63 (69)	64 (72)
Middle	21 (23)	18 (20)
High	7 (8)	8 (9)
Employment status (%)		
Paid employment	24 (26)	25 (26)
Unemployed or incapacitated	8 (9)	7 (8)
Retired	62 (66)	61 (66)
Alcohol use >2 units/day (%)	12 (13)	12 (13)
Current smoker (%)	15 (16)	13 (14)
Physical activity (%)		
< 2 hours/week	31 (33)	22 (24)
2-5 hours/week	40 (43)	52 (56)
> 5 hours/week	23 (25)	19 (20)
Sun exposure (%)		
< 5 hours/week	34 (36)	37 (40)
5-10 hours/week	46 (49)	44 (47)
> 10 hours/week	14 (15)	12 (13)
Season of blood collection (%)		
Spring	12 (13)	8 (9)
Summer	23 (25)	20 (22)
Autumn	43 (46)	49 (53)
Winter	16 (17)	16 (17)
Clinical characteristics		
BMI (kg/m2)	27.7 (26.0 - 31.2)	27.5 (25.3 - 30.6)
HbA1c (%)	6.8 (6.4 - 7.2)	6.8 (6.4 - 7.0)
HbA1c (mmol/mol)	51 (46 - 55)	51 (46 - 53)
Serum 25(OH)D (nmol/l)	59.0 (43.0 - 75.0)	60.0 (44.0 - 74.0)
Serum PTH (pmol/l)	5.1 (3.8 - 6.8)	5.2 (4.0 - 6.5)
Health-related quality of life		
Physical functioning	85 (70 - 95)	85 (65 - 95)
Role limitations physical	100 (50 - 100)	100 (50 - 100)
Bodily pain	74 (52 - 100)	74 (62 - 100)
General health perceptions	67 (47 - 77)	62 (47 - 72)
Mental health	88 (76 - 92)	80 (64 - 92)
Role limitations emotional	100 (100 - 100)	100 (100 - 100)
Vitality	75 (60 - 85)	70 (55 - 85)
Social functioning	100 (88 - 100)	100 (75 - 100)
Physical component summary	80 (60 - 91)	76 (63 - 87)
Mental component summary	87 (74 - 91)	82 (70 - 90)

25(OH)D, 25-hydroxyvitamin D; BMI, body mass index; PTH, parathyroid hormone
Continuous variables are presented in mean ± SD or median (IQR) depending on normality.
Categorical variables are presented in numbers (%).
**Including retinopathy, nephropathy and neuropathy*

During the trial, 17 patients met the withdrawal criteria for premature termination due to change in antidiabetic medication (n = 10), HbA1c > 8.5% (> 69 mmol/mol) (n = 5) or serum 25(OH)D <15 or > 250 nmol/l (n = 2) and nine patients were lost to follow up. SF-36 response rate at baseline was 88% (241/275) and 89% at six months of follow-up (191/215), total SF-36 response rate was 70% (191/275). Linear imputation was executed in four patients at baseline and two patients at follow-up for the SF-36 domains role limitations due to physical problems, general health perceptions and role limitations due to emotional problems. Four patients were excluded because information on two or more SF-36 domains were missing, resulting in 187/275 (68%) patients with complete data.
Baseline demographic, clinical characteristics and HRQOL of all patients included in the vitamin D group and in the placebo group are presented in Table 1. Mean age was 68 years ± 8 and 67% of the patients were men. The median diabetes duration was 6 years (3 - 8) with a median HbA1c of 6.8 (6.4 - 7.1%) [51 (46 - 54 mmol/mol)]. Overall mean serum 25(OH)D was 61.1 ± 22.6 nmol/l. At baseline 63 patients (34%) had a serum 25(OH)D level of 50 nmol/l or less; a serum 25(OH)D level between 50 and 75 nmol/l was present in 79 patients (42%) and 45 patients (24%) had a serum 25(OH)D > 75 nmol/l. After six months of vitamin D supplementation, the median 25(OH)D level almost doubled in the vitamin D group from 58.5 (43.0 - 75.0) to 106.0 (85.0 - 117.0) nmol/l whereas in the placebo group the 25(OH)D level remained stable (serum 25(OH)D: 60.0 (44.0 - 74.0) to 61.5 (37.0 - 85.5) nmol/l). In the intervention group, 73% of the patients achieved a serum 25(OH)D level ≥ 75 nmol/l at three months, and 84% after six months of vitamin D supplementation. No differences in baseline characteristics were seen between the patients who were randomised (n = 275) and those finally analysed (n = 187) (data not shown).

Serum 25(OH)D and HRQOL

The present study revealed that vitamin D supplementation did not affect HRQOL (Table 2) in patients with type 2 diabetes. No effect modification by gender was seen (data not shown). A small significant difference, to the detriment of the vitamin D group, was observed in the SF-36 domain role limitations due to physical problems (adjusted B: -8.90; 95% CI: -17.16 to -0.65).
In the group patients with 25(OH)D < 50 nmol/l (34%) mean age was 67 years ± 8, 56% of the patients were men and mean serum 25(OH)D was 38 ± 8 nmol/l. Linear regression revealed no differences in HRQOL between the vitamin D and placebo group in this pre specified subgroup analysis (data not shown).

Table 2. Health-related quality of life (SF-36 domains) (n = 187)

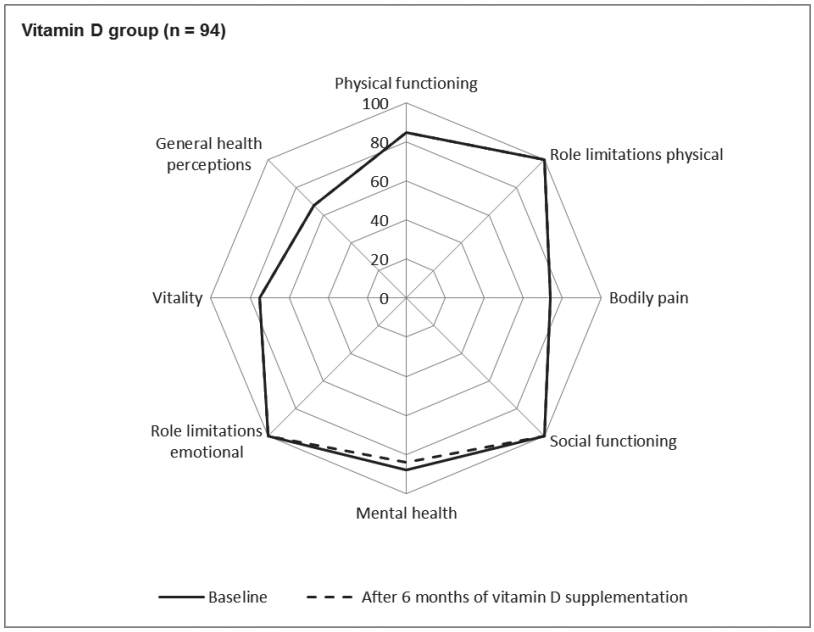
	Δ Vitamin D group (n = 94)	Δ Placebo group (n = 93)	β	B*	95% CI	p
Physical functioning	-0.55 ± 12.77	1.21 ± 11.70	-0.062	-1.51	-4.99; 1.96	0.39
Role limitations physical	-5.32 ± 32.77	4.84 ± 32.61	-0.138	-8.90	-17.16; -0.65	0.04†
Bodily pain	-0.24 ± 19.33	2.40 ± 16.59	-0.070	-2.52	-7.30; 2.27	0.30
General health perceptions	0.37 ± 13.39	3.10 ± 13.61	-0.063	-1.71	-5.44; 2.02	0.37
Mental health	-1.68 ± 11.78	-0.12 ± 13.09	-0.033	-0.83	-4.42; 2.77	0.65
Role limitations emotional	-3.72 ± 34.92	1.08 ± 33.50	-0.063	-4.31	-13.00; 4.37	0.31
Vitality	-2.71 ± 13.35	-1.00 ± 12.17	-0.064	-1.62	-5.11; 1.88	0.36
Social functioning‡	0.00 (-12.50 - 0.00)	0.00 (-12.50 - 0.00)	0.95	0.95	0.80; 1.11	0.49
Physical component summary	-1.50 ± 13.82	2.89 ± 11.39	-0.150	-3.77	-7.26; -0.28	0.04†
Mental component summary‡	0.79 (-6.38 - 6.00)	0.00 (-4.50 - 7.50)	0.93	0.97	0.91; 1.04	0.34

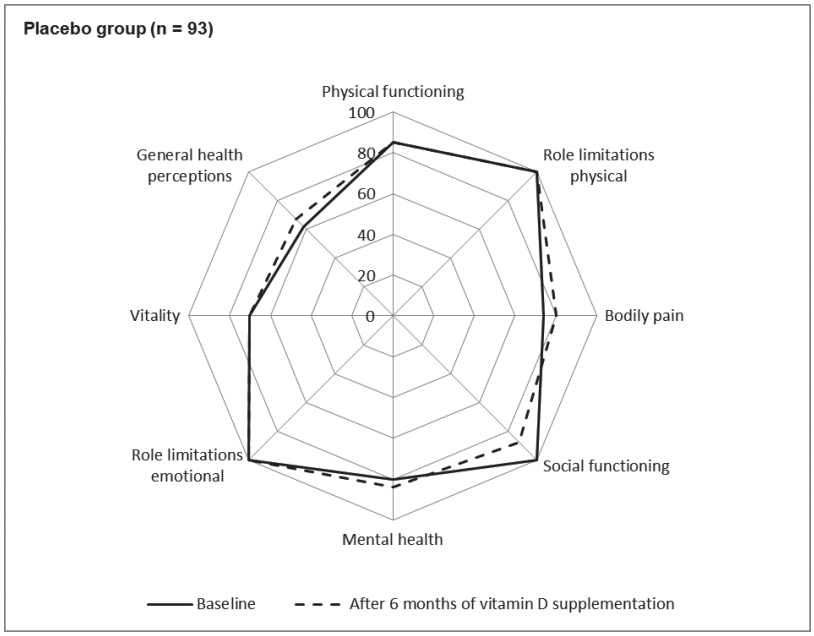
A positive β value indicates an increase in the SF-36 domain in the vitamin D group compared to the placebo group.

**Adjusted for age, gender, BMI, baseline SF-36 domain, baseline 25-hydroxyvitamin and season of blood collection*

† $p < 0.05$; ‡ Using log-transformed values.

Figure 2. Health-related quality of life domains (SF-36) in the vitamin D group (upper) and placebo group (lower); baseline versus after six months of vitamin D supplementation





DISCUSSION

In this randomised, double-blind, placebo-controlled trial in Dutch patients with well-controlled type 2 diabetes treated in general practice, we found a statistically significant decline (B: -8.90; 95% CI: -17.16 to -0.65) in the SF-36 domain "role limitations due to physical problems" after six months of vitamin D supplementation. However, concerning the remaining SF-36 domains no effect of vitamin D supplementation was found.

Before interpreting the results of our study it should be emphasised that the SF-36 domain-scores were not standardized, they are calculated from different numbers of questions with different types of set response choices resulting in a fixed value per question which is domain-specific. Considering the SF-36 domain role limitations due to physical problems which represent only four yes or no questions, thus valuing every question with twenty-five points, we interpret the statistically significant finding with a beta of only 0.138 (B: -8.90; 95% CI: -17.16 to -0.65) as clinically not relevant (30).

Other studies exploring the effect of vitamin D on HRQOL in patients with diabetes are scarce. A recent systematic review from Hoffmann et al. (33), categorised fifteen articles (of which seven randomised placebo-controlled trials) which examined the effect of vitamin D supplementation on HRQOL according to length of intervention (more or less than six months) and study population (healthy versus disease subjects; no studies focusing on specially diabetes were included). In contrast to our results, in four of the seven studies, which were derived from the group with diseased subjects and vitamin D intervention for six months or less, an improvement of HRQOL (especially in the domains role limitations due to physical problems, bodily pain, vitality and physical functioning, however only two studies used (a variation of) the SF-36) after vitamin D supplementation was found, which was interpreted by the researchers as evidence for an small to moderate positive effect of short term vitamin D supplementation on HRQOL in diseased subjects (33). However, no meta-analysis could be done due to the great heterogeneity in study samples, dose and type of vitamin D supplementation and the variation of HRQOL instruments that had been used. Earlier study results should be viewed with caution as the quality of evidence is low due to poor methodological quality. Also, many of the differences in HRQOL that have been reported were small and not likely to be of value in the clinical setting. In addition, the only randomised placebo-controlled trial in this review with the maximal points for methodology, found no effect of vitamin D supplementation (daily oral 800 IU vitamin D3) on the physical component summary or mental component summary in elderly subjects > 70 years with previous low trauma osteoporotic fracture using the SF-12 (shortened version of the SF-36) after 24 - 62 months of follow-up (34).

Moreover, one recent double-blind, placebo-controlled study including 60 patients receiving hemodialysis of whom 55% had a history of diabetes, did not demonstrate an effect of vitamin D supplementation (cholecalciferol 50.000 IU/week for eight weeks followed by 50.000 IU/month for four months) on HRQOL (using KDQOL-36, a kidney disease-specific measure of HRQOL including several parts of the SF-36 questionnaire) after six months of follow-up (35).

The main limitation of our study, which could explain that we found no positive effect of vitamin D supplementation on HRQOL in the present study, is the relatively good baseline HRQOL of several SF-36 domains in our study population that may have resulted in ceiling effects. In addition, the SF-36 domain scores in our study population are comparable with the SF-36 domain scores in the general Dutch population (29), suggesting low disease burden with few mental and physical

limitations, leaving almost no opportunity for improvement. The low disease burden in our study population is also reflected in the small number of patients with one or more than one microvascular complications (n = 38, 20%) and the good glycaemic control with a median HbA1c of 6.8 (6.4 - 7.1%) [51 (46 - 54) mmol/mol].

Furthermore, when expecting a positive effect of vitamin D supplementation on HRQOL by reducing systemic low-grade inflammation or improving glycaemic control leading to reduced or less severe diabetes-specific complications, the relatively short duration of the trial could be another reason for not finding an improvement of HRQOL after vitamin D supplementation.

The strengths of our study are the randomised, double-blind, placebo-controlled design, the use of a well-validated questionnaire to determine HRQOL and the large study population.

In conclusion, six months of vitamin D supplementation did not improve HRQOL in patients with tight controlled type 2 diabetes derived from general practices. Longitudinal studies in patients with poorly controlled type 2 diabetes, with multiple measurements over time concerning physical limitations, mental health and vitality and factors possible affecting these domains including low 25(OH) D level, inflammation factors, diabetes-specific treatment and complications and lifestyle factors are necessary to understand and eventually affect, the relationship between diabetes and a reduced (health-related) quality of life.

