Anna Vitezova

Vitamin D and Cardio metabolic Health in the Elderly
The Rotterdam Study

To my son Maximiliaan

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Vitamin D and Cardio-metabolic Health in the Elderly
The Rotterdam Study

Vitamine D en cardiometabole gezondheid in ouderen: De Rotterdam Studie

Thesis

to obtain the degree of Doctor from the
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Prof.dr. H.A.P. Pols

and in accordance with the decision of the Doctorate Board.
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by

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born in Novi Sad, Serbia

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Copromotors:	Dr. J.C. Kiefte- de Jong
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BMJ. 2014 Apr 1;348:g1903

Chapter 3
Anna Vitezova, M. Carola Zillikens, Thijs T. van Herpt, Eric J.G. Sijbrands, Albert Hofman, Andre G. Uitterlinden, Oscar H. Franco, Jessica C. Kiefte – de Jong. Vitamin D status and metabolic syndrome in the elderly: the Rotterdam Study.
Eur J Endocrinol. 2015 Mar;172(3):327-35

Anna Vitezova, Taulant Muka, M. Carola Zillikens, Trudy Voortman, Andre G. Uitterlinden, Albert Hofman, Fernando Rivadeneira, Jessica C. Kiefte- de Jong, Oscar H. Franco. Vitamin D and body composition in the elderly.
Clinical Nutrition (under review)

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Chapter 1
General Introduction

1.1 Vitamin D metabolism
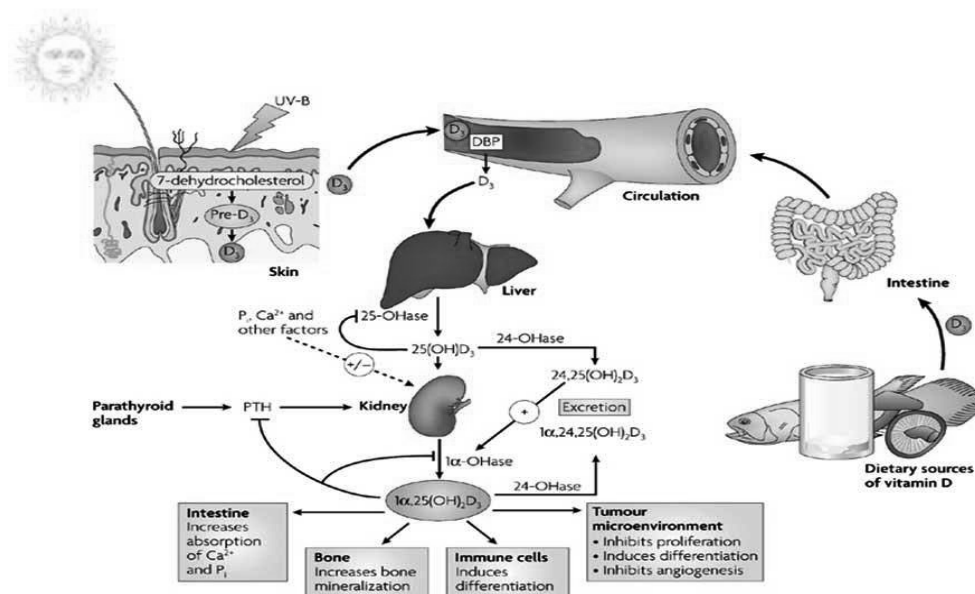
Figure 1.1.1. Vitamin D metabolism(1).

Vitamin D has been discovered at the beginning of the 20th century as a cure for rickets, a disease softening the bones in children (2, 3). Since then it has been established as an important factor in bone health, more specifically in bone metabolism and calcium homeostasis(3). Vitamin D is a lipid soluble vitamin which has a function of a hormone in the human body(4). There are two forms of vitamin D present in nature: vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol)(3). Vitamin D2 is found naturally in some mushrooms while D3 comes from animal foods as fish and eggs and can be produced in the skin under the effect of solar irradiation(3, 5, 6). Regardless of the form, both vitamin D2 and D3 when entered in the bloodstream bind to a protein called vitamin D binding protein (VBP) which is the main transporter of vitamin D in the circulation(3). Both of these forms of vitamin D are biologically inert and need to undergo chemical transformations in order to get activated. The first transformation is 25-hydroxylation which takes place in the liver. As a result 25-hydroxyvitamin D is produced. This is the main circulating form of vitamin D in the human body(7). It is used to assess the vitamin D status of the individual(7). Even though in recent years scientists acknowledge that it has certain functions in the human body, it is

still not considered as an active vitamin D metabolite. In order to get activated 25-hydroxyvitamin D needs to undergo further transformation in the kidney and as a product of this reaction 1,25-dihydroxyvitamin D is synthesized(3). This is the active form of vitamin D which has a function of a hormone in the human body. This active form binds to a member of the nuclear receptor family called vitamin D receptor (VDR) present in many different tissues and by this manner influences among other things calcium metabolism in the human body, such as calcium absorption in the intestines or calcium reabsorption in the kidneys (see Figure 1.1.1)(8). Finally, the excess active vitamin D can stimulate its own destruction by upregulating the expression of enzyme 25-hydroxyvitamin D-24-hydroxylase which metabolizes 25-hydroxyvitamin D but also the active form into inactive forms which are soluble in water(6, 9).

1.2 Vitamin D sources, recommendations and optimal status

Naturally, humans obtain their vitamin D from two sources: by consuming foods containing vitamin D and by endogenous production under the effect of solar irradiation in their skin(6). Some foods such as fatty fish and egg yolks naturally contain vitamin D while others like milk and margarine are being fortified with vitamin D in specific countries(4). However, the amount of vitamin D coming from diet only contributes 10 to 20 percent of the recommended daily allowance of vitamin D. The major source of vitamin D is cutaneous production under the effects of UVB irradiation(4). The change in lifestyle nowadays, preventing people from going outside in the sun and thus producing enough vitamin D, resulted in a high prevalence of vitamin D deficiency globally(4). Additionally to sun exposure, factors like skin pigmentation, use of sun screen, season, latitude, age, obesity, liver and kidney diseases, malabsorption, and use of different medications are determinants of vitamin D insufficiency(6). Currently, there is still a controversy in defining the optimal vitamin D status and subsequently in defining the recommendation for daily intake of vitamin D. The Institute of Medicine (IoM) report (10) defines levels of 20 ng/ml of 25-hydroxivitamin D as adequate while a working group of the Endocrine Society collected evidence why the IoM recommendations are flawed and why levels of 30ng/ml are to be considered as optimal for vitamin D status(4, 11). In line

with this controversy there are also different recommendations for daily vitamin D intake. While the IoM report recommends 600IU/day to be sufficient for optimal bone health, others suggest more is needed for the prevention of vitamin D deficiency and maintaining the optimal status(4, 11). According to these reports vitamin D intake as much as 1500IU/day to 2000IU/day is needed to treat vitamin D deficiency since supplementation with 100IU per day raises the levels of 25-hydroxivitamin D only with 0.7ng/ml(11).

1.3 Extra-skeletal effects of vitamin D

VDRs are present in almost all of the cells and tissues of the human body(6). Research into so called extra-skeletal effects of vitamin D demonstrated that 1,25-dihydroxyvitamin D has an effect on approximately 3,000 genes of the human genome e.g., genes regulating cellular proliferation, cellular differentiation and cellular apoptosis(6, 12, 13). With this in mind numerous studies have been performed examining the associations between vitamin D and diseases like diabetes mellitus, cardio-vascular disease, different cancers, autoimmune diseases, infections, depression and schizophrenia. However, most of the evidence on the extra-skeletal effects of vitamin D still comes from observational studies and also animal model studies, while high quality randomized controlled trials on vitamin D supplementation are still scarce.

With the extra-skeletal effects of vitamin D in mind we explored in more depth the effects of vitamin D on mortality. Since there have already been many studies published on this topic, including reports from supplementation trials, however yielding contradictory results, we reviewed the literature systematically and performed a meta-analysis of published results. For this purpose we used only data from observational cohort studies and intervention trials.

1.4 The Rotterdam Study

The Rotterdam Study (14, 15) is a prospective population-based cohort study designed to investigate the determinants of the incidence and progression of the disease in the elderly. It has been ongoing since 1990 in Rotterdam, the Netherlands. Briefly, in 1990 all inhabitants of Ommoord, district of Rotterdam, aged 55 years or older were invited to participate in the study.

From 11,850 eligible inhabitants 7,983 agreed to participate in the study. The study has been extended twice since; in 2000 when all inhabitants aged 55 years or older and also those who have migrated into the district were invited to participate; and in 2006 when all inhabitants aged 45 years and older and had not been examined earlier were invited. All participants were interviewed at home and were subsequently invited for extensive examinations to the research center. The participants were invited to the research center every three to four years for further examinations of the characteristics that change over time. These examinations focused also on collecting bodily fluids and imaging. The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus Medical Center and by the Ministry of Health, Welfare and Sport of the Netherlands, implementing the "Wet Bevolkingsonderzoek: ERGO" (Population Studies Act: Rotterdam Study). All participants provided written consent to participate in the study and to obtain information from their treating physicians (14, 15).