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CHAPTER 8

Vitamin D status is associated with skin autofluorescence in patients with type 2 diabetes mellitus: a preliminary report

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ABSTRACT

Background

Skin autofluorescence is a non-invasive measurement of advanced glycation end product (AGE), which are suggested to be one of the major agents in the pathogenesis and progression of diabetes related cardiovascular complications. Recently, low vitamin D status has been linked to the progression of type 2 diabetes mellitus and cardiovascular disease. The aim of this study is to investigate the association between vitamin D status and skin autofluorescence in patients with type 2 diabetes.

Methods

In this preliminary report skin autofluorescence was measured non-invasively with an AGE-reader in 245 patients with type 2 diabetes treated with lifestyle advice, metformin and/or sulphonylurea-derivatives. All patients were randomly assigned to receive either vitamin D 50,000 IU/month or placebo for six months. .

Results

Skin autofluorescence was significantly higher in patients with a serum 25(OH)D < 50 nmol/l compared to patients with a serum 25(OH)D > 75nmol/l (2.81 versus 2.41; $p < 0.001$). Mean serum 25(OH)D was 60.3 ± 23.4 nmol/l and was independently associated with skin autofluorescence (β -0.006; $p < 0.001$). Mean vitamin D increased from 60.8 to 103.6 nmol/l in the intervention group, however no effect was seen on accumulation of skin AGEs after six months compared to placebo.

Conclusions

Vitamin D status is independently associated with skin auto fluorescence in patients with tight controlled type 2 diabetes. No effect was seen on the amount of skin AGEs after a short period of six months vitamin D supplementation. Further research with circulating advanced glycation end products is scheduled to elucidate these results.

INTRODUCTION

One of the chronic consequences of hyperglycaemia is the accelerated formation of advanced glycation end products (AGEs), which are suggested as one of the major pathogenic mechanisms causing end organ damage in diabetes (1,2). AGEs are formed nonenzymatically by the modification of proteins, lipids and nucleic acids by glucose. AGEs are highly reactive pro-inflammatory molecules and accumulate slowly over a persons' lifetime with an estimated lifetime of 15 years (3,4). Diabetes mellitus, chronic renal failure and aging accelerate the generation of AGEs (5). AGEs act by binding to the receptor for AGEs (RAGE), which is expressed on neutrophils, T-lymphocytes, macrophages and synovial fibroblasts. Upon binding of AGE to its receptor the transcription of pro-inflammatory genes is stimulated, leading to the up-regulation of endothelial adhesion molecules contributing to the development of atherosclerosis (6). In patients with type 2 diabetes mellitus AGEs seem to represent inflammatory, oxidative and cumulative metabolic stress (3). Earlier research demonstrated an increased skin autofluorescence, as a measure of AGE accumulation, in patients with type 2 diabetes compared to healthy controls (7). In addition, skin autofluorescence predicts both micro- and macrovascular complications in patients with type 2 diabetes, and has a strong association with the severity of diabetes-related complications and mortality (2,8-14).

Similar to the consequences of AGE accumulation, low vitamin D status has been linked to numerous biochemical and clinical disturbances, including the pathogenesis and progression of type 2 diabetes and cardiovascular disease (15-18). The prevalence of vitamin D deficiency is high worldwide, especially in patients with type 2 diabetes compared to healthy persons (19,20). The underlying mechanism explaining the association between vitamin D status, type 2 diabetes and cardiovascular disease is not clarified yet. Earlier data suggest a potential independent role for vitamin D in the regulation of glucose metabolism in a setting of obese patients (21). It is known that besides the classical role of vitamin D in calcium and bone homeostasis, vitamin D is linked to numerous non-skeletal diseases due to the elucidation that most cells, including the pancreatic beta-cells and cardiomyocytes contain the vitamin D receptor (VDR), and most of them also have the capability to produce the biologically active 1,25-dihydroxyvitamin D for paracrine functions (22,23). Experimental studies have established a role for vitamin D in type 2 diabetes and cardiovascular disease by interacting with inflammation, insulin resistance, renin-angiotensin system, thrombosis and gene regulation (24-28). To our knowledge no literature is available about the association between AGEs and vitamin D status in patients with type 2 diabetes. Hypothetically, vitamin D may reduce inflammation, oxidative stress and insulin resistance, and thereby reducing the accumulation of AGEs. Interestingly, in diabetic rats vitamin D supplementation attenuated the deposition of AGEs in the vascular system (29) and decreased the diabetic effects on the receptor of AGEs in rats (30). Therefore, the aim of our study is to investigate the association between vitamin D status and skin auto fluorescence in patients with type 2 diabetes.

METHODS

We performed a preliminary cross-sectional and longitudinal analysis of 245 patients with type 2 diabetes. The patients were included between July 2012 and April 2013 in a randomised placebo-controlled trial ("the SUNNY trial"), in which the effect of 50,000 IU/month vitamin D supplementation during six months versus similar looking placebo was examined on glycaemic control in patients with type 2 diabetes (31). The trial was approved by the Medical Ethics Committee of North-Holland, the Netherlands. The trial protocol is described in greater detail elsewhere (32). In brief, adult patients (≥ 18 years), treated with lifestyle advice, metformin or sulphonylurea-derivatives, whether or not in combination, were invited for participation in the trial. The main exclusion criteria were previous treatment with insulin, serum 25-hydroxyvitamin D (25(OH)D) < 15 nmol/l or > 150 nmol/l, hypercalcaemia (serum calcium > 2.65 nmol/l), impaired renal function (estimated glomerular filtration rate [eGFR] < 30ml/min, measured by the MDRD formula) urolithiasis, and no signed informed consent. Throughout the study no drug alterations regarding hypoglycaemic agents and statins, and no vitamin D supplements were allowed. Informed consent was obtained from all patients before start of the trial.

Outcome measures

Outcome measures were obtained at baseline (immediately prior to dosing), and after six months. The following data were collected during the first visit: age, gender, ethnicity, social status, diabetes duration, medical history, family history of type 2 diabetes, medication use, diabetic complications, previous cardiovascular disease, co-morbidity, smoking status, alcohol use, diet (especially fish and dairy products), physical activity, sun exposure, and season of blood collection. Cardiovascular disease was defined as coronary artery disease / ischemic heart disease, stroke, hypertensive heart disease, aortic aneurysms, cardiomyopathy and peripheral artery disease. Standard anthropometric data (height, weight, waist and hip circumference, blood pressure) were obtained from each patient. Venous blood collection for serum 25(OH)D, HbA1c, fasting blood glucose and insulin, lipid profile, serum calcium, albumin, estimated glomerular filtration rate (eGFR) and parathyroid hormone (PTH) were collected after an overnight fast at 8.00 – 9.30 am. Serum 25(OH)D was measured on an iSYS automated immunoanalyser (IDS GmbH, Frankfurt, Germany). All samples are stored at -20°C for future research on circulating AGE levels.

Skin autofluorescence

Skin autofluorescence levels were measured with an AGE reader (Diagnoptics Technologies B.V., Groningen, the Netherlands). This non-invasive method utilises the characteristic fluorescent properties of AGEs, and has been validated with specific AGE measurements in skin biopsies (33). Autofluorescence was defined as the average light intensity per nanometer in the range between 420 and 600nm, divided by the average light intensity per nanometer in the range between 300 and 420 nm. It was expressed in arbitrary units (AU) using the AGE reader software. Skin autofluorescence was measured at room temperature while patients were in a seated position, at the volar side of the lower arm. Measurements were not specifically performed in a fasting state. Previous research has demonstrated that repeated AGE measurements on one day showed an overall Altman error percentage <6.0%. Intra-individual seasonal variance showed an Altman error percentage <6.0% (33).

Table 1. Baseline demographic and clinical characteristics*

	All	Serum 25 (OH)D *15-49 nmol/l*	Serum 25 (OH)D *50-74 nmol/l*	Serum 25 (OH)D *75-150 nmol/l*	P-value
N	245	89	96	60	
Male (%)	156 (63)	49 (55)	66 (69)	41 (68)	0.09
Age (years)	67 ± 8	67 ± 9	67 ± 9	67 ± 7	0.82
White skin colour (%)	234 (95)	80 (90)	93 (97)	60 (100)	0.01
Diabetes duration (years)	6.0 (3.0 – 8.0)	5.0 (3.5 – 7.0)	6.0 (3.0 – 8.0)	6.0 (3.0 – 8.0)	0.59
Antidiabetic treatment					0.55
Lifestyle adjustments	11 (5)	5 (6)	4 (4)	2 (3)	
Metformin	155 (63)	50 (56)	62 (65)	43 (72)	
SU-derivative	9 (4)	3 (3)	4 (4)	2 (3)	
Metformin + SU-derivative	70 (28)	31 (35)	26 (27)	13 (22)	
DM-complications (%)					
Retinopathy	11 (5)	5 (6)	4 (4)	2 (3)	0.80
Neuropathy	32 (13)	11 (12)	11 (12)	10 (17)	0.63
Microalbuminuria	30 (12)	16 (18)	9 (9)	5 (8)	0.13
Antihypertensive drugs (%)					
Diuretics	88 (36)	38 (42)	28 (30)	22 (37)	0.20
Calcium channel blockers	44 (18)	19 (21)	14 (15)	11 (18)	0.53
ACE inhibitors / AT-II receptor antagonists	135 (55)	56 (63)	45 (47)	34 (57)	0.34
Beta blockers	92 (38)	35 (39)	35 (37)	22 (37)	0.95
Statins (%)	205 (84%)	75 (83%)	80 (84%)	50 (83%)	0.99
Cardiovascular disease (%)	67 (27)	23 (26)	32 (33)	12 (20)	0.16
Smoking status					
Current (%)	36 (15)	17 (19)	12 (12)	7 (12)	0.20
Alcohol use (%) ≤ 2 units/day	216 (89)	75 (83)	84 (88)	66 (92)	0.03
Exposure to sun					<0.001
<5 hours/week	102 (42)	51 (57)	36 (37.5)	15 (25)	
5-10 hours/week	103 (42)	33 (37)	45 (47.9)	25 (42)	
>10 hours/week	40 (16)	5 (6)	15 (15.6)	20 (33)	
Physical activity					0.03
< 2 hours/week	78 (32)	38 (43)	30 (31)	10 (17)	
2-5 hours/week	112 (45)	38 (43)	46 (48)	28 (47)	
>5 hours/week	55 (23)	13 (14)	20 (21)	22 (36)	
Season of blood collection					0.047
Spring	24 (10)	10 (11)	11 (11)	3 (5)	
Summer	66 (27)	16 (18)	24 (25)	26 (43)	
Autumn	119 (48)	48 (54)	45 (47)	26 (43)	
Winter	36 (15)	15 (17)	16 (17)	5 (9)	

Table 1. Continued

	All	Serum 25 (OH)D *15-49 nmol/l*	Serum 25 (OH)D *50-74 nmol/l*	Serum 25 (OH)D *75-150 nmol/l*	P-value
Body Mass Index (kg/m2)	28.8 ± 4.5	29.9 ± 4.4	28.0 ± 4.6	28.2 ± 3.9	0.009
WTH ratio	0.98 (0.93 – 1.04)	0.99 (0.92 – 1.04)	0.97 (0.93 – 1.04)	1.00 (0.94 – 1.03)	0.77
AGE-value	2.64 ± 0.6	2.81 ± 0.6	2.63 ± 0.6	2.41 ± 0.5	<0.01
Fasting glucose (mmol/L)	7.5 (6.9-8.2)	7.4 (6.9 – 8.2)	7.5 (6.9 – 8.6)	7.7 (6.9 – 8.4)	0.84
Fasting insulin (mU/L)	14.5 (9.0-21.0)	14.9 (9.5 – 21.8)	13.8 (8.8 – 21.0)	14.6 (8.7 – 20.9)	0.78
HbA1c (mmol/mol)	51 (47 - 55)	51 (49 – 54)	52 (45 - 56)	51 (46 – 55)	0.96
HbA1c (%)	6.8 (6.5 – 7.2)	6.8 (6.6 – 7.1)	6.9 (6.3 – 7.3)	6.8 (6.4 – 7.2)	0.96
LDL-cholesterol (mmol/L)	2.5 ± 0.9	2.5 ± 1.0	2.5 ± 1.0	2.4 ± 0.7	0.77
eGFR (ml/min/1.73m^2)	81 ± 18	82 ± 20	81 ± 17	82 ± 17	0.94
Calcium (mmol/L)	2.33 ± 0.08	2.32 ± 0.08	2.33 ± 0.08	2.34 ± 0.07	0.22
Albumine (g/L)	40 (38-41)	39 (38 – 41)	40 (38 – 41)	40 (39 – 41)	0.07
Serum 25(OH)D (nmol/l)	60.3 ± 23.4	36.9 ± 8.0	61.5 ± 7.2	92.9 ± 13.4	<0.01
PTH (pmol/L)	5.1 (4.0 – 6.8)	5.2 (4.4 – 7.0)	5.4 (4.1 – 6.9)	4.3 (3.6 – 5.9)	0.01
AP (U/L)	72.6 ± 20.6	74.6 ± 24.0	70.1 ± 19.3	73.3 ± 16.6	0.38

** Data are presented as numbers (%), mean ± SD or median (ICR).*
25(OH)D, 25-hydroxyvitamin D; ACE, angiotensin converting enzyme; AGE, advanced glycation endproducts; AP, alkaline phosphatase; AT-II, angiotensin II; DM, diabetes mellitus; e-GFR, estimated glomerular filtration rate; LDL, low density lipoprotein; PTH, parathyroid hormone; WTH, waist to hip;

Statistical analysis

All data were analysed using the statistical package SPSS software (version 20.0, SPSS Inc, Chicago, IL). For the purpose of our study and consistent with the widely used cut-off values of vitamin D, the patients were stratified into three vitamin D groups: 1) serum 25(OH)D: 15 - 49 nmol/l, 2) serum 25(OH)D: 50 - 74 nmol/l, and 3) serum 25(OH)D: 75 -150 nmol/l (34). Baseline characteristics were compared using χ2-test for categorical variables and ANOVA or Kruskall-Wallis for continuous variables depending on normal distribution. Data are presented as means ± standard deviation if normally distributed and otherwise as median and interquartile range. Multiple linear regression analysis was used to determine independent associations between vitamin D status and skin autofluorescence. We adjusted for confounders based on the literature and regression correlation coefficient difference > 10%: age, season of measurement, duration of diabetes, renal function, gender, ethnicity, smoking behaviour, lipid profile, HbA1c, and the presence of cardiovascular disease. Linear regression analysis was used to assess the mean difference between intervention and placebo groups after six months (mean difference is reported as beta). A p-value < 0.05 was considered as statistically significant.

RESULTS

Baseline skin autofluorescence level was determined in 245 of 275 patients included in the SUNNY trial. Skin autofluorescence was not measurable in 30 patients, mainly due to low reflection caused by dark coloured skin. Demographic, anthropometric and clinical characteristics of all 245 patients, and stratified to vitamin D level are presented in Table 1.