1.5 Objectives of the thesis

The broad objective of this thesis was to investigate the association of vitamin D serum levels with human cardio-metabolic health. Firstly, the objective was to study the effect of vitamin D status on mortality in relation to cause of death. For this purpose we performed a systematic review and meta-analysis. Secondly, the objective was to assess the association between vitamin D and metabolic health. Thirdly, the objective was to investigate the effect of vitamin D on cardio-vascular health. Additionally we aimed to explore if the effects of vitamin D might be mediated by serum magnesium status. Finally, we wanted to explore the directionality of the associations between vitamin D and serum lipids.

1.6 Thesis outline

Chapter 1 presents the introduction to the thesis focusing on vitamin D and its metabolism. The next chapter (chapter 2) includes the systematic review and meta-analysis of observational cohort studies and randomized intervention studies on vitamin D and risk of death. In chapter 3 the association between vitamin D and metabolic health is explored; chapter 3.1 describes the association between vitamin D and metabolic syndrome while chapter 3.2

focuses on the association between vitamin D and body composition. In chapter 4 the focus is on cardio-vascular health. More specifically, in this chapter the association between vitamin D and incidence of atrial fibrillation is presented. Further, chapter 5 explores two novel approaches in vitamin D research, namely potential effect modification by magnesium levels and path analyses. Chapter 5.1 describes possible effect modification of the effects of vitamin D by serum magnesium status. This chapter focusses on the question whether the association between vitamin D and the incidence of type 2 diabetes mellitus might be modified by serum magnesium levels. Chapter 5.2 presents a novel approach in analyzing vitamin D data by using path analysis. With this approach bidirectional associations between vitamin D and serum lipids were investigated. The results from chapters 3 to 5 were derived from the data from the Rotterdam Study. Finally, chapter 6 gives overview of all main findings and the main conclusions of this thesis.

Table 1.1. Overview of studies reported in this thesis

Chapter	Study design/study group	Research aim
2.1	Systematic review and meta-analysis	Association of vitamin D with the risk of mortality in observational cohort studies and in intervention trials
3.1	The Rotterdam Study (N=3240)	Association of vitamin D with prevalence of metabolic syndrome in the elderly
3.2	The Rotterdam Study (N=2158)	Association of vitamin D status with differential distribution of body fat in the elderly
4.1	The Rotterdam Study (N=3395)	Association of vitamin D status with incidence of atrial fibrillation in the elderly
5.1	The Rotterdam Study (N=8481)	Interaction between serum magnesium levels and 25-hydroxyvitamin D in incidence of type 2 diabetes mellitus
5.2	The Rotterdam Study (N=1165)	Bidirectional associations between circulating 25-hydroxyvitamin D and serum cholesterol

Chapter 2
Vitamin D and Mortality

2.1. Vitamin D and risk of cause-specific death: a systematic review and meta-analysis of prospective observational and randomised intervention studies

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* Denotes equal contribution.

Abstract

Objective To evaluate the extent to which circulating biomarker and supplements of vitamin D is associated with mortality from vascular, cancer or other conditions, under various circumstances.

Design Systematic review and meta-analysis of observational studies and randomised controlled trials (RCTs).

Data sources Medline, Embase, Cochrane Library, reference lists of relevant studies to August 2013, and email contact with investigators.

Study selection Prospective cohort studies and RCTs in human adults, that had reported associations between vitamin D (measured as circulating 25-hydroxyvitamin D (25(OH)D) concentration or vitamin D supplement given singly) and cause-specific mortality outcomes.

Data extraction Data were extracted by two independent investigators and a consensus was reached with involvement of a third. Study-specific relative risks (RRs) from 73 cohort studies (848,870 participants) and 22 RCTs (vitamin D given alone vs. placebo or no treatment; 34,023 participants) were meta-analysed using random-effect models, and were grouped by study and population characteristics.

Results In the primary prevention observational studies, comparing bottom versus top thirds of baseline circulating 25(OH)D distribution, pooled RRs (95% confidence intervals) were 1.33 (1.11 to 1.59) for vascular death, 1.14 (1.01 to 1.29) for cancer death, 1.30 (1.07 to 1.59) for nonvascular-noncancer death and 1.39 (1.25 to 1.54) for all-cause mortality. Subgroup analyses in the observational studies indicated that mortality risk was significantly higher in studies with lower baseline use of vitamin D supplements. In RCTs, RRs for all-cause mortality were 0.89 (0.80 to 0.99) and 1.04 (0.97 to 1.11), for vitamin D_3 and vitamin D_2 supplementations, respectively. The effects observed for vitamin D_3 supplementation remained unchanged when grouped by various characteristics; however, differed for vitamin D_2 supplementation, for which increased risks of mortality were observed in studies with lower intervention dose and shorter average intervention period.

Limitations *Potential biases owing to preferential publication, unmeasured confounding or lack of serial measurements.*

Conclusion *Evidence from observational studies show inverse associations of circulating 25(OH)D with risks of vascular, cancer and nonvascular- noncancer deaths. Supplementation with vitamin D_3 reduces overall mortality among older adults significantly; however, further investigations are required to establish the optimal dose and duration, and whether vitamin D_3 affects mortality risk differently than vitamin D_2.*

2.1.1. Introduction

Vitamin D is a group of fat-soluble vitamins responsible for intestinal absorption of calcium and phosphate.(16) There are two major forms of vitamin D. Vitamin D_2 (ergocalciferol), found in plants, is produced by ultraviolet B (UVB) irradiation of ergosterol, and can be consumed as a supplement or in fortified foods.(17) Vitamin D_3 (cholecalciferol), on the other hand, a product of UVB irradiation of 7-dehydrocholesterol, is synthesized in the human epidermis or consumed in the form of natural (e.g. fish) or fortified food sources, or as a supplement.(17) Supplementing with vitamin D has shown to benefit skeletal conditions such as rickets, fractures, and falls (18-20), although a similar effect on bone mineral density was not evident in a recent review of trials. A growing body of evidence indicate that vitamin D may reduce risks of a wide range of diseases including multiple sclerosis,(21) autoimmune disorders,(22) infections,(23) cardiometabolic(24, 25) and cancer outcomes(26) – indicating a possible pleiotropic effect across extraskeletal systems. Nonetheless, the evidence for vitamin D reducing risk of nonskeletal diseases is still being debated.(27)

Suboptimal levels of vitamin D have also been implicated as a potential determinant of mortality because of its wide-ranging anti-inflammatory and immune-modulating effects.(17, 28, 29) However, available observational studies examining this intriguing link are yet to be rigorously reviewed and the extent to which vitamin D deficiency confers risk of death from vascular, cancer or other conditions remains uncertain. While several individual reports(30, 31) and reviews(32-35) have been published on the topic, they vary greatly and lack sufficient detail (eg, associations for diverse causes of death; primary versus secondary prevention settings). Additionally, interpretation of the earlier quantitative reviews(32, 35) of randomised trials is challenging as they typically include (1) studies with mixed interventions (e.g. combined with calcium intake, which has been associated with vascular risk(36) and (2) lack detailed assessments to distinguish the effects across important characteristics (e.g. geographical location, intervention dosage, duration and follow-up time). There is a need, therefore, for adequately powered, comprehensive assessment of associations of vitamin D levels with the risk of mortality across primary versus secondary prevention settings and from a broad range of causes. This is of particular significance as mortality

risk estimates remains a cornerstone in formulating health policies to prevent or reduce premature deaths and improve quality of life, and in this sense Vitamin D might play a key role.

In the present study, we have attempted a large-scale synthesis of the available observational and intervention evidence under one updated systematic review and meta-analysis to: (1) determine the associations of 25-hydroxyvitamin levels with the risk of cause-specific mortality outcomes in observational cohort studies, (2) quantify effects of vitamin D supplementation (overall and by subtypes), when given alone compared to placebo or no treatment, on mortality outcomes in the randomised controlled trials (RCTs), and (3) examine all associations under a wide range of study-level circumstances.

2.1.2. Methods
2.1.2.1. Data sources, search strategy and eligibility criteria

This review was conducted using a predefined protocol and in accordance with the PRISMA and MOOSE guidelines (37, 38) (**eAppendix 1** and **2**). Two independent authors, in duplication, sought studies published before August 1, 2013 (date last searched) using Medline, Embase and Cochrane databases. The computer-based searches combined terms related to the exposure (eg, *vitamin D, 25-hydroxyvitamin D*) and outcomes (eg, *mortality, all-cause mortality, death*), without any language restriction. Details on the search strategy are provided in **eAppendix 3**. Studies were sought that had reported on associations of circulating vitamin D [measured as 25-hydroxyvitamin D or 25(OH)D] or vitamin D supplements with all-cause mortality (defined as deaths from any causes) and/or cause-specific mortality (defined as deaths due to cardiovascular, cancer, and other nonvascular-noncancer causes), where fatal outcomes were registered according to the primary cause (or, in its absence, the underlying cause), on the basis of coding from the *International Classification of Diseases (ICD)*, or according to study-defined classifications; ascertainment was based on death certificates.

2.1.2.2. Study selection

Observational cohort studies were eligible for inclusion if they followed participants prospectively, assessed association of circulating 25(OH)D with

cause-specific and/or all cause deaths in adults, and recruited participants from any of the following categories: (1) free-living, healthy participants with no previous chronic disease at entry including vascular, metabolic, malignant or renal disorders (i.e., primary prevention cohorts); or (2) people with pre-existing baseline conditions mentioned above (i.e., secondary prevention cohorts). Intervention studies were eligible if they: (1) were randomised; (2) assessed effects of vitamin D supplements singly (i.e., RCTs with a "vitamin D alone" intervention group) in adults compared to a placebo or no treatment; and (3) collected cause-specific or all-cause mortality endpoints (as defined before). Two independent reviewers working in pairs screened the titles and abstracts of all initially identified studies according to the selection criteria. Full texts were retrieved from studies that satisfied all selection criteria. Reference lists of selected studies and relevant reviews identified on the topic were searched for additional publications.

2.1.2.3. Data extraction

Data were extracted by two independent authors and a consensus was reached with involvement of a third. A predesigned data abstraction form was used to extract relevant information. This included questions on study size; study design; baseline population; location; age at baseline; duration of follow-up; reported degree of adjustment [defined as '+' when RRs were adjusted for established vascular risk factors (eg, age, sex, smoking status, lipids, hypertension, history of cardiometabolic disease); '++' adjustment for other potential risk factors (eg, physical activity, body mass index, social status) and '+++' when adjusted for other additional variables (eg, bone minerals)]; type and numbers of mortality outcomes and reported relative risks (RRs). Where appropriate, information of subtypes of vitamin D supplement, baseline level in nanogram per millilitre (ng/mL), assay method, blinding status, composition of supplement or placebo were extracted. If risk estimates were unavailable from a published report, we collected relevant data by corresponding with the authors,(39-41) abstracting from other published reviews,(33, 35, 42) or hand-calculating based on the available information from the paper,(43-45) where appropriate. Additionally, in the case of multiple publications, the most up-to-date or comprehensive information was included.(46, 47)

2.1.2.4. Assessing the risk of bias

For prospective observational studies, the Newcastle-Ottawa Scale[48] was used to assess the risk of bias. This scale uses a star system (with maximum of nine stars) to evaluate a study in three domains: selection of participants; comparability of study groups; and the ascertainment of outcomes of interest. Studies that received a score of nine stars were judged to be of at low risk of bias; studies that scored seven or eight stars were considered at medium risk; those that scored six or less were considered at high risk of bias. Similarly, for the randomised trials, the Cochrane Collaboration's tool [49] was used for assessing the risk of bias. This tool evaluates seven possible sources of bias: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting and other bias. For each individual domain, studies were classified into low, unclear and high risk of bias.

2.1.2.5. Data synthesis and analysis

To enable a consistent approach to meta-analysis and interpretation of findings in this review, relative risk estimates for association of circulating 25(OH)D and mortality outcomes that were often differently reported by each study (e.g. per unit change, or per 1-SD change, or comparing quintiles, quartiles, thirds, and other groupings) were transformed to consistently correspond to comparison of the top vs. bottom third of baseline 25(OH)D distribution in each study, using methods previously described.Briefly, log risk ratios were transformed assuming a normal distribution, with the comparison between top and bottom thirds being equivalent to 2.18 times the log risk ratio for 1 standard deviation increase (or equivalently as 2.18/2.54 times the log risk ratio for a comparison of extreme quarters). Standard errors of the log risk ratios were calculated using published confidence limits and were standardised in the same way. Hazard and odds ratios were assumed to approximate the same measure of RRs. Study-specific RRs were combined using a random effects model that included between-study heterogeneity (and additionally using fixed-effect models). Where studies reported RRs with varying degrees of adjustments, the maximum adjusted estimate was used. Subsidiary assessments involving circulating 25(OH)D cut-offs (defined as 21-29, 10-20 and <10 ng/mL)[17, 50, 51] compared to the reference category

($\geq$30 ng/mL) were based on combining comparable RR estimates across studies using random effects meta-analyses (and additionally using fixed-effect models).(52, 53)For randomised intervention trials, we used reported RR or calculated study-specific unadjusted RR for overall vitamin D supplementation (and individually by supplements of vitamins D_3 and D_2 subtypes). Hazard ratios and odds ratios were assumed to approximate the same measure of relative risk. Summary RRs were calculated by pooling the study-specific estimates using a random-effects model that included between-study heterogeneity (parallel analyses used fixed-effect models). Consistency of findings across studies was assessed by standard χ^2 tests and the I^2 statistic.(54) Heterogeneity was quantified by comparing results from studies grouped according to study-level characteristics using random-effects meta-regression. Additionally, univariate meta-regression analyses were conducted to investigate the impact of study level characteristics such as daily intervention dose of supplement and duration of intervention or follow-up on the size of the effect estimates for both supplementation trials and observational cohort studies. The natural logarithm of the RR was used as the dependent variable and the study level characteristic was used as the explanatory factor. Evidence of publication bias was assessed using funnel plots and Egger test.(55) Population attributable risk (PAR) was calculated by the following equation: *PAR%=100xPe(RR−1)/(Pe[RR−1]+1)*(56) **[eAppendix 5]**. All statistical tests were two-sided and used a significance level of p<0.05. Analyses were performed using Stata release-12 (StataCorp, College Station, Texas).

2.1.3. Results

The search strategy identified 2704 unique citations. Following initial screening based on titles and abstracts 320 articles remained for further evaluation. Of these articles, 225 were excluded in the subsequent detailed assessments for reasons shown in eFigure 1. The remaining 95 unique study reports met our inclusion criteria and were included in the meta-analysis (eAppendix 6). In aggregate, these included studies comprised of 882,893 unique individuals and 71,332 mortality outcomes (including 10,544 deaths from cardiovascular disease (CVD) and 6,911 deaths from cancer) (Table 2.1.1.; eTables 1-3).

2.1.3.1. Association of circulating 25(OH)D levels with cause-specific mortality

Circulating 25(OH)D in relation to subsequent risk of death was reported in 73 observational cohort studies, involving 848,870 participants and 66,218 mortality events recorded during an average follow-up ranging from 0.3 to 29 years (Table 2.1.1; eTable 1). Out of these observational cohort studies, 38 studies involved participants from Europe, 26 from North America, 8 from the Asia-Pacific region, and 1 from South America. Average age of all included participants ranged from 29 to 77 years. Eight studies were judged to be at low risk of bias, 41 at medium risk, while 24 studies were at high risk of bias (eTable 1). Of the medium quality studies, all showed a potential bias in the participant selection. Median baseline level of 25(OH)D in these studies was 20.7 (IQR: 17.5 to 24.3) ng/mL. For the primary prevention cohorts, pooled RRs (95% CIs) in comparisons of people in the bottom versus top thirds of the population distribution of baseline circulating 25(OH)D, adjusted for several potential risk factors, were 1.33 (1.11 to 1.59) for CVD death (6,143 events), 1.14 (1.01 to 1.29) for cancer death (5,003 events), 1.30 (1.07 to 1.59) for other nonvascular-noncancer death (1,444 events) and 1.39 (1.25 to 1.54) for all-cause mortality (48,488 events) (Figure 2.1.1 and eFigure 2).

The corresponding pooled RRs were broadly similar in the secondary prevention cohorts. Additional analyses by various circulating 25(OH)D cut-offs, showed a significantly inverse association with all-cause mortality (P<0.05, Figure 2.1.2). Assuming linearity, each 10 ng/mL decline of 25(OH)D was associated with a 16% (95% CI: 8-23%) increased risk of all cause mortality (Figure 2.1.2).

In subsidiary analyses, there were significant inverse associations for various cause-specific mortality outcomes, including deaths due to coronary disease, lymphoma, upper digestive tract cancer, and respiratory diseases (eFigure 3). There was moderate level of heterogeneity observed in observational studies, which was partly explained by between-study differences in the baseline usage of vitamin D supplements, average duration of longitudinal follow up, level of multivariate adjustments and study quality (Pmetaregression <0.05 for all; Figure 2.1.3).

Table 2.1.1. Summary characteristics of the included studies.