The mean age of the patients was 67 ± 8 years and 64% were male, with a median diabetes duration of 6.0 (3.0 – 8.0) years. Overall mean serum 25(OH)D was 60.3 ± 23.4 nmol/l and mean skin autofluorescence 2.64 ± 0.6. Vitamin D deficiency (serum 25(OH)D < 50 nmol/l) was present in 89 patients (37%), 96 patients (39%) had a serum 25(OH)D level between 50 - 74 nmol/l and 60 patients (24%) had a serum 25(OH)D > 75 nmol/l.
Skin autofluorescence values were significantly higher in the vitamin D deficient group compared to the group with a serum 25(OH)D > 75 nmol/l (2.81 ± 0.6 versus 2.41 ± 0.5; p < 0.001). Skin autofluorescence significantly raised with increasing age (2.44 ± 0.49 to 2.91 ± 0.61 in patients aged < 60 years and > 70 years, respectively, data not shown). No difference in skin autofluorescence was seen in patients treated with metformin (n = 225) compared to patients without metformin treatment (n = 20) (data not shown).
Linear regression analyses were performed to determine the association between serum 25(OH)D and skin autofluorescence. A significant association between serum 25(OH)D and skin autofluorescence (β = -0.007; p < 0.01) was demonstrated. Confounders for this association were age, ethnicity, season, sun exposure, diabetes duration, presence of cardiovascular disease, eGFR, alkaline phosphatase and LDL cholesterol. No effect of HbA1c, sex, smoking behaviour or the use of statins and/or antihypertensive drugs was measured on the association between serum 25(OH)D and skin auto fluorescence. After adjustment for above mentioned confounding risk factors, the association between serum 25(OH)D and skin autofluorescence remained statistically significant (β = -0.006; p < 0.01) (Table 2).

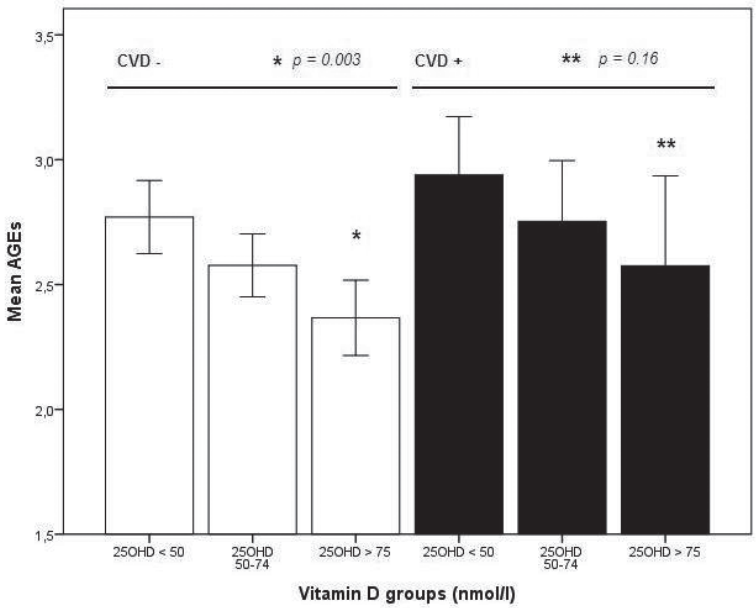
Table 2. Linear regression analysis of serum 25(OH)D (independent variable) and skin auto fluorescence (dependent variable)

	β (95% CI)	SE B	p-value
Model 1: crude analysis	-0.007 (-0.010 - -0.004)	0.002	< 0.01
Model 2: Model 1 + age, ethnicity, season, smoking, BMI	-0.007 (-0.010 - -0.004)	0.002	< 0.01
Model 3: Model 2 + sun exposure, diabetes duration, CVD, HbA1c, eGFR, AP, LDL cholesterol	-0.006 (-0.009 - -0.003)	0.002	< 0.01

25(OH)D, 25-hydroxyvitamin D; AP, alkaline phosphatase; BMI, body mass index; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; LDL, low density lipoprotein

In patients with previous cardiovascular disease (n = 67) mean skin autofluorescence was significantly higher compared to patients without cardiovascular disease (n = 178) (AGEs: 2.79 ± 0.57 and 2.59 ± 0.62; p = 0.02, respectively). In patients without previous cardiovascular disease mean skin autofluorescence was significantly higher in patients with a serum 25(OH)D level < 50 nmol/l compared to patients with a serum 25(OH)D level > 75 nmol/l (2.77 ± 0.60 and 2.37 ± 0.52; p = 0.003, respectively). This result was also seen in patients with cardiovascular disease, although this difference was not statistically significant (2.94 ± 0.54, and 2.58 ± 0.57 in patients with a serum 25(OH)D level < 50 nmol/l and > 75 nmol/l; p = 0.16, respectively) (Figure. 1).

Figure 1. Mean skin autofluorescence in vitamin D subgroups stratified by cardiovascular disease.



Longitudinal analysis

210 out of 245 (85%) patients completed the trial and conducted a skin AGE measurement after six months. The majority of the excluded patients throughout the study had changed their hypoglycaemic agents on own initiative or due to an HbA1c level > 69 mmol/mol (n = 19), two patients had a serum 25(OH)D < 15 nmol/l, one patient suffered from new onset urolithiasis, and eight patients did not show at their last visit. Serum 25(OH)D raised from 60.8 to 103.6 nmol/l in the vitamin D group (n = 107) (p = < 0.01) and decreased from 61.0 to 60.3 nmol/l in the placebo group (n = 103) (p = 0.78). Mean skin autofluorescence significantly increased (2.63 ± 0.53 to 2.74 ± 0.56; p = 0.02) in the placebo group, whereas in the vitamin D group mean skin autofluorescence increased less profoundly which was not significant (2.67 ± 0.64 to 2.73 ± 0.69; p = 0.19). Comparing the change in mean skin autofluorescence from baseline to six months, no significant difference was found between both

groups (β -0.05, 95% CI: -0.17 – 0.08, p = 0.47). No significant change over time in HbA1c was seen in the intervention group whereas mean BMI increased significantly (Table 3).

Table 3. Comparison of the main outcomes before and after intervention between both groups.

	Vitamin D group (n = 107)		Placebo group (n = 103)		β 95% CIs)	p
Variable	**0 m**	**6 m**	**0 m**	**6 m**		
Serum 25(OH)D (nmol/l)	60.8 ± 23.0	103.6 ± 25.7	61.0 ± 23.4	60.3 ± 26.5	42.5 (35.8 – 49.6)	< 0.01
AGE-value	2.67 ± 0.64	2.73 ± 0.69	2.63 ± 0.53	2.74 ± 0.56	-0.05 (-0.17 – 0.08)	0.47
BMI (kg/m^2)	28.8 ± 4.8	29.1 ± 4.4	28.7 ± 4.7	28.8 ± 4.8	0.23 (0.01 – 0.46)	0.04
HbA1^c (mmol/mol)	51.1 ± 6.0	50.0 ± 6.3	50.3 ± 5.3	49.4 ± 6.2	1.2 (-0.1 – 2.6)	0.07

25(OH)D, 25-hydroxyvitamin D; AGE, advanced glycation endproducts; BMI, body mass index

DISCUSSION

To our knowledge this is the first study examining the relationship between vitamin D status and AGE accumulation in the skin in patients with type 2 diabetes. We found a significant inverse association between serum 25(OH)D and skin autofluorescence independent of major confounders, including age, season, diabetes duration and renal function in a group of patients with relatively tight controlled type 2 diabetes. For each 10 nmol/l increment in serum 25(OH)D level skin autofluorescence decreases with 0.06 in patients with type 2 diabetes. Patients with a serum 25(OH)D < 50 nmol/l had a significantly higher skin autofluorescence compared to patients with an adequate serum 25(OH)D level > 75 nmol/l (2.81± 0.6 and 2.41 ± 0.5, respectively). Six months of vitamin D supplementation did not alter the amount of skin auto fluorescence significantly compared to the placebo group. However, the duration of the intervention could be too short for this outcome measure, as skin AGEs have a mean half life of 10 to 15 years (35).

A possible underlying mechanism for the association found between skin AGEs and vitamin D status, could be a reduction of oxidative stress by vitamin D and thereby a decrease in the formation of AGEs and pro-inflammatory cytokines that mediate microvascular and macrovascular complications in type 2 diabetes. A recent in vitro study demonstrated a decrease of cytokine-mediated endothelial inflammation after addition of calcitriol, supporting the concept that calcitriol may act as a vascular protective agent counteracting the probable deleterious actions of AGEs on endothelial cell activities (13,36). Moreover, several studies have shown protective effects of vitamin D

on vascular wall function and cell membranes by maintaining steady levels of certain intracellular antioxidants, by reducing lipid peroxidation and by reducing the overproduction of reactive oxygen species (37). However, a recent study performed by Stürmer et al. (38) among 119 healthy en 27 hypertensive non-diabetic participants with a mean serum 25(OH)D 56.2 ± 22.2 nmol/l, revealed no association between vitamin D status and skin autofluorescence.

Our results regarding the significantly higher skin autofluorescence found in patients with previous cardiovascular disease compared to patients without cardiovascular disease (mean skin autofluorescence: 2.79 ± 0.57 versus 2.59 ± 0.62) are in line with previous studies which demonstrated mean skin autofluorescence values of 2.57 in patients with type 2 diabetes without diabetes-related complications, and skin AGEs of 3.12, 2.91 and 2.71 in patients with type 2 diabetes and both micro- and macrovascular complications, solely macrovascular complications and solely microvascular complications, respectively (7).
Interestingly, the patients in the highest vitamin D group in our study had a mean skin autofluorescence comparable with the non-diabetic population demonstrated in a study by Koetsier et al (39). The authors generated a formula for calculating the mean skin autofluorescence in the general population without diabetes mellitus: 0.024 x age + 0.83. When this formula is applied to our study population with a mean age of 67 years, the calculated mean skin autofluorescence is 2.44. In our study results the patients with type 2 diabetes in the highest vitamin D group had a lower mean autofluorescence level: 2.41 ± 0.5. This could imply a protective effect of vitamin D on the formation and accumulation of AGEs in patients with type 2 diabetes. However, an important limitation in our study is that we measured the accumulated AGEs in the skin using an AGE-reader, and other known AGE types, such as circulating AGEs and AGEs without fluorescent properties could have provided different results within the vitamin D subgroups. In addition from this point of view the association between vitamin D status and skin autofluorescence found in our study could be explained by the fact that in theory cutaneous AGE accumulation could hinder the photoconversion of the provitamin D into vitamin D. Nevertheless, our results show that vitamin D sufficiency is mainly found in patients with type 2 diabetes without high cardiovascular risk profile as indicated by the age-adjusted AGE levels within the normal range. Furthermore, we plan to conduct future analyses with the stored serum of all patients to measure circulating AGEs.
In contrast to previous studies, smoking was not correlated to skin autofluorescence in our study. This may be due to the small group of smokers in our study population (n = 36; 15%). HbA1c, which is also an end product of glycation, was not associated with skin autofluorescence. This is not surprisingly as earlier research demonstrated only a weak effect between skin autofluorescence and HbA1c. A logical explanation is that HbA1c represents AGE accumulation over a short period of approximately 6 – 8 weeks, while skin AGEs accumulate over ten to 15 years (35).
In conclusion, this preliminary report demonstrates an independent association between vitamin D status and skin autofluorescence in patients with tight controlled type 2 diabetes. A short intervention of vitamin D supplementation, however did not have any effect on the amount of skin autofluorescence. Future analyses are planned to measure circulating AGEs for further exploration of the association found.

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CHAPTER 9

The effect of vitamin D supplementation on glycaemic control in patients with Type 2 Diabetes Mellitus: a systematic review and meta-analysis

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ABSTRACT

Objective

Epidemiologic studies suggest that vitamin D status plays a role in glycaemic control in patients with type 2 diabetes. However, intervention studies yielded inconsistent results. The aim of this study is to systematically review the effect of vitamin D supplementation on glycaemic control in patients with type 2 diabetes

Methods

Systematic review and meta-analysis. We searched Medline, Embase and the Cochrane Library for RCTs examining the effect of vitamin D supplementation on glycaemic control in patients with type 2 diabetes. A random-effect model meta-analysis was performed to obtain a summarised outcome of vitamin D supplementation on HbA1c, fasting glucose and homeostatic model assessment – insulin resistance (HOMA-IR).

Results

Twenty-three RCTs were included in this systematic review representing a total of 1797 patients with type 2 diabetes. Mean change in serum 25-hydroxyvitamin D varied from 1.8 ± 10.2 nmol/l to 80.1 ± 54.0 nmol/l. Nineteen studies included HbA1c as outcome variable. Combining these studies no significant effect in change of HbA1c was seen after vitamin D intervention compared to placebo. A significant effect of vitamin D supplementation was seen on fasting glucose in a subgroup of studies with a mean baseline HbA1c ≥ 8% (64 mmol/mol) (standardized difference in means: 0.36; 95% CI: 0.12 to 0.61, p = 0.003)

Conclusions

Current evidence of RCTs shows no evidence to support short-term vitamin D supplementation in a heterogeneous population with type 2 diabetes. However, in patients with poorly controlled diabetes a favourable effect of vitamin D is seen on fasting glucose.

Introduction

Vitamin D is a key factor for the maintenance of calcium and bone homeostasis. Over the past decade, vitamin D has attracted substantial interest towards extra-skeletal roles in various disease conditions, including diabetes mellitus (1). This interest has arisen due to the identification that most cells, including the pancreatic beta-cells, contain the vitamin D receptor (VDR). Most of these cells also have the capability to produce the biologically active form of vitamin D: 1,25-dihydroxyvitamin D for paracrine functions (1-3). Furthermore, vitamin D is known to have immuno-modulatory and anti-inflammatory effects, which could improve peripheral insulin resistance by altering low-grade chronic inflammation that has been implicated in insulin resistance in type 2 diabetes mellitus (3-5). Observational studies have demonstrated a link between vitamin D deficiency and the onset of and progression of type 2 diabetes (6-9). Furthermore low vitamin D status is associated with future macrovascular events in patients with type 2 diabetes mellitus (10). This association may be the result of the link between vitamin D status and renin-angiotensin system (11), endothelial function (12), blood pressure (13), or chronic inflammation (4).