Eligible studies	Observational cohort studies	Intervention studies
No. of unique studies	73	22
Average follow-up (years), median (IQR)	6 (3-9.5)	1.39 (0.5-3.0)
Participants		
Total no. of participants	848 870	34 023
No. of participants, median (IQR)	1 073 (510 -2 312)	343 (124-2578)
Total no. of deaths	66 218	5 114
No. of deaths, median (IQR)	224 (106-633)	22.5 (7-471)
Male (%), median (IQR)	51 (35-62)	-
Age (years), median (range)	63 (59-71)	77 (56-85)
Baseline population, No. of studies (No. of participants)		
Not selected based on prior chronic disease	29 (787 682)	9 (24 828)
With pre-existing chronic disease	44 (61 188)	13 (9 195)
Location, No. of studies (No. of participants)		
Europe	38 (330 631)	13 (28 464)
North America	26 (89 742)	5 (2 182)
Asia-Pacific	8 (427 515)	4 (3 377)
South America	1 (982)	-
Sample, No. of studies (No. of participants)		
Serum	57 (821 798)	-
Plasma	16 (27 072)	-
Assay method, No. of studies (No. of participants)		
Radioimmunoassay	34 60 471	-
Automated immunoassays	19 753 285	-
Chromatographic methods	20 35 114	-
25-hydroxy vitamin D Level		
Pooled average level at baseline (ng/ml), median (IQR)	20.7 (17.5-24.3)	15.2 (10.4-21.3)
Outcome, No. of studies (No. of events)†		
All cause mortality	68 (64 636)	22 (5 114)
Cardiovascular mortality	29 (9 970)	3 (574)
Cancer mortality	17 (6 620)	2 (291)
Other nonvascular-noncancer mortality	10 (2 565)	-

†, are not unique studies; CPBA, competitive-binding protein assay

Figure 2.1.1. Association of circulating 25- hydroxyvitamin D with cause-specific mortality in the observational cohort studies.

	No. of Studies	No. of Participants	No. of deaths		Relative Risk (95% CI)* for cause-specific mortality
Cardiovascular death					
	19	80,062	6,143		1.33 (1.11, 1.59)
	10	21,045	3,827		1.60 (1.32, 1.94)
	29	101,107	9,970		1.42 (1.24, 1.62)
Cancer death					
	12	103,753	5,003		1.14 (1.01, 1.29)
	5	16,382	1,617		1.57 (1.15, 2.14)
	17	120,135	6,620		1.25 (1.10, 1.43)
Nonvascular, noncancer death					
	7	38,526	1,444		1.30 (1.07, 1.59)
	3	13,035	1,121		1.49 (0.94, 2.35)
	10	51,561	2,565		1.34 (1.13, 1.60)
All cause mortality					
	27	780,390	48,488		1.39 (1.25, 1.54)
	41	59,976	16,148		1.50 (1.36, 1.65)
	68	840,366	64,636		1.44 (1.34, 1.55)

Primary prevention cohorts □
Secondary prevention cohorts △
All cohorts ○

0.50   1.00   2.50   5.00
RR (95% CI) for bottom versus top thirds of baseline 25(OH)D levels

*Pooled estimates are based on random effects meta-analysis. Using fixed effects models, the estimates were [1.39 (1.32, 1.47), 1.50 (1.35, 1.66), 1.41 (1.35, 1.48)], [1.10 (1.02, 1.17), 1.44 (1.27, 1.64), 1.16 (1.10, 1.24)], [1.28 (1.12, 1.47), 1.38 (1.09, 1.75), 1.30 (1.16, 1.47)], and [1.45 (1.41, 1.49), 1.49 (1.42, 1.56), 1.46 (1.42, 1.49)] for cardiovascular, cancer, nonvascular noncancer, and all cause deaths respectively in the order: primary prevention cohorts to all cohorts; Size of the data marker is proportional to the inverse of the variance of relative risk (RR) and horizontal line represents 95% confidence interval (CI). Corresponding forest plots and I² (95% CI) estimates are provided in Supplementary Material

Specifically, all-cause and cancer mortality risk for low baseline circulating 25(OH)D was significantly higher in studies where less than 10% of the population used vitamin D supplements. Additionally, all-cause mortality risk in subjects with low 25(OH)D levels was significantly higher in studies with <5 years of average follow-up (Figure 2.1.3).The overall associations observed, however, were similar across other subgroups such as latitude of study location, gender, assay methods, adjustments for seasonality or socioeconomic status, and geographical location (Figure 2.1.3 and eFigure 4). Results from univariate meta-regression analyses showed no evidence of associations of the duration of follow-up with risk of CVD death, cancer death, other nonvascular-noncancer death, and all-cause mortality (*P*-values > 0.05 for all) (eFigure 6).
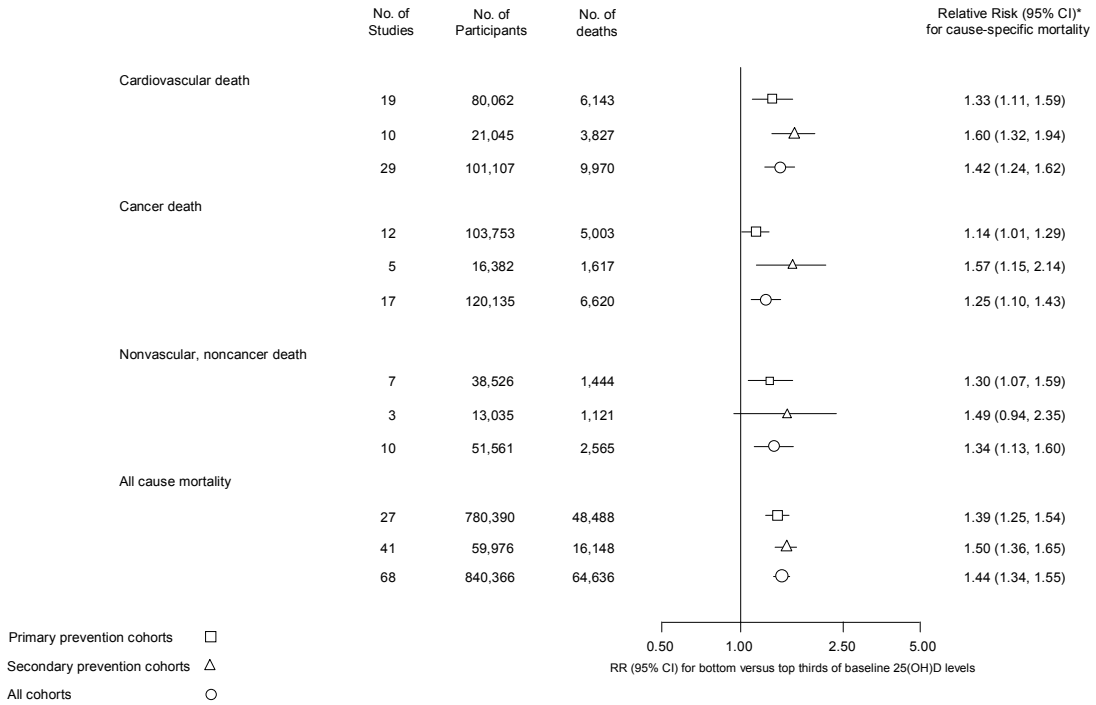
In subsidiary analyses, there were significant inverse associations for various cause-specific mortality outcomes, including deaths due to coronary disease, lymphoma, upper digestive tract cancer, and respiratory diseases (eFigure 3). There was moderate level of heterogeneity observed in observational studies, which was partly explained by between-study differences in the baseline usage of vitamin D supplements, average duration of longitudinal follow up, level of multivariate adjustments and study quality (Pmetaregression <0.05 for all; Figure 2.1.3).

Figure 2.1.2. Association of circulating 25-hydroxyvitamin D levels with all cause mortality, based on primary prevention cohorts.
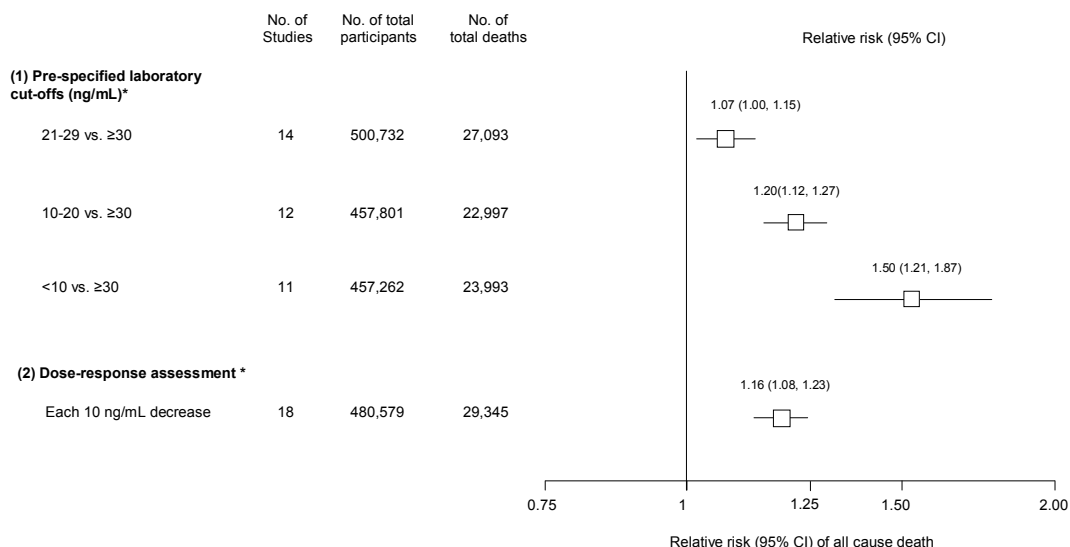
*Indirect comparisons based on available studies with relevant information in each category; the summary estimates presented were calculated using random effects models; Using fixed effects models, the estimates were 1.09 (1.06, 1.11), 1.20 (1.15, 1.26), 1.23 (1.20, 1.26), and 1.19 (1.18, 1.21) for clinical cut-offs 21-29 vs. ≥ 30, 10-20 vs. ≥ 30, < 10 vs. ≥ 30, and per 10 ng/Ml decrease respectively.

Figure 2.1.3. Association of circulating 25-hydroxyvitamin D and risk of cause-specific mortality in the primary prevention cohorts, according to various characteristics.

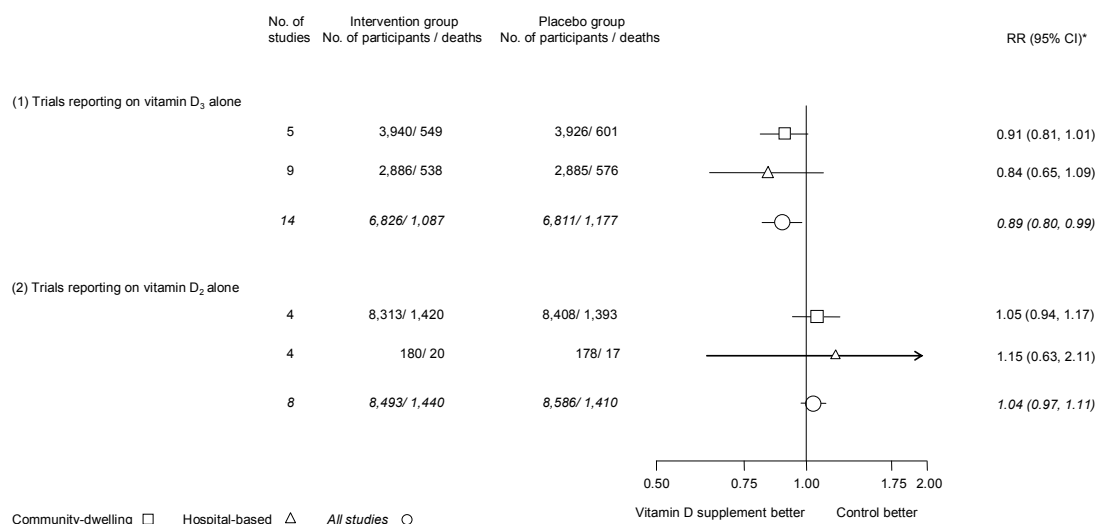| Subgroups	Cardiovascular death				Cancer death				All cause mortality			
	Studies	Participants	Deaths	RR (95% CI)	Studies	Participants	Deaths	RR (95% CI)	Studies	Participants	Deaths	RR (95% CI)
Latitude of study location												
Above 40 N and below 40 S	10	47,695	3,644	1.37 (1.04, 1.81)	4	27,079	1,421	1.16 (0.91, 1.46)	16	295,232	27,646	1.35 (1.21, 1.51)
Between 40 N and 40 S	9	32,367	2,499	1.29 (1.09, 1.53)	8	76,674	3,582	1.14 (0.97, 1.35)	11	485,158	20,842	1.37 (1.15, 1.62)
Average age of participants, yrs												
<70	12	68,878	4,944	1.24 (1.06, 1.45)	9	98,431	4,499	1.07 (1.00, 1.15)	17	766,853	44,040	1.39 (1.21, 1.59)
≥70	7	11,184	1,199	1.41 (1.01, 1.96)	3	5,322	504	1.32 (0.63, 2.73)	10	13,537	4,448	1.36 (1.20, 1.54)
Sex												
Male	2	2,684	306	1.55 (1.05, 2.27)	3	50,484	2,311	1.16 (0.64, 2.11)	3	3,466	1,096	1.36 (0.91, 2.05)
Female	2	3,300	142	1.15 (0.81, 1.63)	1	1,829	62	1.33 (0.90, 1.96)	4	6,027	522	1.32 (1.02, 1.69)
Both	15	74,078	5,695	1.34 (1.09, 1.64)	8	51,440	2,630	1.10 (0.97, 1.25)	20	770,897	46,870	1.40 (1.24, 1.58)
Baseline vitamin D supplement use ‡												
≥10%	4	18,187	1,340	1.12 (0.94, 1.32)	1	13,331	424	0.92 (0.67, 1.26)	8	23,169	4,299	1.26 (1.16, 1.37)
None or <10%	1	1,194	196	1.70 (0.96, 3.00) *	1	1,194	189	2.35 (1.37, 4.04) *	3	424,414	12,993	1.80 (1.72, 1.88) *
Sample type												
Plasma	2	11,364	1,718	1.41 (1.24, 1.61)	2	48,994	2,214	1.57 (0.79, 3.11)	4	13,910	8,118	1.38 (1.26, 1.52)
Serum	17	68,698	4,425	1.31 (1.07, 1.62)	10	54,759	2,789	1.09 (0.96, 1.25)	23	766,480	40,370	1.38 (1.21, 1.57)
Assay method												
Radioimmunoassay	9	35,740	2,208	1.43 (1.08, 1.90)	6	74,230	3,008	1.19 (1.03, 1.38)	11	32,714	4,649	1.32 (1.14, 1.53)
Automated assays	5	29,044	3,027	1.21 (0.93, 1.58)	3	17,693	1,408	1.01 (0.91, 1.11)	7	728,695	39,712	1.44 (1.17, 1.76)
Chromatographic plus CBPA	5	15,278	908	1.22 (0.99, 1.51)	3	11,830	587	1.18 (0.60, 2.32)	9	18,981	4,127	1.33 (1.17, 1.51)
Average follow up, yrs												
≥5	17	71,469	5,533	1.29 (1.07, 1.56)	10	95,268	4,464	1.12 (0.98, 1.29)	22	71,073	17,618	1.29 (1.18, 1.40)
<5	2	8,593	610	1.70 (0.90, 3.22) *	2	8,485	539	1.29 (0.97, 1.72)	5	709,317	30,870	1.78 (1.71, 1.86) *
Events ascertained, n												
≥500	5	37,943	3,983	1.31 (1.09, 1.58)	1	47,800	2,025	1.16 (1.02, 1.33)	14	758,654	46,162	1.36 (1.18, 1.55)
<500	14	42,119	2,160	1.33 (1.02, 1.74)	11	55,953	2,978	1.14 (0.98, 1.33)	13	21,736	2,326	1.40 (1.22, 1.61)
Level of adjustment												
+++	9	46,923	4,008	1.21 (1.01, 1.44)	5	32,754	1,641	1.02 (0.80, 1.29)	10	47,824	13,666	1.24 (1.11, 1.39)
++/+	10	33,139	2,135	1.40 (1.07, 1.83)	7	70,999	3,362	1.21 (1.10, 1.34) *	17	732,566	34,822	1.46 (1.28, 1.67)
Controlled for seasonality												
Yes	15	68,987	5,745	1.31 (1.08, 1.61)	8	45,492	2,637	1.13 (0.93, 1.37)	20	738,356	45,365	1.41 (1.24, 1.59)
No	4	11,075	398	1.39 (0.92, 2.10)	4	58,261	2,366	1.17 (1.05, 1.31)	7	42,034	3,123	1.33 (1.05, 1.68)
Adjusted for socioeconomic status												
Yes	12	56,643	3,615	1.40 (1.09, 1.80)	8	45,862	2,201	1.18 (0.95, 1.46)	17	481,332	22,014	1.43 (1.23, 1.67)
No	7	23,419	2,528	1.18 (0.94, 1.49)	4	57,891	2,802	1.12 (1.01, 1.24)	10	299,058	26,474	1.30 (1.13, 1.50)
Risk of bias score §												
9	6	34,661	3,352	1.22 (0.98, 1.50)	2	20,492	922	0.96 (0.80, 1.15)	7	282,422	26,767	1.28 (1.18, 1.38)
<9	13	45,401	2,791	1.37 (1.06, 1.76)	10	83,261	4,081	1.19 (1.03, 1.38)	20	497,968	21,721	1.43 (1.23, 1.66)

RR (95% CI) for bottom vs. top thirds of baseline 25(OH)D concentrations

Based on available studies with relevant subgroup information; *P <0.05 from meta-regression analyses; § Based on Newcastle-Ottawa scale

Figure 2.1.4. Effects of vitamin D supplementation on all cause mortality when given alone, derived from the available randomized control trials.