A recent meta-analysis performed in 2012 by George et al. (14) demonstrated a weak positive effect of vitamin D supplementation on fasting glucose and insulin resistance in patients with type 2 diabetes mellitus. However, overall the authors concluded that there was insufficient evidence of a beneficial effect to recommend vitamin D supplementation as a means of improving glycaemic control in patients with type 2 diabetes, impaired fasting glucose or normal glucose tolerance. Inconsistency in these results may be due to the different study populations (normal glucose tolerance, impaired glucose tolerance and type 2 diabetes), small sample sizes, and different dosage regimes of vitamin D supplementation. Additionally, in 2014 a meta-analysis published by Seida et al. which included RCTs among adults with normal glucose tolerance, prediabetes and/or type 2 diabetes, demonstrated no effect of vitamin D supplementation on improving glucose homeostasis and preventing diabetes including only RCTs. Definitive conclusion could not be drawn in the context of heterogeneity, short-term follow-up duration and variable risk of bias (15).

Due to the ongoing increased interest in the effect of vitamin D on glycaemic control in type 2 diabetes, many more studies have been published since these meta-analyses were performed.

Taken together, it is still unclear whether vitamin D supplementation has a beneficial effect on glycaemic control in patients with type 2 diabetes mellitus. We present an up to date analysis of the effect of vitamin D supplementation on glycaemic indices (HbA1c, insulin resistance and fasting glucose) in patients with type 2 diabetes mellitus.

METHODS

Search strategy and selection criteria

A systematic literature search (MEDLINE, Embase and The Cochrane Library) was performed to identify articles from January, 1976, to 15 October 2015 that assessed the effect of vitamin D supplementation on glycaemic indices in patients with type 2 diabetes. The search terms included type 2 diabetes mellitus AND [vitamin D OR vitamin D deficiency OR vitamin D2 OR vitamin D3 OR cholecalciferol OR ergocalciferol]. References of the retrieved articles were scanned for additional studies. The objective was to systematically review the evidence that vitamin D can improve glycaemic

indices (HbA1c, insulin resistance and fasting glucose) in patients with type 2 diabetes. One author (YK-P) performed an initial screening of titles and abstracts. Full-text articles of the selected titles were screened using the inclusion criteria described below. If there was a doubt to whether a particular article should be included, the author discussed the article with the last author (SS) until consensus was reached.

We included randomised controlled trials (RCT) in the following groups: vitamin D supplementation versus placebo, vitamin D supplementation and calcium supplementation versus calcium alone and / or placebo. Additional inclusion criteria were: 1) the study population consisted of patients with type 2 diabetes; 2) supplementation of vitamin D2 (ergocalciferol) or vitamin D3 (cholecalciferol) for intervention; 3) HbA1c or parameters of glycaemic control (fasting glucose, fasting insulin or homeostatic model assessment - insulin resistance [HOMA-IR]) had to be a primary or secondary outcome; 4) the authors report data of an original clinical study (i.e. no review, commentary, case reports, or editorial); 5) study performed in adults ≥ 18 years; 6) published in English. We excluded studies using 1,25 dihydroxyvitamin D and studies performed in patients other than type 2 diabetes mellitus, or patients on dialysis.

Quality assessment and data extraction

The quality of selected articles was assessed by two reviewers using a checklist from the Dutch Cochrane Collaboration (Fig 1) (16). The checklist consists of 11 criteria which has three answer options: yes (adequate information/approach); no (no adequate information/approach); or little information. Each criteria answered with yes scored one point, we considered a total score ≥ 9 points as a good quality study.

Data were extracted by one author (YK-P) and controlled by the last author (SS) using a self composed form including the following items of studies included: country, design, publication year, participants, therapy duration, type and dose of vitamin D supplementation, primary outcome, baseline and change in serum 25-hydroxy vitamin D (25(OH)D) and parameters of glycaemic control (HbA1c, fasting glucose, fasting insulin and homeostasis model of assessment – insulin resistance (HOMA-IR)). For studies lacking a reported standard deviation of change in outcome between baseline and follow-up, we derived standard deviation of change as the mean of the baseline and follow-up standard deviations for each treatment group. This method was used successfully in the meta-analysis from George et al. performed on this subject (14).

Statistical analysis

To obtain a summarised outcome of the effect of vitamin D supplementation on glycaemic control, we compared the mean change between baseline and follow-up of each variable of the intervention and control group. Studies in which the mean change and/or standard deviation was not reported or could not be derived, were excluded in the meta-analysis. If a study included more than two groups, we used the data of the group in which the highest dose of vitamin D supplementation was given for the meta-analysis compared to placebo. If studies compared both vitamin D and/or calcium supplementation versus placebo, the data of the group with solely vitamin D supplementation was used for the meta-analysis.

The results of the included studies were pooled and meta-analyses were carried out using random-effects models as some heterogeneity of outcome was expected. To compare the intervention and

placebo group, the results are presented as between group standardized mean differences with 95% CI. Subgroup analyses were performed for studies with a baseline mean serum 25(OH)D < 50 nmol/l and < 30 nmol/l, and for studies having a mean baseline HbA1c ≥ 8% (64 mmol/l) in the intervention group. We assessed statistical heterogeneity between studies with I2 statistic (with 95% CIs). The I2 is the proportion of total variation contributed by between-study variation. In general, I2values greater than 60-70% indicate the presence of substantial heterogeneity (17). In the presence of heterogeneity between studies, we assessed potential publication bias using formal tests, being the funnel plot and Egger test (18). Meta-analyses were performed using comprehensive meta-analysis version 3.0 (http://www.meta-analysis.com). A p-value < 0.05 was considered statistically significant.

Figure 1. Quality checklists randomised controlled trials

Quality assessment RCTs studies

a. Was assigning of the intervention done by randomisation?
b. The person who includes patients should not know the randomisation sequence? Was that the case?
c. Were patients blinded for the treatment?
d. Were treating physicians blinded for the treatment?
e. Were effect assessors blinded for the treatment?
f. Were the groups similar at baseline? Extra answer option: a) no, but corrected for or b) no and not corrected for.
g. Is a complete follow-up period available for a sufficient proportion of the included patients? If the answer is no: is selective loss to follow-up appropriately accounted for?
h. Were al included patients analysed in the group were they were randomised in (intention to treat population)?
i. Were the groups equally treated, apart from the intervention?
j. Is selective publication of results sufficiently ruled out?
k. Is adverse influence of sponsors sufficiently ruled out?

RESULTS

The initial systematic search yielded 1489 articles. Of those, 328 were duplicates and 1074 articles were excluded based on abstract and title. The most common reasons for exclusion of these articles were no inclusion of patients with type 2 diabetes or no intervention with vitamin D. Eighty-seven articles were selected for full text review as shown in Fig. 2. Finally, 23 trials were selected for quality assessment and included in this systematic review.

Description of the studies

Twenty-three RCTs representing a total of 1797 patients with type 2 diabetes were included in this systematic review. The quality assessment of the studies resulted in 14 out of 23 studies having a good quality (appendix 1) (12,19-31). Table 1 represents the main characteristics and main outcomes of the included studies. All studies had a randomised controlled trial design of which 18 studies used a placebo for control (12,19,20,22-25,27,29-38), three studies compared vitamin D fortified yoghurt versus plain yoghurt (21,28,39), one study used oral calcium supplementation for control (26), and one study used vitamin C supplementation for control (40). Besides from two studies, which solely

included post-menopausal women (21,33), all studies included both men and women. Mean age varied from 44 to 67 years (24,25). Mean HbA1c varied from 6.2% (44 mmol/mol) (20) to 8.7% (71 mmol/mol) (28) in the intervention group. Six studies had a mean baseline HbA1c ≥ 8% (64 mmol/mol) in the intervention group (27,28,32,33,36,40). Different assays were used for measurement of serum 25(OH)D with most studies using an enzyme-immunoassay (12,20,21,23,24,26,27,29-37), three studies measures serum 25(OH)D using high-performance liquid chromatography (22,28,39), two studies used a radio-immunoassay method (25,38), one study used a competitive protein-binding assay (19), and one study did not report the method of measurement (40).

A wide variety was seen in mean baseline serum 25(OH)D in the intervention group, with the lowest value of 21.5 ± 23.7 nmol/l (34) and a highest value of 117.3 ± 86.7 nmol/l (35). Four studies included only vitamin D deficient (serum 25(OH)D < 50 nmol/l) patients (12,23,26,27). Many different intervention regimes were used. The mean change in serum 25(OH)D between the intervention and control group is summarised for each study in Fig. 3. Except for two studies performed by Breslavsky (19) and Cavalcante (33), all studies observed a significant increase in serum 25(OH)D in the intervention group compared with the placebo group with an overall mean difference: 30.2 nmol/l; 95% CI: 23.1 to 37.3, $p < 0.01$). Five studies could not be included in this analysis due to missing data (20,29,31,38,40).

Figure 2. Flow chart of literature search

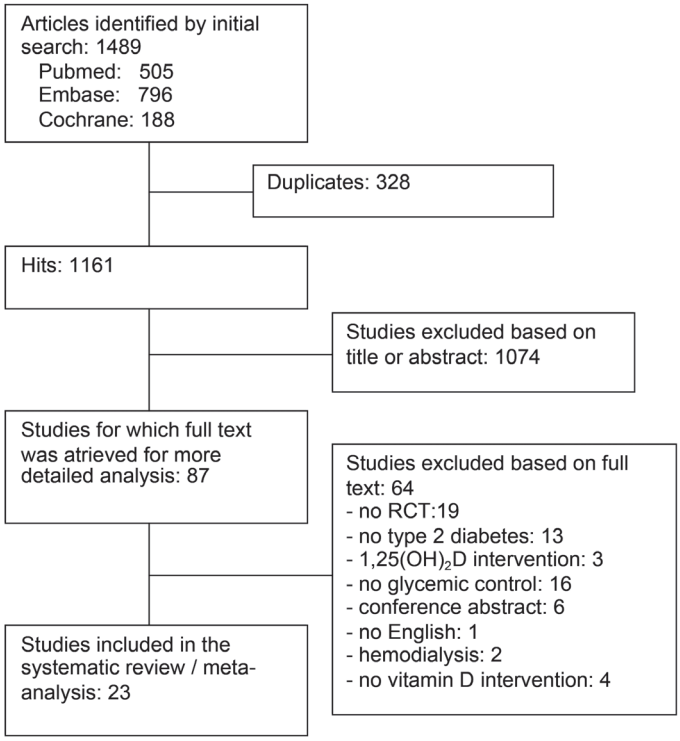


Table 1. Summary of the intervention studies included in this systematic review

Name, (year), ref	Location	Cohort T2DM (n)	Intervention	Control	Duration	Primary outcome	25(OH)D nmol/l*	Baseline HbA1c (%)**	Main results
Al-Zahrani (2014), (32)	Saudi Arabia	183, 25(OH)D < 75 nmol/l	Vitamin D3 45000 IU/week	Placebo	3 m	Metabolic parameters	25.3 ± 15.8 to 82.8 ± 31.7	8.5 ± 1.6	↓ diastolic blood pressure = HbA1c, fasting glucose, lipid profile
Breslavsky (2013), (19)	Israël	47	Vitamin D3 1000 IU/day	Placebo	12 m	Metabolic parameters	29.5 ± 27.2 to 43.9 ± 28.7	7.3 ± 1.1	= HbA1c, fasting glucose, insulin, HOMA-IR, lipid profile
Cavalcante (2015), (33)	Brazil	38 post-menopausal women, 25(OH)D < 75 nmol/l,	Vitamin D3 6600 IU/week	Placebo	3 m	Metabolic parameters and muscle strength	55.5 ± 9.9 to 57.4 ± 10.5	8.2 ± 2.1	↑ handgrip strength = HbA1c, fasting glucose, insulin, lipid profile
Elkassaby (2014), (20)	Australia	50 T2DM duration < 1 year	Vitamin D3 10000 IU/day for 2 weeks followed by 6000 IU/day for 6 months	Placebo	6 m	Change in C-peptide	59 (42 – 75) to 128 (111 – 146)	6.2 (6.0 – 6.6)	= C-peptide = HbA1c, fasting glucose, insulin, HOMA-IR
Ghavamzadeh (2013), (34)	Iran	51, non insulin	Vitamin D3 400 IU/day	Placebo	14 w	HbA1c, TNF-α, leptin	21.5 ± 23.7 to 46.4 ± 35.1	6.8 ± 0.4	= HbA1c ↑ serum leptin ↓TNF-α
Heshmat (2012), (35)	Iran	42, non insulin	Vitamin D3 300.000 IU single dose	Placebo	3 m	Glycaemic parameters	117.3 ± 86.7 to 173.2 ± nr	6.5 ± 0.9	= HbA1c ↑ HOMA-IR, fasting glucose
Jafari (2015), (21)	Iran	59 post-menopausal women, non insulin	Vitamin D3 fortified yoghurt (2000 IU/day)	Plain yoghurt	12 w	Metabolic parameters	62.2 ± 24.6 to 86.8 ± 26.7	7.2 ± 1.3	= HbA1c ↓ fasting glucose, insulin, HOMA-IR, lipid profile
Jehle (2014), (22)	Switzerland	55 T2DM duration > 10 years	Vitamin D3 300000 IU single dose i.m.	Placebo	6 m	Change in HbA1c	36.0 ± 18.1 to 84.9 ± 16.0	7.0 ± 1.1	↓HOMA-IR = fasting insulin and glucose Significantly less increase in HbA1c in the intervention group

Table 1. Continued

Name, year, ref	Location	Cohort T2DM (n)	Intervention	Control	Duration	Primary outcome	25(OH)D nmol/l*	Baseline HbA1c (%)**	Main results
Jorde (2009), (36)	Norwegian	36, insulin treatment	Vitamin D3 40000 IU/week	Placebo	6 m	Glycaemic parameters	60.0 ± 14.0 to 118.3 ± nr	8.0 ± 1.3	= HbA1c, HOMA-IR, lipid levels
Kampmann (2014), (23)	Denmark	16, 25(OH)D < 50 nmol/l	Vitamin D3 11200 IU/day for 2 weeks followed by 5600 IU/day for 10 weeks	Placebo	12 w	Glycaemic parameters†	31.0 ± 13.6 to 104.9 ± 53.7	nr	= insulin sensitivity, HbA1c, lipid profile, 24h blood pressure
Krul-Poel (2015), (24)	Netherlands	261, non insulin	Vitamin D3 50000 IU/month	Placebo	6 m	HbA1c	60.6 ± 23.3 to 101.4 ± 27.6	6.8 ± 0.5	= HbA1c, HOMA-IR, lipid levels ↓ HbA1c (subgroup: 25(OH)D ≤ 30 nmol/l)
Nasri (2013), (37)	Iran	60	Vitamin D3 50000 IU/week	Placebo	12 w	Glycaemic parameters	83.9 ± 52.0 to 164.0 ± 57.0	7.7 ± 0.4	↓ HbA1c in male subjects
Nikooyeh (2011), (39)	Iran	90	1. Vitamin D3 fortified yoghurt (1000 IU/day) 2. Vitamin D3 + Ca fortified yoghurt (1000 IU / 500 mg/day)	Plain yoghurt	12 w	Metabolic parameters	44.4 ± 28.7 to 77.7 ± 28.6	7.4 ± 1.8	↓ HbA1c, HOMA-IR, fasting glucose and insulin, BMI = lipid levels
Parekh (2010), (25)	India	28, non insulin	Vitamin D3 300000 IU single dose i.m.	Placebo	4 w	Glycaemic parameters; OGTT	37.2 ± 16.9 to 103.8 ± 30.5	7.6 ± 0.6	= HbA1c, HOMA-IR, fasting glucose, insulin
Ryu (2013), (26)	Korea	158, non insulin, 5(OH)D < 50 nmol/l	Vitamin D3 1000 IU/day + Ca 100mg bid	Ca 100mg bid	24 w	Glycaemic parameters	27.0 ± 12.7 to 75.4 ± 27.0	7.3 ± 0.6	= HbA1c, HOMA-IR
Sadiya (2014), (27)	United Arab Emirates	8,7 25(OH)D < 50 nmol/l, BMI > 30	Vitamin D3 6000 IU/day for 3 months followed by 3000 IU/day for 3 months	Placebo	6 m	Metabolic parameters	28.5 ± 9.5 to 62.3 ± 20.8	8.3 ± 1.3	= HbA1c, fasting glucose, lipid levels Subgroup 25(OH)D < 30nmol/l: no difference
Shab-Bidar (2011), (28)	Iran	100, non insulin	Vitamin D3 fortified doogh (1000 IU/day + 340 mg Ca/day)	Plain doogh (340 mg Ca)	12 w	Metabolic parameters, endothelial biomarkers	38.5 ± 20.2 to 72.0 ± 23.5	8.7 ± 1.8	↓ fasting glucose, insulin, lipid profile, endothelial biomarkers = HbA1c