	No. of studies	Intervention group No. of participants / deaths	Placebo group No. of participants / deaths		RR (95% CI)*
(1) Trials reporting on vitamin D$_3$ alone					
	5	3,940/ 549	3,926/ 601		0.91 (0.81, 1.01)
	9	2,886/ 538	2,885/ 576		0.84 (0.65, 1.09)
	14	6,826/ 1,087	6,811/ 1,177		0.89 (0.80, 0.99)
(2) Trials reporting on vitamin D$_2$ alone					
	4	8,313/ 1,420	8,408/ 1,393		1.05 (0.94, 1.17)
	4	180/ 20	178/ 17		1.15 (0.63, 2.11)
	8	8,493/ 1,440	8,586/ 1,410		1.04 (0.97, 1.11)

Community-dwelling □   Hospital-based △   *All studies* ○

*Pooled estimates are based on random effects meta-analysis. Using fixed effects models, the estimates were [0.91 (0.81, 1.01). 0.88 (0.77, 1.01), 0.90 (0.82, 0.98)] and [1.05 (0.94, 1.17), 1.15 (0.63, 2.11), 1.03 (0.97, 1.09)] for vitamins D3 and vitamin D2 trials respectively in the order: community-dwelling to overall; the overall fixed effect estimate for all trials was 0.98 (0.94, 1.03); Size of the data marker is proportional to the inverse of the variance of relative risk (RR) and horizontal line represents 95% confidence interval (CI). Corresponding forest plots and I^2 (95% CI) estimates are provided in Supplementary Material

Specifically, all-cause and cancer mortality risk for low baseline circulating 25(OH)D was significantly higher in studies where less than 10% of the population used vitamin D supplements. Additionally, all-cause mortality risk in subjects with low 25(OH)D levels was significantly higher in studies with <5 years of average follow-up (Figure 2.1.3).The overall associations observed, however, were similar across other subgroups such as latitude of study location, gender, assay methods, adjustments for seasonality or socioeconomic status, and geographical location (Figure 2.103 and eFigure 4). Results from univariate meta-regression analyses showed no evidence of associations of the duration of follow-up with risk of CVD death, cancer death, other nonvascular-noncancer death, and all-cause mortality (*P*-values > 0.05 for all) (eFigure 6).

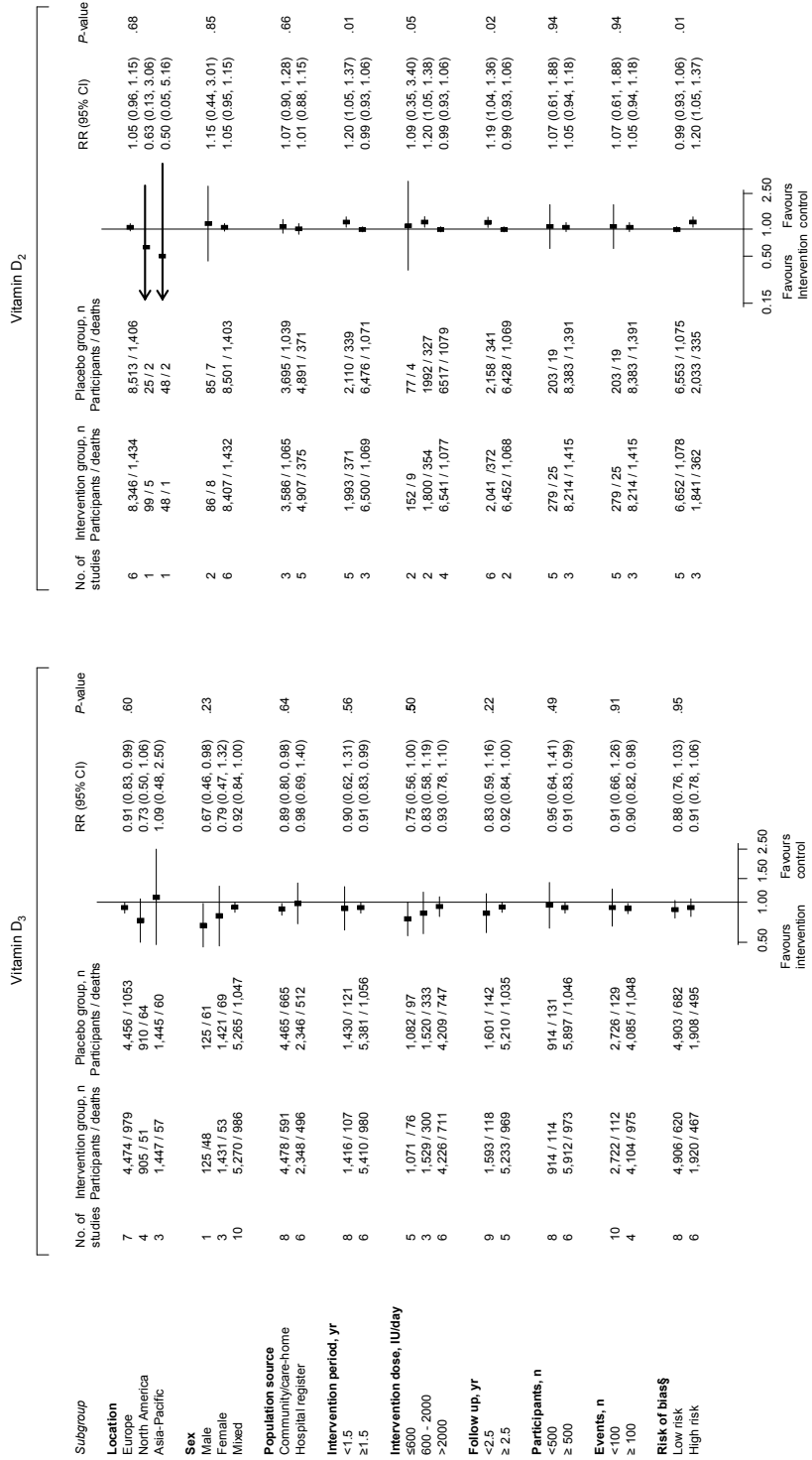## 2.1.3.2. Effects of vitamin D supplementation on all-cause mortality

Twenty-two RCTs reported effects of vitamin D supplementation in isolation on mortality outcomes, including a total of 34,023 participants (Table 2.1.1; eTable 3). Fourteen of these RCTs assessed effect of vitamin D_3 whereas 8 studies reported effects of vitamin D_2. Thirteen trials involved participants from Europe, five from North America, and four from the Asia-Pacific region. Average age of included participants in these trials ranged from 56 to 85 years. Eleven trials included individuals from community-based registers (6 from general population and 5 from care or residential homes), whereas the rest recruited participants from clinical registers. Risk of bias assessment in each trial is reported in eAppendix 4. The majority of the trials had low risk of bias for the random sequence generation, allocation concealment, participants' blinding and selective reporting. Seven trials had a high risk of bias for blinding of outcome assessment and eight had high bias in outcome data completion. Among the vitamin D_3 studies, participants in the intervention arm received vitamin D_3 supplementation ranging from 10 to 6000 international units (IUs) per day, where oral tablet

was the principle form of supplementation. The corresponding range was 208 to 4500 IU for vitamin D_2. After an average follow-up ranging from 0.38 to 6.8 years, a total of 2,527 all-cause mortality events occurred among participants in the intervention group compared to 2,587 events in the control group, with a combined RR (95% CI) of 0.98 (0.94 to 1.02) in all studies. The corresponding RRs according to type of vitamin D supplementation was 0.89 (0.80 to 0.99) for with vitamin D_3 and 1.04 (0.97 to 1.11) for vitamin D_2 (Figure 2.1.4).

There was no evidence of heterogeneity across vitamin D_3 (eFigure 5a: $P_{heterogeneity}$ = 0.34) or vitamin D_2 trials (eFigure 5b: $P_{heterogeneity}$ = 0.38). For vitamin D_3, the overall effect did not vary significantly across location, gender, population source, daily dose, and intervention or follow up duration ($P_{metaregression}$>0.05; Figure 2.1.5). The effect, however, differed importantly for vitamin D_2 supplementation, for which increased risk of mortality were observed in the RCTs that used lower intervention dose (600-200 IU per day), had shorter average intervention period (<1.5 years vs. ≥1.5 years) and had high risk of bias ($P_{metaregression}$ <0.05 for all; Figure 2.1.5). Similarly, results from univariate meta-regression analysis showed no evidence of an

association of daily intervention dose or average intervention period with treatment effect for vitamin D_3 supplementation (*P*-values of 0.47 and 0.50 respectively). The evidence was however suggestive of associations of daily intervention dose and average intervention period with the treatment effect for vitamin D_2 supplementation, though these were nominally significant (*P*-values of 0.06 and 0.07 respectively) (eFigure 7). There was insufficient data to meaningfully combine the effects of vitamin D supplementation alone on cause-specific mortality outcomes. There was no evidence of publication bias across all included studies in this review ($P_{Egger's\ asymmetry}$ >0.05 for all) (eFigure 8).

Figure 2.1.5. Effects of vitamin D supplementation on all cause mortality, derived from the available randomized control trials and according to various characteristics.