Table 1. Continued

Name, year, ref	Location	Cohort T2DM (n)	Intervention	Control	Duration	Primary outcome	25(OH)D nmol/l*	Baseline HbA1c (%)**	Main results
Soric (2012), (40)	US	37	Vitamin D3 1200 IU/day	Vitamin C 500mg /day	12 w	HbA1c	nr	8.6 ± 1.2	= HbA1c (total group) ↓ HbA1c (subgroup: HbA1c ≥ 9.0 %)
Strobel (2013), (38)	Germany	86, non insulin	Vigantol oil (vitamin D3 1904 IU/day)	Placebo	6 m / 12m	Glycaemic parameters	30.2 ± nr to 87.4 ± nr	nr	= HbA1c, HOMA-IR, fasting insulin and glucose
Sugden (2008), (12)	UK	34, 25(OH)D < 50 nmol/l	Vitamin D3 100000 IU single dose	Placebo	8 w	Endothelial function	40.2 ± 10.3 to 63.1 ± nr	7.5 ± 1.6	↑ FMD brachial artery = HbA1c
Tabesh (2014), (29)	Iran	118, 25(OH)D < 75 nmol/l	1. Vitamin D3 50000 IU /week 2. Ca 1000mg/day 3. Vitamin D3 50000 IU / week + Ca 1000 mg/day	Placebo	8 w	Metabolic parameters	28.0 ± 13.9 to nr	6.6 ± 0.8	↓ HbA1c, HOMA-IR, fasting glucose and insulin, LDL-cholesterol in Calcium + Vitamin D group. No change in the vitamin D group
Witham (2010), (30)	UK	61	Vitamin D3 single dose: 1. 100.000 IU 2. 200.000 IU	Placebo	16 w	Metabolic parameters	48.0 ± 21.0 to 76.0 ± 30.0	6.9 ± 0.8	= HbA1c, HOMA-IR, lipid levels
Yiu (2013), (31)	China	100, 25(OH)D < 75 nmol/l	Vitamin D3 5000 IU/day	Placebo	12 w	Endothelial function	52.7 ± 11.0 to 146.3 ± nr	7.4 (6.8 – 8.5)	= FMD, HbA1c, lipid levels

25(OH)D, 25-hydroxy vitamin D; BMI, body mass index; Ca, calcium; FMD, flow-mediated dilatation; HOMA-IR, homeostatic model assessment – insulin resistance; nr, not reported; OGTT, oral glucose tolerance test; T2DM, type 2 diabetes mellitus.

** serum 25(OH)D before and after treatment*

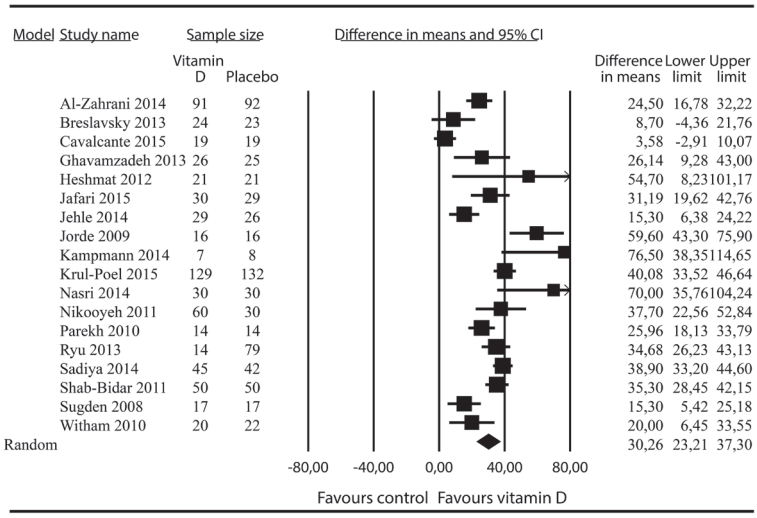
*** Baseline values of the intervention groups.*

↑ *= increasement*

↓ *= decreasement*

† *hyperinsulinemic euglycaemic clamp method*

Figure 3. Mean change from baseline in serum 25(OH)D (nmol/l) between intervention and control



The effect on HbA1c

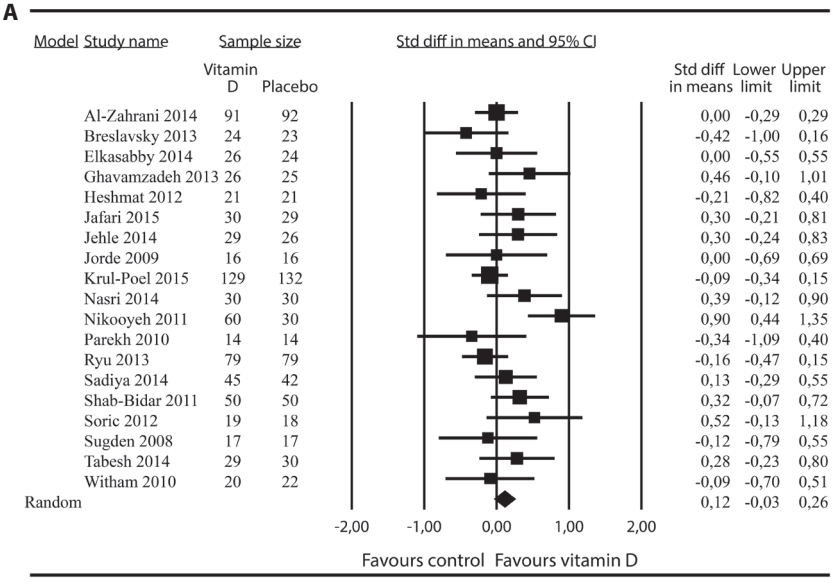
Nineteen studies reported sufficient data for inclusion in the meta-analysis to measure the overall effect of vitamin D on HbA1c (12,19-22,24-30,32,34-37,39,40). Four studies were excluded from this analysis by the following reasons: 1. no post-intervention HbA1c of the control group (33); 2. glycaemic control was measured by hyperinsulinemic euglycaemic clamp method (23); 3. no baseline HbA1c was available in the intervention group (38), and 4. no standard deviations were reported (31). The total number of included patients was 1475 of whom 755 were included in the treatment group and 720 in the placebo group. One out of these 19 studies reported a significant reduction in HbA1c after vitamin D intervention compared to placebo (39). In a study among 118 patients who were randomised to either vitamin D with or without calcium, or placebo, a significant decrease in HbA1c was seen in the vitamin D plus calcium group versus placebo. However, this study failed to reach a significant reduction in HbA1c in the group with solely vitamin D supplementation (29). A pilot RCT performed by Soric et al. (40) showed a trend towards a greater reduction in mean change of HbA1c in the vitamin D group compared to the control group, however, this difference was not statistically significant. In a subgroup analysis among patients with an HbA1c > 9.0%, a significantly greater reduction in HbA1c was observed in the intervention group (mean change: - 1.4%; 95% CI: -2.4 to -0.4, p = 0.01) compared to placebo (40). In our own study population, a significant effect of vitamin D supplementation on HbA1c was seen in patients with a serum 25(OH)D level ≤ 30 nmol/l (n = 19, mean change: -0.34%; 95% CI: -0.65 to -0.04, p = 0.02) (24). Furthermore, Nasri et al. (37) reported a significant difference in HbA1c between the intervention and control group only in male patients.

Based on a random-effect meta-analysis, comparing the mean change in HbA1c from baseline between the intervention and placebo group, no overall effect was seen on HbA1c after vitamin D intervention (standardized difference in means: 0.12; 95% CI: -0.03 to 0.26, p = 0.11) (Fig. 4a). Heterogeneity was present (I2 = 42%, p = 0.03) However, there was no evidence for publication bias (Egger's test: p = 0.38).

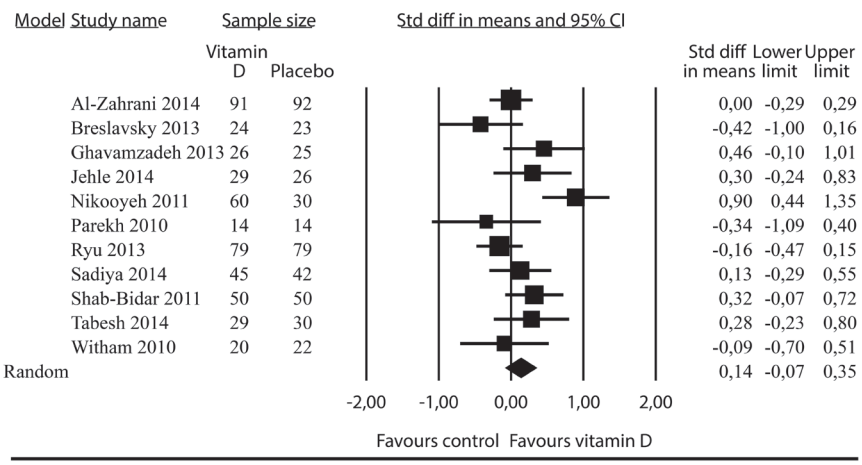
Including only the studies with a mean baseline 25(OH)D < 50 nmol/l did not change the effect of vitamin D intervention on HbA1c (standardized difference in means: 0.14; 95% CI: -0.07 to 0.35, p = 0.20) (Fig 4b) (12,19,22,23,25,27-30,32,34,38,39). In addition no difference was seen including only the studies with a mean baseline serum 25(OH)D < 30 nmol/l (standardized difference in means: 0.02; 95% CI: -0.18 to 0.23, p = 0.82) (Fig 4c). Including the studies with a baseline mean HbA1c ≥ 8% (64 mmol/mol) a trend towards a positive effect of vitamin D supplementation was seen, but this was not significant (standardized difference in means: 0.14; 95% CI -0.05 to 0.33, p = 0.14) (Fig 4d). Furthermore, inclusion of the studies which were labelled as good quality did not alter the results (standardized difference in means: 0.01; 95% CI -0.12 to 0.14, p = 0.90) (Fig 5). Heterogeneity was not present (I2 = 1%).

Figure 4. Meta-analysis of effects on HbA1c in all studies (a) and in studies with a baseline mean serum 25(OH)D < 50 nmol/l (b) or < 30 nmol/l (c), and mean baseline HbA1c ≥ 8% (d)

A



B


Model Study name
Sample size
Vitamin D
Placebo
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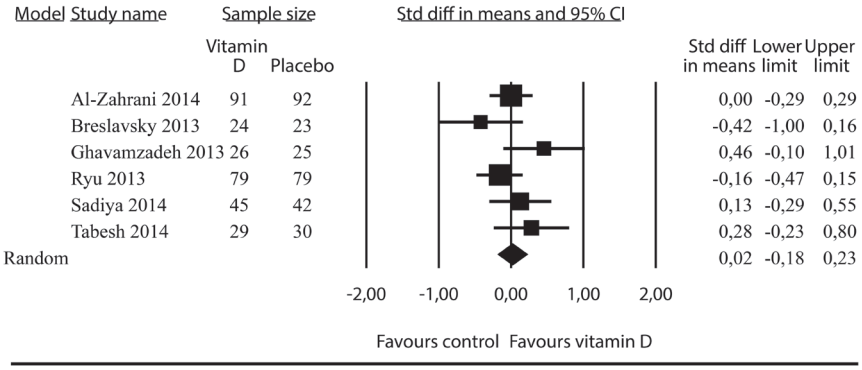
Std diff in means
Lower limit
Upper limit
Al-Zahrani 2014 91 92 0,00 -0,29 0,29
Breslavsky 2013 24 23 -0,42 -1,00 0,16
Ghavamzadeh 2013 26 25 0,46 -0,10 1,01
Jehle 2014 29 26 0,30 -0,24 0,83
Nikooyeh 2011 60 30 0,90 0,44 1,35
Parekh 2010 14 14 -0,34 -1,09 0,40
Ryu 2013 79 79 -0,16 -0,47 0,15
Sadiya 2014 45 42 0,13 -0,29 0,55
Shab-Bidar 2011 50 50 0,32 -0,07 0,72
Tabesh 2014 29 30 0,28 -0,23 0,80
Witham 2010 20 22 -0,09 -0,70 0,51
Random 0,14 -0,07 0,35
-2,00 -1,00 0,00 1,00 2,00
Favours control Favours vitamin D

C


Model Study name
Sample size
Vitamin D
Placebo
Std diff in means and 95% CI
Std diff in means
Lower limit
Upper limit
Al-Zahrani 2014 91 92 0,00 -0,29 0,29
Breslavsky 2013 24 23 -0,42 -1,00 0,16
Ghavamzadeh 2013 26 25 0,46 -0,10 1,01
Ryu 2013 79 79 -0,16 -0,47 0,15
Sadiya 2014 45 42 0,13 -0,29 0,55
Tabesh 2014 29 30 0,28 -0,23 0,80
Random 0,02 -0,18 0,23
-2,00 -1,00 0,00 1,00 2,00
Favours control Favours vitamin D