Subgroup	Vitamin D₃ No. of studies	Intervention group, n Participants / deaths	Placebo group, n Participants / deaths	RR (95% CI)	P-value	Vitamin D₂ No. of studies	Intervention group, n Participants / deaths	Placebo group, n Participants / deaths	RR (95% CI)	P-value
Location										
Europe	7	4,474 / 979	4,456 / 1053	0.91 (0.83, 0.99)	.60	6	8,346 / 1,434	8,513 / 1,406	1.05 (0.96, 1.15)	.68
North America	4	905 / 51	910 / 64	0.73 (0.50, 1.06)		1	99 / 5	25 / 2	0.63 (0.13, 3.06)	
Asia-Pacific	3	1,447 / 57	1,445 / 60	1.09 (0.48, 2.50)		1	48 / 1	48 / 2	0.50 (0.05, 5.16)	
Sex										
Male	1	125 / 48	125 / 61	0.67 (0.46, 0.98)	.23	2	86 / 8	85 / 7	1.15 (0.44, 3.01)	.85
Female	3	1,431 / 53	1,421 / 69	0.79 (0.47, 1.32)		6	8,407 / 1,432	8,501 / 1,403	1.05 (0.95, 1.15)	
Mixed	10	5,270 / 986	5,265 / 1,047	0.92 (0.84, 1.00)						
Population source										
Community/care-home	8	4,478 / 591	4,465 / 665	0.89 (0.80, 0.98)	.64	3	3,586 / 1,065	3,695 / 1,039	1.07 (0.90, 1.28)	.66
Hospital register	6	2,348 / 496	2,346 / 512	0.98 (0.69, 1.40)		5	4,907 / 375	4,891 / 371	1.01 (0.88, 1.15)	
Intervention period, yr										
<1.5	8	1,416 / 107	1,430 / 121	0.90 (0.62, 1.31)	.56	5	1,993 / 371	2,110 / 339	1.20 (1.05, 1.37)	.01
≥1.5	6	5,410 / 980	5,381 / 1,056	0.91 (0.83, 0.99)		3	6,500 / 1,069	6,476 / 1,071	0.99 (0.93, 1.06)	
Intervention dose, IU/day										
≤600	5	1,071 / 76	1,082 / 97	0.75 (0.56, 1.00)	.50	2	152 / 9	77 / 4	1.09 (0.35, 3.40)	.05
600 - 2000	3	1,529 / 300	1,520 / 333	0.83 (0.58, 1.19)		2	1,800 / 354	1992 / 327	1.20 (1.05, 1.38)	
>2000	6	4,226 / 711	4,209 / 747	0.93 (0.78, 1.10)		4	6,541 / 1,077	6517 / 1079	0.99 (0.93, 1.06)	
Follow up, yr										
<2.5	9	1,593 / 118	1,601 / 142	0.83 (0.59, 1.16)	.22	6	2,041 / 372	2,158 / 341	1.19 (1.04, 1.36)	.02
≥2.5	5	5,233 / 969	5,210 / 1,035	0.92 (0.84, 1.00)		2	6,452 / 1,068	6,428 / 1,069	0.99 (0.93, 1.06)	
Participants, n										
<500	8	914 / 114	914 / 131	0.95 (0.64, 1.41)	.49	5	279 / 25	203 / 19	1.07 (0.61, 1.88)	.94
≥500	6	5,912 / 973	5,897 / 1,046	0.91 (0.83, 0.99)		3	8,214 / 1,415	8,383 / 1,391	1.05 (0.94, 1.18)	
Events, n										
<100	10	2,722 / 112	2,726 / 129	0.91 (0.66, 1.26)	.91	5	279 / 25	203 / 19	1.07 (0.61, 1.88)	.94
≥100	4	4,104 / 975	4,085 / 1,048	0.90 (0.82, 0.98)		3	8,214 / 1,415	8,383 / 1,391	1.05 (0.94, 1.18)	
Risk of bias§										
Low risk	8	4,906 / 620	4,903 / 682	0.88 (0.76, 1.03)	.95	5	6,652 / 1,078	6,553 / 1,075	0.99 (0.93, 1.06)	.01
High risk	6	1,920 / 467	1,908 / 495	0.91 (0.78, 1.06)		3	1,841 / 362	2,033 / 335	1.20 (1.05, 1.37)	

Vitamin D₃ scale: 0.50 1.00 1.50 2.50 — Favours intervention / Favours control

Vitamin D₂ scale: 0.15 0.50 1.00 2.50 — Favours Intervention / Favours control

2.1.3.3. Prevalence of vitamin D deficiency and estimated absolute risk

In supplementary analyses, based on data from available cohorts, prevalence (and 95% CI) of vitamin D insufficiency (defined as 25(OH)D level of <30 ng/mL) was 69.5% (62.1 to 77.7%) for the United States (US) and 86.4% (78.4 to 95.2%) for Europe. Furthermore, using 25(OH)D concentrations of <10 ng/mL as the criterion, 4% and 15% of the general population were severely deficient in the Europe and US , respectively (eFigure 9). Additionally, using the most recent mortality statistics for the US and Europe,(57, 58) the estimated absolute risk differences for all-cause mortality associated with vitamin D deficiency were 75.4 events in Europe and 96.6 events in the US, per 100,000 individuals, per year (eAppendix 5). Using the population prevalence estimates of vitamin D deficiency from the current study, 9.4% and 12.8% of all deaths in Europe and in the US, respectively, could be attributed to vitamin D deficiency.

2.1.4. Discussion

Findings of this review indicate that there is a moderate, however, significant, inverse association between circulating vitamin D levels and the risk of all-cause mortality in the primary prevention cohort studies. The inverse association was evident generally for all broad causes of death and more specifically for deaths due to coronary disease, lymphoma, upper-digestive cancer and respiratory disorders. In all RCTs combined, vitamin D supplementation, when given alone, did not reduce overall mortality significantly among older adults. However, when stratified by type of supplementation, vitamin D_3, given singly, reduced all-cause mortality significantly by 11%. By contrast, there was no apparent benefit of vitamin D_2 supplementation in either clinical or community-based trials.

The inverse association between vitamin D and mortality can be explained by several different mechanisms. First, activated vitamin D may influence a range of biological responses from cellular growth, proliferation, apoptosis and immune system functions [2 8]. Vitamin D receptors and the enzyme required for its activation is present in most human cells and tissues, indicating a major role for vitamin D in "non-skeletal" physiological processes. Second, ~3K binding sites for the vitamin D receptor have been found throughout the human genome ,(59) indicating regulation of a very

large number of genes (currently estimated to be ~3% of the human genome [2]) either directly or indirectly responsive to vitamin D receptors. This, along with the potential adverse consequences of low 25(OH)D levels, such as CHD, cancer and mortality,(60) found in people with 25(OH)D-related genetic variants, reinforces the importance of an endocrine system beyond extracellular calcium and phosphate homeostasis.(59) Third, the positive association between vitamin D concentrations and longer leukocyte telomere length, a potential determinant of age-related disorders and overall longevity,(61) emphasises the possible beneficial effects of vitamin D on healthy ageing and associated adverse outcomes Fourth, as primary causes of vitamin D deficiency include insufficient exposure to sunlight, poor diet, increased adiposity, and reduced synthesis or absorption,(62) it is possible that a poor vitamin D status essentially reflects suboptimum lifestyle and socioeconomic circumstances. These individual-level factors may, in turn, influence risk for their potential roles on several established determinants of morbidity and mortality such as smoking, blood pressure, body mass index, and use of supplements.(63) Although the majority of the included studies in this review controlled for these characteristics and our pooled estimates were largely unchanged when they were further stratified by adjustment for standard socioeconomic factors, potential residual and unmeasured confounding by differences in diet, lifestyle and socioeconomic status remains a concern. Such unaccounted confounding could partly explain the discrepancy of findings observed earlier between observational and intervention studies of other dietary factors.(64) Finally, our study indicates that vitamin D is inversely and moderately associated with risk of death from coronary disease, lymphoma, cancers of the upper digestive tract and respiratory disease. Although causality could not be established for these associations, local expression of vitamin D receptors(65, 66) and systemic immunomodulatory(67, 68) roles of vitamin D have been proposed to explain them.

Subgroup analyses among observational studies indicated that the inverse associations of circulating 25(OH)D level with all-cause and cancer-specific mortality were significantly stronger in the populations with a low prevalence of vitamin D supplement use. This suggests that the effect of vitamin D may be dependent on baseline vitamin D status. Given that baseline circulating

25(OH)D levels in a population with low prevalent vitamin D supplement use is likely to be low(69) and the risk of mortality outcomes is known to be greater in the lower levels of 25(OH)D,(41) the current findings are not unexpected. In addition, a threshold effect in 25(OH)D levels up to 112 nmol/L has been suggested in a previous study, which can be achieved by daily use of 600 IU of vitamin D_3.(70) Additionally, we found a significant higher mortality risk of low 25(OH) D levels in studies with a follow-up duration shorter than 5 years. This may be attributed to reverse causality, where individuals have underlying diseases that are associated with low 25(OH)D levels such as cardiometabolic diseases.