D

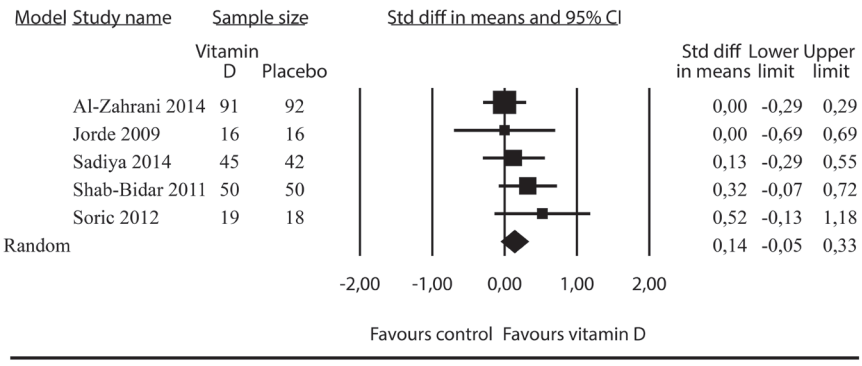
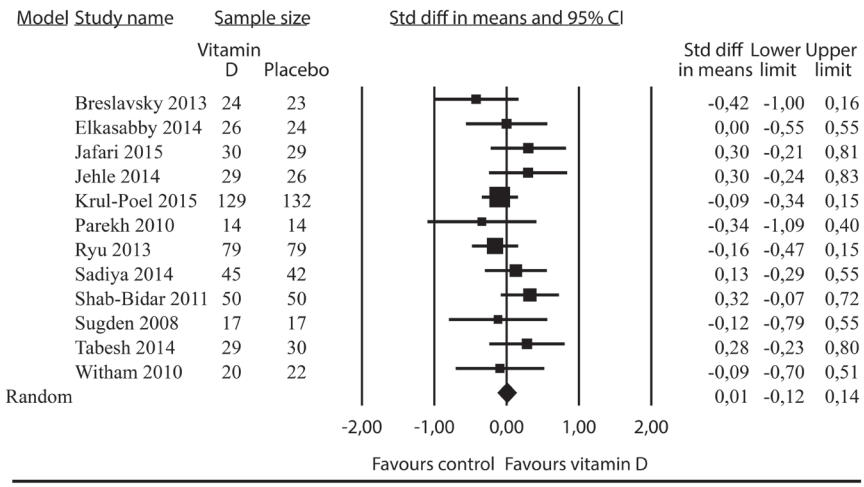

Model Study name
Sample size
Vitamin D
Placebo
Std diff in means and 95% CI
Std diff in means
Lower limit
Upper limit
Al-Zahrani 2014 91 92 0,00 -0,29 0,29
Jorde 2009 16 16 0,00 -0,69 0,69
Sadiya 2014 45 42 0,13 -0,29 0,55
Shab-Bidar 2011 50 50 0,32 -0,07 0,72
Soric 2012 19 18 0,52 -0,13 1,18
Random 0,14 -0,05 0,33
-2,00 -1,00 0,00 1,00 2,00
Favours control Favours vitamin D

Figure 5. Meta-analysis of effects on HbA1c in studies labelled as good quality

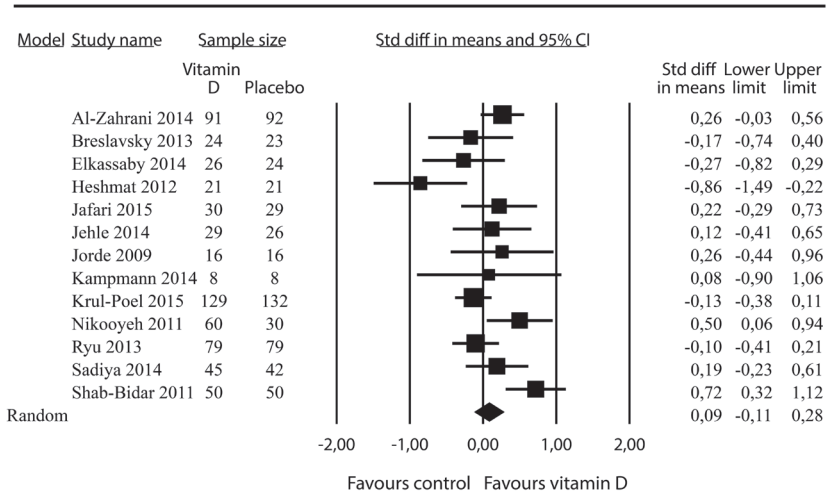


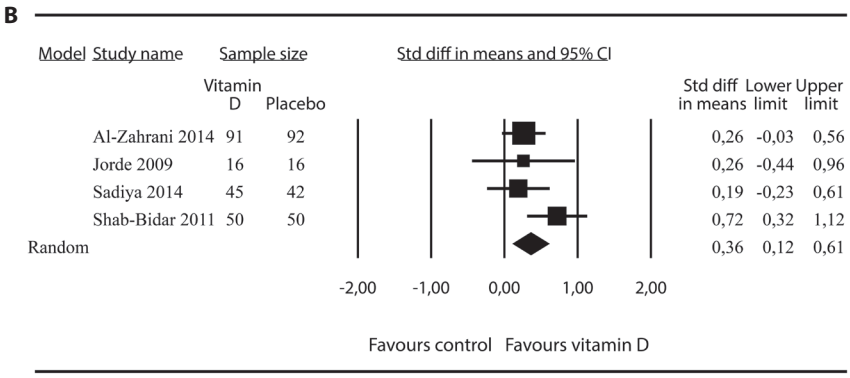
The effect on fasting glucose

Of the 23 studies that were included in the systematic review, 13 reported fasting glucose as primary or secondary outcome measure (19-24,26-28,32,35,36,39). Three studies reported a significant reduction of fasting glucose after vitamin D supplementation (21,28,39). groups did not reveal an overall effect of vitamin D supplementation on fasting glucose (between group standardized mean difference: 0.09; 95% CI: -0.11 to 0.28, p = 0.39, I2 = 60%) (Fig. 6a). No evidence for publication bias was found using a funnel plot and Egger's test (p = 0.97). Including only the good quality studies did not alter the effect on fasting glucose. A pooled meta-analysis with the inclusion of the studies with a mean baseline HbA1c ≥ 8% (64 mmol/mol) shows a significant effect of vitamin D on fasting glucose (standardized difference in means: 0.36; 95% CI: 0.12 to 0.61, p = 0.003, I2 = 0%) (Fig 6b).

Figure. 6 Meta-analysis of effects on fasting glucose in all studies (a) and in studies with a baseline mean HbA1c ≥ 8% (b)

A



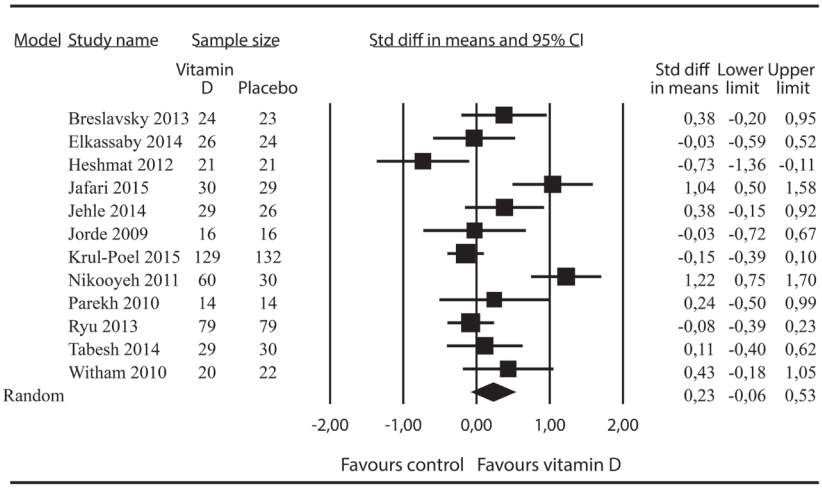


The effect on insulin resistance

Thirteen studies reported data on insulin resistance of which twelve studies used the HOMA-IR to quantify insulin resistance (19-22,24-26,29,30,35,36,39), and one study measured insulin resistance through hyperinsulinemic euglycaemic clamp method (23). Two studies observed a significant reduction of insulin resistance after vitamin D supplementation (21,39), and one study found a negative effect of vitamin D supplementation on insulin resistance compared to placebo (35).

Twelve studies were compared in a random effects meta-analysis model, demonstrating no significant effect of vitamin D supplementation on insulin resistance compared to controls (between group standardized difference in means: 0.23; 95% CI: -0.06 to 0.53, p = 0.12; I2 77%, p = 0.04) (Fig 7). No evidence for publication bias was found using a funnel plot and Egger's test (p = 0.26). Inclusion of the studies which were qualified as good did not alter the results. Only one study reported data of HOMA-IR with a baseline HbA1c ≥ 8%. The study performed by Kampmann et al. (23) which measured insulin resistance by using the hyperinsulinemic euglycaemic clamp method, which is the golden standard, did not find a positive effect of vitamin D on glycaemic control in 16 patients with type 2 diabetes.

Figure 7. Meta-analysis of effects on HOMA-IR



DISCUSSION

Our systematic review and meta-analysis examined the effect of vitamin D supplementation on glycaemic indices in patients with type 2 diabetes mellitus. Combining all studies no effect was seen of vitamin D supplementation on parameters of glycaemic control (i.e. HbA1c, fasting glucose and HOMA-IR) in patients with type 2 diabetes. Including only studies with a mean baseline serum 25(OH)D < 50 nmol/l or < 30 nmol/l did not change these results. Including only the studies with a mean baseline HbA1c ≥ 8% (64 mmol/mol) revealed a significant effect of vitamin D supplementation on fasting glucose.

The main challenge of this systematic review was the heterogeneity between the studies. To level for this challenge we only included RCTs. However, still heterogeneity was present with a wide variety of intervention schemes and follow-up duration used in the included studies, which resulted in a varying increase in serum 25(OH)D as was shown in Figure 2. To resolve the problem of heterogeneity we applied a quality assessment of all included studies. Including only good quality studies did not alter the effect of vitamin D supplementation on glycaemic indices.
Still no consensus has been reached in the optimal value of serum 25(OH)D and the best supplementation regime. Nowadays vitamin D deficiency is commonly defined by a serum 25(OH)D less than 30 nmol/l. This threshold level has been confirmed by the Institute of Medicine at the end of 2010 and the Endocrine Society Guideline (41,42). Optimal serum 25(OH)D is defined as a level above 50 nmol/l according to the Institute of Medicine and above 75 nmol/l according to the Endocrine Society.

A possible explanation for the lack of effect found in most studies could be an underrepresentation of vitamin D deficient patients. It is possible that vitamin D could only be effective in vitamin D deficient patients, and especially in those with poor glycaemic control (43,44). This hypothesis was confirmed in the study performed by Soric et al. (40) who showed a 1.4% decrease in HbA1c in patients with a baseline HbA1c level ≥ 9.0% after 12 weeks with a daily consumption of 2.000 IU vitamin D in contrast to patients with a HbA1c < 9.0% where no effect on glycaemic control was seen after vitamin D treatment. Additionally, in our previous RCT among 275 patients with type 2 diabetes, in 19 patients with a serum 25(OH)D below 30 nmol/l a significant decrease in HbA1c was seen after six months of vitamin D supplementation compared to placebo (24). Another important note is the wide range in follow-up duration between the studies. As HbA1c is representing the glycosylated haemoglobin which has a life time around 100 days, a follow-up duration of more than three months is favourable.

Of interest is the possibility that vitamin D could only be beneficial in patients with normal glucose tolerance or impaired glucose tolerance. The pathogenesis of type 2 diabetes consists of a progressive insulin resistance, which is initially compensated by enhanced insulin secretion by the pancreatic beta-cells. At the time of onset of type 2 diabetes the beta-cell mass is reduced by 25-50% (45). The direct effect of vitamin D on the pancreatic beta-cell might be negligible at this time. In this line, our systematic review including only studies examining patients with type 2 diabetes is a limitation of this study.

Individual variability explained by vitamin D receptor polymorphisms may also play a role in the study results. Earlier research demonstrated an association between vitamin D receptor polymorphisms and the risk for type 2 diabetes, suggesting that timing of vitamin D supplementation is critical (46,47). In addition, a study performed by Wang et al. demonstrated that the vitamin D binding protein polymorphism, and thus vitamin D bioavailability, was moderately associated with increased susceptibility to type 2 diabetes in Asians, but not in Caucasians, suggesting that ethnicity might be a potential factor associated with heterogeneity (48) .

Another relevant note is the different vitamin D assays which were used in the included studies. Much discussion is going on about the comparability and accuracy of the different assays, which raises concerns (49). Most of the studies included in this review used an enzyme-immunoassay method for measurement of serum 25(OH)D, where the liquid chromatography-mass spectrometry (LC-MS) method is the golden standard.

The strength and limitations of our study needs to be mentioned. First, our initial search was performed by only one author, which may cause that eligible studies have not been included. However, our negative findings suggest that unpublished studies (which also tend to be negative) would be very unlikely to alter our conclusions. We found no evidence for publication bias from the funnel plots. For the meta-analysis we performed a quality assessment according to the checklist of the Dutch Cochrane Collaboration which has some limitations, especially when trying to decide on the relative importance of the different criteria (16). Another note is that we did not have access to all original data, which is the best method to perform a meta-analysis. A strength of our study is that we included only RCT's to assess the strength of evidence and limit the role of bias.

In conclusion, current evidence of RCTs shows no evidence to support short-term vitamin D supplementation in a heterogeneous population with type 2 diabetes. However, in patients with poorly controlled diabetes a favourable effect of vitamin D is seen on fasting glucose. Future research among this subgroup is mandatory.

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PART IV

GENERAL DISCUSSION AND SUMMARY

GENERAL DISCUSSION

The classical role of vitamin D is to promote calcium absorption in the gut and maintain adequate serum calcium and phosphate concentrations to enable normal bone mineralisation. Besides this, vitamin D has been shown in the past decades to have multiple biological targets mediated by the vitamin D receptor (VDR) which is localized in over 35 cell types. In epidemiological studies, vitamin D status has been associated with many non-skeletal outcomes including, insulin resistance, type 2 diabetes, several auto-immune diseases, cardiovascular diseases and depression (1).
This thesis highlights the role of vitamin D in glycaemic control in different populations (i.e. gestational diabetes, polycystic ovary syndrome (PCOS) and type 2 diabetes). Type 2 diabetes represents a worldwide epidemic with significant co-morbidity and mortality (2). Insulin resistance and decreased beta cell function are major contributing factors in the onset of type 2 diabetes. Although therapies for type 2 diabetes have improved over the last few decades, new insights in the prevention and management remain necessary.

Decades ago, a seasonal variation of glycaemic control with worsening during winter and spring first highlighted a possible link between vitamin D and glycaemic indices (3,4). In addition, a large number of observational studies has been performed, most of them demonstrating an inverse association between vitamin D status and glycaemic control and emphasizing the clinical relevance of determining vitamin D status in high risk patients as well as patients with prediabetes and type 2 diabetes (5,6). However, the confounding effect of environmental factors (i.e. physical activity, obesity, dietary habits, sun exposure, and seasonal variation) could not be ruled out. Intervention studies among animal models have demonstrated a favourable effect of vitamin D on glucose homeostasis. Cholecalciferol supplementation in spontaneously hypertensive rats and Wistar rats with diet-induced diabetes reduced the blood glucose level (7).
In humans, intervention studies yielded conflicting results. A large prospective study performed by Pittas et al. (8) followed 83,779 women with no history of diabetes for the development of type 2 diabetes. Women with the highest vitamin D and calcium intake showed the lowest incidence of type 2 diabetes. In contrast in the Woman's Health Initiative trial in which 33,951 healthy postmenopausal women were followed over a period of 7 years, a daily intake of 400 IU of vitamin D3 daily and 1000 mg of calcium did not reduce the incidence of diabetes (9). However, this study was not designed to investigate diabetes risk in this rather healthy population. A summary and a meta-analysis of the intervention studies investigating the effect of vitamin D on glycaemic control in type 2 diabetes is given in Chapter 9.

In Chapter 1 of this thesis a random effect meta-analysis indicates a significant inverse association of serum 25(OH)D with the incidence of gestational diabetes. Intervention trials in pregnant women at risk for, or having gestational diabetes are scarce. A recently performed RCT among 54 pregnant women with gestational diabetes demonstrated a beneficial effect of two capsules of 50.000 IU cholecalciferol within three weeks on glycaemic indices (10). However, the blood glucose level in the intervention group was significantly higher at baseline than in controls. Yap et al. (11) conducted a RCT among 179 pregnant women with normal glucose tolerance. The main findings were that supplementation with 5000 IU vitamin D3 daily during pregnancy could safely and effectively elevate

serum 25(OH)D concentrations into the desired target range in 90% of the included women, but this did not lead to an improvement in maternal glucose metabolism, compared with a control group taking a standard pregnancy supplemental dose of 400 IU vitamin D3 daily.
A large European multicentre RCT (DALI), examining the effect of vitamin D supplementation and lifestyle in preventing gestational diabetes in obese pregnant women with a normal glucose tolerance, is currently ongoing (12). Hopefully, this study will elucidate whether vitamin D is effective in preventing gestational diabetes in high risk women.

The role of vitamin D on glycaemic control in women suffering from PCOS is described in part II of this thesis. A significant inverse association between vitamin D status and glycaemic indices in a large PCOS cohort is described in Chapter 3. Until now, seven intervention studies examining the effect of vitamin D supplementation on metabolic disturbances in PCOS have been performed, which are summarised in a recently published systematic review (13). A significant inverse association was found between serum 25(OH)D and insulin resistance in the included observational studies, which is in line with our results. However, no significant improvement in metabolic functions was found among PCOS women supplemented with vitamin D. An important drawback is that most of the intervention studies were not randomised, and were conducted with relatively small samples sizes with a short follow-up. The largest study with a RCT design among 104 obese, vitamin D deficient PCOS women did reveal a positive effect of weekly 50.000 IU vitamin D plus calcium 1000mg/day on insulin resistance (14).
Of interest is a study performed by Irani et al. among 45 PCOS women and 22 controls, in which the participants were randomised to either vitamin D 50.000 IU/week or placebo for 8 weeks. The primary outcome of this study was the change in soluble receptor for advanced glycation end products (sRAGE) that binds to serum pro-inflammatory advanced glycation end products (AGEs), which are in turn involved in the pathogenesis of PCOS (15). The authors found an increase in circulating sRAGE in the intervention group suggesting a protective effect of vitamin D in PCOS (16). This result is in line with our findings described in Chapter 8 in which a positive association between vitamin D status and skin AGEs is found. This may imply a protective anti-inflammatory effect of vitamin D. However, to prove causality further research with randomised clinical trials is necessary.

In summary, in line with the results of this thesis we can conclude that a clearly disparity exists between observational studies showing vitamin D deficiency as a risk factor for impaired glycaemic control, and intervention trials that have largely failed to prove that vitamin D supplementation has significant benefits.
There are many possible reasons for this disparity; the design of the intervention trials may not be adequate (i.e. sample size of the subjects, vitamin D status at baseline, vitamin D dose, study population, follow-up duration).

Study population
The sample sizes of the study populations are very different in the intervention studies performed until now. To detect a small effect of vitamin D supplementation large studies are necessary.
When considering the results of our intervention trial in particular, demonstrating a small positive effect of vitamin D supplementation on HbA1c in severely vitamin D deficient patients (serum

25(OH)D < 30 nmol/l), it is imaginable that vitamin D supplementation would only be beneficial in severely vitamin D deficient patients. Studies including only severely vitamin D deficient patients with glycaemic indices as primary outcome are not available until now. Furthermore, the degree of irreversible tissue damage at baseline of the trial affects outcomes; for example, rickets bone deformities are irreversible, as defective insulin secretion may also be. Our intervention trial was conducted in patients with type 2 diabetes that is characterized by insulin resistance in combination with pancreatic beta-cell dysfunction. While serum 25(OH)D can directly stimulate insulin release, this is not effective in case of already exhausted pancreatic beta-cell in type 2 diabetes. For this, it would be preferable to conduct studies among subjects with a 'prediabetic' state.
Moreover, it is likely that vitamin D supplementation is more effective in patients with type 2 diabetes who are poorly regulated. This hypothesis was confirmed in the study performed by Soric et al. (17) who showed a 1.4% decrease in HbA1c in patients with a baseline HbA1c level ≥ 9.0% after 12 weeks with a daily consumption of 2.000 IU vitamin D in contrast to patients with a HbA1c < 9.0% where no effect on glycaemic control was seen after vitamin D treatment. In addition, this results was confirmed by our meta-analysis, described in Chapter 9, in which an overall significant effect was seen of vitamin D supplementation on fasting glucose including four studies with a baseline HbA1c ≥ 8%.

Intervention dose

The lack of effect could also be due to an insufficient supplementation dose. To date, the vitamin D experts worldwide are still discussing about the optimal serum 25(OH)D level and the required supplementation dose. For example in pregnancy, national health councils recommend vitamin D supplementation, ranging from 400 – 600 IU/day, which often is not sufficient for an optimal vitamin D status during pregnancy according to experts (18,19). The Institute of Medicine has determined that serum 25(OH)D levels greater than 50 nmol/l are sufficient based on the current studies available, although other experts consider that optimal levels should be higher (greater than 75 nmol/l) (19,20).

Follow-up duration

The wide range in follow-up duration between the studies, from several weeks to one year, is a matter of concern. As HbA1c represent the glycosylated haemoglobin that has a life time around 100 days, a follow-up duration of more than three months is favourable.

Measurement of insulin resistance

The method used to assess insulin resistance in the conducted trials could have influenced the outcome of the studies. Most of the studies, including our intervention study, measured insulin resistance through homeostasis model assessment of insulin resistance (HOMA-IR) using fasting glucose and fasting insulin that is inferior to the golden standard of the euglycaemic hyperinsulinemic clamp technique and oral glucose tolerance test as second best (21).

Serum 25(OH)D assay

Another key note is the diversity in 25(OH)D assays which are used in all studies. Much discussion is ongoing about the comparability and accuracy of the different assays, which raises concerns for the

comparability and interpretation of all study results (22). Currently, the liquid chromatography-mass spectrometry (LC-MS) method is the golden standard to measure serum 25(OH)D.

Genetics

Individual variability explained by the presence of vitamin D receptor (VDR) polymorphisms and polymorphisms in genes affecting vitamin D and glucose homeostasis could also play a role in the diverging results. Recent literature demonstrated an association between VDR polymorphisms and the risk for type 2 diabetes(23). In addition Wang et al. (24) demonstrated an association between vitamin D binding protein polymorphism and increased susceptibility to type 2 diabetes in Asians, but not in Caucasians, suggesting that ethnicity might be a contributing factor in the scattered results. Moreover, certain VDR polymorphisms have been associated with reduced response to vitamin D supplementation in terms of improvement of serum 25(OH)D, insulin sensitivity and inflammation(25,26).

Taken these limitations together, much heterogeneity is present in the current studies which makes it difficult to interpret the conclusions from meta-analyses carried out in this field. Finally, the reason for the discrepancies between observational and intervention studies raises the question whether or not vitamin D status and glycaemic indices are causally related? The reason for the discrepancies could be that there is no causal relationship. In line with this question, a recent mendelian randomisation study performed by Ye et al. suggested that the association between circulating serum 25(OH)D and incident type 2 diabetes is unlikely to be causal. The authors conclude that efforts to increase serum 25(OH)D might not reduce the risk of type 2 diabetes as would be expected on the basis of cross-sectional findings. They additionally suggest to identify causal factors that might increase serum 25(OH)D concentration and also reduce the risk of type 2 diabetes (27). In contrast to this, Pilz et al. (28) reply that evidence from genetic data suggest that health benefits from vitamin D may require adequate lifelong supplementation. Continuing on the assumption that vitamin D and glycaemic control are not causally related, vitamin D might be a marker of frailty or ill health. This would explain why low vitamin D status is related with a wide range of diseases (29).

In conclusion, so far no definitive conclusion can be drawn about the relationship between vitamin D status and glycaemic indices. Probably, a small effect of vitamin D supplementation exists on glycaemic indices in severely vitamin D deficient patients and a poorly regulated type 2 diabetes. The discrepancy between the positive observational studies and the lack of effect found in most intervention studies is remarkable. Whether this is due to the limitations in all studies (i.e. low intervention dose, inclusion criteria of the study population, follow-up duration, small sample size), or that there is simply no causal relationship between vitamin D status and glycaemic control, is still not clear. Hopefully, upcoming large trials, which are summarised in table 1 will give an answer to this uncertainty.

Future perspectives

Future studies among severely vitamin D deficient subjects (serum 25(OH)D < 30 nmol/l) and poorly controlled diabetes are highly recommended. First, the study design should be a double-blind randomised placebo-controlled trial with sufficient power and a follow-up duration for at least six months.

Serum 25(OH)D should be measured using the LC-MS method. Concerning the intervention, a sufficient vitamin D supplementation dose is necessary, which should be ≥ 2000 IU vitamin D/day. The study population to examine a subject of discussion. It would be interesting to perform a study in a population at risk for diabetes (i.e. subjects with impaired glucose tolerance, PCOS women, pregnant women at high risk for gestational diabetes), as the pancreatic beta-cell will not be exhausted in this phase of hyperinsulinemia and insulin resistance. To carry out the 'perfect' study additional genotyping of the VDR gene and genes involved in glucose and vitamin D metabolism of all participants would be necessary. However, to examine the effects of genotyping, first more research is needed to elucidate the influence of the vitamin D related polymorphisms on glycaemic indices.
On the other hand, more studies are needed to elucidate the association between vitamin D status and skin AGEs. This recently observed association may be a new pathway clarifying the association between vitamin D and cardiovascular morbidity.

Table 1. Upcoming intervention trials examining vitamin D supplementation on glycaemic indices in pregnancy, PCOS women, prediabetes and type 2 diabetes, with study size > 150 participants.

Study Centre	Population	Intervention	Primary outcome	Trial register	Trial status
Multicentre European study, DALI	880 pregnant women at risk for GDM	1600 IU/d vs lifestyle vs placebo	Glycaemic indices Weight gain	ISRCTN70595832	Completed
Saudi Arabia	340 PCOS women	50.000 IU/w for 8 w, followed by 1000 IU/d for 16 w vs placebo	Insulin resistance	NCT02164552	Recruiting
Graz University, Austria	150 PCOS versus 150 control women, 25(OH)D < 75nmol/l	20.000 IU/w vs placebo 24 weeks		NCT01721915	Recruiting
Tufts Medical Centre, D2d	2382 prediabetes adults	4000 IU/d vs placebo 3 years	Time to development of diabetes	NCT01942694	Recruiting
HaEmek Medical Centre, Israel	180 T2DM, HbA1c > 7.5%, serum 25(OH) D < 50 nmol/l	120.000 IU/m for 6 months vs placebo	Change in HbA1c	NCT01991054	Recruiting
Qatar	200 adults with high diabetes risk, 25(OH) D < 75 nmol/l	4000 IU/d 24 weeks vs placebo	Change in insulin resistance	NCT02098980	Recruiting
US, VITAL	28875 adults	2000 IU/d versus omega-3 fatty acids	Incident T2DM, cancer, CVD	NCT1169259	Recruiting
Canada, EVIDENCE	160 adults with increased diabetes risk, 25(OH)D < 65 nmol/l	Vitamin D enriched cheese (28.000 IU/w) vs normal cheese; 24 weeks	Insulin resistance (OGTT)	NCT01726777	Completed
King Faisal Specialist Hospital, Saudi Arabia	500 prediabetes, 25(OH)D between 10-30 nmol/l	5000 IU/d vs placebo for 2 years	Incidence of T2DM	NCT01170468	Recruitment closed

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SUMMARY

This thesis highlights the role of vitamin D in glycaemic control in various populations. The aim of this thesis is to explore the role of vitamin D in patients with gestational diabetes mellitus (GDM), polycystic ovary syndrome (PCOS) and type 2 diabetes mellitus.

Part I

Chapter 1 provides a systematic literature review and meta-analysis of the association between maternal vitamin D status and the onset of gestational diabetes mellitus (GDM). Seven observational studies were included, in which overall a significant inverse association was found between maternal vitamin D status and the incidence of GDM. Serum 25-hydroxyvitamin D (25(OH)D was significantly lower in patients with GDM compared to normal glucose tolerance.

Part II

The second part of this thesis focuses on the association between vitamin D status and metabolic disturbances in women with polycystic ovary syndrome (PCOS). This part is divided into two chapters. First, in Chapter 2 a summary of the literature is provided in a systematic literature review about the association between vitamin D and metabolic disturbances in women suffering from polycystic ovary syndrome (PCOS). Current evidence including 29 observational studies suggests an inverse association between vitamin D status and metabolic disturbances in PCOS. However, significancy disappeared in PCOS women after correcting the results for BMI.

Second, in Chapter 3 the association between vitamin D and metabolic disturbances was explored in women with PCOS (Rotterdam PCOS cohort) compared to controls. A total of 639 PCOS women and 449 control women were included. The results demonstrated a significant lower serum 25(OH) D in PCOS versus control women (serum 25(OH)D 49.0 versus 64.5 nmol/l, respectively). As expected from the earlier systematic review a significant higher insulin resistance, measured using the homeostasis model assessment (HOMA-IR), was found in the lowest vitamin D group compared to PCOS women in the highest vitamin D group. Additionally, a significant adjusted assocation was seen between serum 25(OH)D and HDL-cholesterol and apolipoprotein A1 in PCOS women. Large randomised controlled trials are necessary to explore the causality of this linkage.

Part III

This part of the thesis presents the data of our randomised placebo-controlled clinical trial in which 275 patients with type 2 diabetes without insulin treatment were randomised to either cholecalciferol 50.000 IU/month or placebo during six months. Chapter 4 presents the study protocol.

In Chapter 5 the primary outcome, the effect of vitamin D supplementation on glycemic control after six months, is described. Mean baseline 25(OH)D was 59.1 versus 59.8 nmol/l in the vitamin D group versus the placebo group, respectively. Mean baseline HbA1c was 6.8% (51 mmol/mol) in both groups. After six months of vitamin D supplementation no improvement of HbA1c, and other indicators of glycaemic control, was found in the intervention group compared to placebo. Subgroup analysis revealed a significant decrease of HbA1c among 19 patients with a baseline serum 25(OH)D < 30 nmol/l.

The next two chapters describe the association between vitamin D status and health related qua-

lity of life in 241 patients with type 2 diabetes. In Chapter 6 cross-sectional analyses showed no association between vitamin D status and health related quality of life. In Chapter 7 longitudinal analyses were performed after six months of vitamin D supplementation versus placebo. Totally, 187 patients completed baseline and follow-up questionnaire (SF-36) after six months of intervention. No improvement of health related quality of life was seen after six months of vitamin D supplementation compared to placebo, despite an adequate rise in serum 25(OH)D from 58.5 to 106.0 nmol/l.
In Chapter 8 the association between vitamin D status and advanced glycation endproducts (AGEs) is examined. AGEs were measured using skin autofluorescence. They are suggested to be one of the major agents in the pathogenesis and progression of diabetes related cardiovascular complications. 245 patients with type 2 diabetes were enrolled in this study. Vitamin D status was independently associated with skin autofluorescence. After six months of intervention no effect was seen on the amount of skin AGEs.
Finally, Chapter 9 provides a systematic review and meta-analysis of all randomised clinical trials examining the effect of vitamin D supplementation on glycaemic control in patients with type 2 diabetes. Combining these studies no significant effect in change of HbA1c, fasting glucose or HOMA-IR, was seen after vitamin D intervention compared to placebo. Subgroup analysis, including only studies with a mean baseline HbA1c ≥ 8.0% (64 mmol/mol), revealed a significant effect on fasting glucose. In conclusion, currently insufficient evidence exists to support vitamin D supplementation in patients with type 2 diabetes with the aim to improve glycaemic control.

NEDERLANDSE SAMENVATTING

Dit proefschrift toont de resultaten van wetenschappelijk onderzoek naar het verband tussen vitamine D en insuline resistentie in drie verschillende groepen: 1) vrouwen met zwangerschapsdiabetes; 2) vrouwen met het polycysteus ovarium syndroom (PCOS) en 3) patiënten met diabetes mellitus type 2. In de laatste groep hebben wij ook gekeken naar kwaliteit van leven in relatie tot vitamine D en de mate van 'advanced glycation endproducts' (versuikerde eiwitten).

Vitamine D

Vitamine D is een vet oplosbaar vitamine wat voornamelijk wordt geproduceerd in de huid onder invloed van zon(UV)licht. Een kleine hoeveelheid vitamine D wordt verkregen uit voeding als vette vis, zuivel en supplementen. Vitamine D wordt nadien gemetaboliseerd in de lever en de nieren tot de actieve vorm van vitamine D: 1,25 dihydroxyvitamine D. De belangrijkste functie van vitamine D is het stimuleren van calciumopname uit de darm om een optimale botmineralisatie te verkrijgen. Ernstig vitamine D gebrek leidt dan ook tot botweekheid (osteomalacie) bij volwassenen of op kinderleeftijd (rachitis / Engelse ziekte).

Sinds de ontdekking dat de vitamine D receptor ook in vele andere weefsels en cellen aanwezig is, wordt vitamine D in verband gebracht met tal van andere aandoeningen, o.a. hart- en vaatziekten, auto-immuun aandoeningen, kanker en ook diabetes. Op dit moment wordt hier veel onderzoek naar verricht. Wereldwijd komt een vitamine D tekort veelvuldig voor. Gezien de grootste productie van vitamine D via zonlicht in de huid plaatsvindt, zijn een donkere huidskleur, lichaamsbedekking, zonnebrandcrème en weinig blootstelling aan zonlicht, belangrijke risicofactoren voor een tekort aan vitamine D.

Vitamine D – insuline resistentie

Insuline resistentie betekent de verminderde gevoeligheid van de receptor (aangrijpingspunt) voor insuline. Dit, samen met een verminderde insuline productie door de alvleesklier, zijn de belangrijkste oorzaken voor het ontstaan van diabetes mellitus type 2. Er zijn meerdere mechanismen hoe vitamine D een invloed kan hebben op zowel de insuline productie in de alvleesklier als het verbeteren van de insuline gevoeligheid.

Er is veel observationeel onderzoek verricht waarbij wordt aangetoond dat patiënten met een lagere vitamine D waarde meer risico hebben op het ontwikkelen van diabetes, meer insuline resistentie vertonen, en een slechter gereguleerde diabetes hebben. Om te bewijzen of dit verband oorzakelijk van aard is, dient klinisch onderzoek te worden verricht waarbij vitamine D suppletie wordt vergeleken met een placebo.

Part I

In het eerste deel van dit proefschrift, hoofdstuk 1, wordt in een systematische review en meta-analyse het verband tussen vitamine D en zwangerschapsdiabetes samengevat. In totaal werden er 7 observationele studies geïncludeerd die de associatie tussen vitamine D en het voorkomen van zwangerschapsdiabetes hebben onderzocht. Alle data bij elkaar tonen een significant verband tussen de hoogte van de vitamine D level en het ontstaan van zwangerschapsdiabetes.

Tevens werd er aangetoond dat patiënten met zwangerschapsdiabetes een significant lagere vitamine D waarde hadden dan controle zwangeren zonder zwangerschapsdiabetes.

Part II

Het tweede deel van dit proefschrift focust zich op het verband tussen vitamine D en metabole kenmerken, in het speciaal insuline resistentie, bij vrouwen met het polycysteus ovarium syndroom (PCOS). PCOS is de meest voorkomende hormonale aandoening bij vrouwen in de vruchtbare levensfase. Het wordt gekenmerkt door anovulatie (uitblijven van de eisprong), hyperandrogenisme (verhoogde waarden van het mannelijk hormoon in het bloed) of hirsutisme (overbeharing) en/of polycysteuze ovaria (de aanwezigheid van veel kleine eiblaasjes in de eierstokken). Bij het merendeel van de patiënten leidt dit tot vruchtbaarheidsproblemen. Daarnaast kenmerkt PCOS zich in het vroeg ontwikkelen van metabole problemen als insuline resistentie, diabetes, en mogelijk ook hart- en vaatziekten.

In hoofdstuk 2 wordt een systematische samenvatting van de literatuur gegeven die het verband tussen vitamine D en metabole kenmerken bij vrouwen met PCOS beschrijft. Negentwintig studies werden in dit artikel samengevoegd wat een verband liet zien tussen een laag vitamine D en toegenomen metabole verstoringen. Echter wanneer dit resultaat werd gecorrigeerd voor het BMI was dit verband niet meer significant.

Hoofdstuk 3 beschrijft het verband tussen vitamine D en metabole kenmerken in 639 vrouwen met PCOS (Rotterdam PCOS cohort). Tevens is de vitamine D waarde bij vrouwen met PCOS vergeleken met controle vrouwen van dezelfde leeftijd. De resultaten toonden een significant lagere vitamine D waarden in vrouwen met PCOS t.o.v. controle vrouwen. Daarnaast werd er een significant hogere insuline resistentie gezien in de groep PCOS vrouwen met de laagste vitamine D waarden t.o.v. de groep met een hogere vitamine D waarde. Het HDL-cholesterol en apolipoproteine A1 was significant hoger in de groep met de hoogste vitamine D waarde t.o.v. de laagste groep.

Part III

Dit deel van het proefschrift presenteert de uitkomsten van de door ons uitgevoerde gerandomiseerde, placebo gecontroleerde studie. In deze studie werden 275 patiënten met diabetes mellitus type 2, die geen insuline gebruikten, geïncludeerd. De patiënten werden gerandomiseerd naar of vitamine D3 50.000 IE per maand, of een identiek uitziende placebo gedurende zes maanden. In hoofdstuk 4 wordt het studie protocol beschreven.

Hoofdstuk 5 toont de primaire uitkomstmaat, het effect van vitamine D op de glycemische controle na zes maanden vergeleken met de placebo groep. De gemiddelde vitamine D waarde bij inclusie was 59.1 versus 59.8 nmol/l in de vitamine D groep versus de placebo groep met een HbA1c van 6.8% (51 mmol/mol) in beide groepen. Na zes maanden suppletie steeg het vitamine D naar 101.4 nmol/l in de interventie groep, in de placebogroep bleef het vitamine D 59.8 nmol/l. Er werd geen verbetering waargenomen van het HbA1c, de meest gebruikt maat voor de regulatie van diabetes type 2, evenals voor de andere glycemische indicatoren (nuchter glucose en insuline resistentie) tussen de interventie en de placebo groep na zes maanden. Een subgroep analyse van 19 patiënten met een vitamine D waarden < 30 nmol/l toonde een significante daling van het HbA1c.

De volgende twee hoofdstukken beschrijven de associatie tussen vitamine D en gezondheid gerelateerde kwaliteit van leven. Hoofdstuk 6 laat zien dat er geen verband wordt aangetoond tussen

gezondheid gerelateerde kwaliteit van leven en de hoogte van de vitamine D waarde aan het begin van het onderzoek. Na zes maanden suppletie (vitamine D of placebo) kon geen verschil worden aangetoond in de gezondheid gerelateerde kwaliteit van leven tussen beide groepen. Dit wordt in hoofdstuk 7 uiteengezet.
Hoofdstuk 8 toont aan dat er een significant verband bestaat tussen de hoogte van vitamine D en de hoeveelheid versuikerde eiwitten onder de huid, ook wel glycation endproducts genoemd. Deze versuikerde eiwitten worden gezien als nieuwe risico indicator in het ontstaan van hart- en vaatziekten op termijn. Eerder onderzoek toonde aan dat hoe hoger deze waarde is, hoe groter de kans wordt op hart- en vaatziekten in de toekomst.
Als laatste, in hoofdstuk 9 worden alle eerder gepubliceerde gerandomiseerde trials naar het effect van vitamine D op glycemische instelling van patiënten met diabetes type 2 samengevat in een systematische review en meta-analyse. Alle studies bij elkaar tonen geen verbetering van het HbA1c, nuchter glucose en/of insuline resistentie na interventie met vitamine D vergeleken met placebo. Een significant verschil werd wel aangetoond voor het nuchter glucose wanneer alleen de studies werden geanalyseerd die bij baseline patiënten met een HbA1c > 8% includeerden.

Interpretatie van de bevindingen en toekomstperspectieven

Dit proefschrift toont een verband aan tussen vitamine D status en insuline resistentie in meerdere onderzoekspopulaties (zwangerschapsdiabetes, PCOS en diabetes mellitus type 2). Het blijft echter onduidelijk of dit verband oorzakelijk van aard is. Onze trial leverde, in overeenkomst met eerder verricht trials, geen duidelijk bewijs voor een effect van vitamine D suppletie op de glycemische controle bij patiënten met type 2 diabetes. In een kleine subgroep van patiënten met een vitamine D waarde < 30nmol/l werd wel een verbetering gevonden van het HbA1c. In de nadien verrichte meta-analyse werd ook een verbetering van het nuchter glucose waargenomen bij studies met patiënten met een HbA1c > 8%.
Op dit moment is er nog onvoldoende bewijs voor het bepalen en toedienen van vitamine D bij patiënten met zwangerschapsdiabetes, PCOS of type 2 diabetes. Gezien de positieve bevindingen in de kleine subgroepen van diabetes patiënten met een vitamine D < 30 nmol/l of een HbA1c > 8% verdient het de aandacht om in deze groep verder onderzoek te verrichten naar het effect van vitamine D suppletie. Grote, goed opgezette klinische trials zijn hiervoor belangrijk waarbij patiënten met een slecht gereguleerde diabetes en een vitamine D tekort dienen te worden geincludeerd. Op dit moment zijn er een aantal internationale trials, waarvan de uitkomsten de komende jaren worden verwacht.

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ABOUT THE AUTHOR

Yvonne Henrica Maria Poel was born in Graft-De Rijp, The Netherlands on the 4th of May 1985. After finishing her secondary school in 2003, she studied medicine at the VU University in Amsterdam. In February 2010 she received her doctor's degree after the internships. After her graduation she worked as a resident at the department of Internal Medicine in the Medical Centre of Alkmaar in Alkmaar under supervision of dr. F. Stam. In 2011 Yvonne started her PhD project at the Medical Centre Alkmaar in collaboration with VU University Medical Centre under supervision of dr. S. Simsek and prof. dr. P. Lips. Since 2013 Yvonne combines her PhD work together with her clinical training in Internal Medicine. After the defence of her thesis she will start her fellowship Endocrinology at the Endocrinology department of VU University Medical Centre.

Yvonne Henrica Maria Poel werd op 4 mei 1985 geboren te Graft-De Rijp waar zij opgroeide samen met haar ouders en oudere broer Martin. De Rijp, met de oude dorpskern en mooie polder, is nu opnieuw de plaats waar zij samen met haar man Maurice Krul, met veel plezier woont.
Na het behalen van haar VWO diploma aan het DaVinci College te Purmerend in 2003, startte zij haar studie Geneeskunde aan de Vrije Universiteit te Amsterdam. In 2007 behaalde zij haar doctoraal examen en in februari 2010 behaalde zij cum laude haar arts examen na het doorlopen van de co-schappen. Hierna startte Yvonne als arts assistent niet in opleiding tot internist in het Medisch Centrum Alkmaar, te Alkmaar onder begeleiding van dr. F. Stam. Tijdens deze periode werd Yvonne enthousiast voor het verrichten van wetenschappelijk onderzoek. In 2011 leidde dit tot het begin van haar promotieonderzoek onder begeleiding van dr. S. Simsek en prof. dr. P. Lips. Gedurende twee jaar heeft Yvonne twee dagen per week onderzoek verricht i.c.m. drie dagen klinische werkzaamheden. Na deze twee jaar is zij aangenomen voor de opleiding tot internist aan de Vrije Universiteit met haar perifere deel in Alkmaar om daarnaast haar promotieonderzoek te kunnen continueren. Per 1 maart 2017 start Yvonne als internist in opleiding tot endocrinoloog aan de Vrije Universiteit, te Amsterdam. Hier kijkt zij erg naar uit.

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