Our meta-analysis of all available RCTs of vitamin D supplements, given singly among principally older adults, suggests that this nutrient may not significantly reduce mortality outcomes. However, when the effects of specific vitamin D metabolites were considered, supplementation in the form of vitamin D_3 (animal-derived, known as *cholecalciferol*) but not vitamin D_2 (plant-based, known as *ergocalciferol*) was associated with reduced mortality. Earlier evidence(71) describe ergocalciferol to be potentially less potent, unit for unit, in maintaining 25(OH)D levels in the circulation; therefore, the expected effect of vitamin D_3 on mortality could be greater. Additionally, previous reviews(72, 73)reported that in the absence of concomitant use of calcium supplements, compared to vitamin D_3, vitamin D_2 was associated with significantly lower overall increase in serum 25(OH)D concentration. Interestingly, concomitant use of 25(OH)D with calcium at baseline was associated with lower increases in 25(OH)D concentrations.(72) Subgroup analyses showed that vitamin D_2 supplementation increased the aggregate risk of mortality in trials that had shorter average intervention periods. Similar higher risks were reported for trials using lower intervention doses. The discrepant findings in our meta-analysis could also be explained by insufficient power (average follow-up duration in the vitamin D_2 trials was about a year less than for D_3 trials) or importantly, factors other than supplements themselves in these studies (such as diversity in population characteristics) and therefore, further RCTs are needed to reinforce these findings.

Findings of this updated meta-analysis generally concur and further extend the previous reviews in several important ways. First, the current study had

enhanced power to examine the associations in greater detail. For example, our meta-analysis of the primary prevention cohort studies involve ~10 times as many participants and ~3 times as many mortality outcomes as previous reviews (24, 33, 34) on this topic combined, include ~10 recent, large-scale prospective cohort studies. Second, in contrast to the earlier reviews, we have performed systematic synthesis of all available primary and secondary prevention cohorts to quantify risk of both composite and various cause-specific death outcomes in a single comprehensive investigation. Third, we have analysed and presented standardised pooled risk estimates comparing extreme thirds of baseline distribution of vitamin, and by pre-specified vitamin D cut-offs. Fourth, unlike previous reviews(32, 35) that included all randomised studies with mixed interventions, our most up-to-date meta-analysis of RCTs include exclusively the studies that adminstered vitamin D alone. Finally, we conducted detailed analyses under a broader range of individual and study-level circumstances to explore the potential sources of heterogeneity. Nonetheless, findings from our trial component is consistent with the earlier meta-analysis (based on RCTs irrespective of concomitant supplementation with calcium) that also reported heterogeneity in efficacy between two forms of supplement.

Our findings may have several implications. They underscore a potentially deleterious role of low vitamin D on all-cause and cause-specific mortality in both primary and secondary prevention cohorts. Additionally, a beneficial effect was observed for supplementation with vitamin D_3 in the RCTs. This is of significant public health importance as the gradual decline in circulating 25(OH)D levels reported globally is likely to continue due to the increase in the proportion of elderly populations, obesity and lack of adequate sun exposure combined with sunscreen use.(74) The current review underscores scientific gaps in the current intervention evidence and, therefore, stimulates further research. For instance, available trials were (i) generally insufficiently powered to reliably assess the optimum dosage, (ii) not able to examine potential toxic effects over prolonged use, and (iii) unable to reliably assess the efficacy in low-risk general populations as the majority of the included community-based studies involves solely older participants. Finally, when compared to other conventional risk factors of ill health, the estimated population attributable risk of death owing to suboptimal vitamin D in our

study appears to be substantial. For example, in the US, while the population attributable risk owing to vitamin D deficiency was estimated as ~13%, the corresponding estimates were ~20% for smoking,(75) ~11% for physical inactivity(76) and ~9% for alcohol consumption(77) in the US. This reinforces the potential importance of scalable, cost-effective public health strategies (such as moderate sun exposure, supplementation and food fortification) in improving the overall vitamin D status to reduce premature deaths worldwide.

The generalisability of our findings has been enhanced by the involvement of data from nearly 900,000 participants in 26 nations. We used standardised estimates to allow consistent comparisons and examined wide-range of clinically-relevant characteristics. However, the current review was limited by the moderate amount of available data on several cause-specific mortality outcomes. For example, even in aggregate, there were generally fewer than 1000 site-specific cancer deaths recorded in the prospective studies. Observational data also provide limited clarity whether observed associations with mortality outcomes are direct (i.e. owing to suboptimal vitamin D) or indirect (due to shared determinants such as obesity, body composition, social status). Furthermore, as all included observational studies lacked serial assessment of circulating 25(OH)D in the same individuals, reliable assessment of the extent of any within-person variability in circulating 25(OH)D concentration was not possible. Because most characteristics of epidemiological studies are measured with a degree of error and are subject to fluctuations within individuals over time, correction of such variability in future studies would help to avoid 'regression dilution'.(78, 79) While the observational studies are unable to assess the causal association, evidence from the intervention studies could provide concluding evidence in this respect. However, such trials are generally sparse, include chiefly elderly individuals (i.e. a population with high competing risk of death owing to comorbidities(80)) and do not typically present data on cause-specific deaths as the primary outcomes of interest. Furthermore, although the majority of trials included in this review appear to have low risk of bias, the current findings should be interpreted with some caution, owing to the relatively small number of trials for each intervention subtype, especially for primary prevention. Therefore, our findings intensify the need for detailed future

intervention studies that (i) involve free-living general populations; (ii) quantify efficacy in important subgroups such as non-white ethnicities; (iii) are adequately powered and sufficiently prolonged to help judge appropriate dosage and safety; (iv) aim to ascertain a broad range of fatal and nonfatal outcomes than has been customary in the RCTs thus far; and (v) study both vitamin D_2 and D_3 to identify which form of vitamin D supplementation can be most efficient and safe.

2.1.5. Conclusion

Evidence from observational studies show inverse associations of circulating 25(OH)D with risks of vascular, cancer and nonvascular-noncancer deaths. Supplementation with vitamin D_3 reduced overall mortality significantly among older adults; however, further study is needed to reliably establish whether vitamin D_3 affects mortality risk differently than vitamin D_2.

Chapter 3
Vitamin D and Metabolic Health

3.1. Vitamin D status and metabolic syndrome in the elderly: the Rotterdam Study.

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Abstract

Objective: The effects of vitamin D in the elderly are inconsistent. The aim of this study was to evaluate the association between vitamin D status and the metabolic syndrome (MetS) in the elderly, as well as between vitamin D status and the components of MetS (i.e. serum glucose, triglycerides (TG), HDL cholesterol (HDL-C), waist circumference (WC), and blood pressure (BP)).

Methods: The study was embedded in the Rotterdam Study, a population-based cohort of middle-aged and elderly adults. We analyzed data from 3240 people (median age 71.2 years) who did not have type 2 diabetes mellitus at baseline.

Results: We found higher 25-hydroxyvitamin D (25(OH)D) concentrations associated with lower prevalence of MetS (Odds Ratio (OR); 95% Confidence Interval (CI): 0.61; 0.49, 0.77 for adequate levels (≥75nmol/l)

versus deficiency (<50nmol/l) . Additionally, in analysis of the individual components, the ORs for adequate versus deficient vitamin D levels were: 0.66 (95%CI 0.53,0.83) for elevated WC, 0.67 (95%CI 0.52,0.86) for reduced HDL-C, 0.69 (95%CI 0.54,0.88) for elevated triglycerides, 0.80 (95%CI 0.65,0.99) for elevated fasting glucose. Vitamin D was not associated with elevated blood pressure, ORs for adequacy versus deficiency were 0.82 (95%CI 0.65,1.03).

Conclusion: Higher 25(OH)D concentrations in the elderly are associated with lower prevalence of MetS and, in particular, with more beneficial HDL-C, TG, WC and serum glucose. Since the prevalence of vitamin D deficiency is common worldwide and its risk increases with age, if causality is proven, benefits of improving vitamin D status among the elderly may be great.

3.1.1. Introduction

Vitamin D was discovered at the beginning of the 20th century when it presented a long awaited cure for rickets, a childhood disease consisting of weak bones(81). Since that time, vitamin D has been established as a major factor influencing metabolism of bones and calcium metabolism (81, 82).

Active vitamin D binds to a receptor from nuclear family called vitamin D receptor (VDR), which, subsequently, increases transcription of its target genes. Recently, scientists discovered that the enzyme needed for the activation of vitamin D, 1α hydroxylase, is present in most cells and tissues in the human body, as are VDRs. This discovery led to additional research into other vitamin D effects beside calcium metabolism and bone health. Currently, we know there are numerous binding sites for vitamin D in the human genome, suggesting a wide range of vitamin D effects(6).

However, the cutoff points for adequate vitamin D status are still being debated. Some experts consider serum 25(OH)D concentrations higher than 75nmol/l as adequate not just for bone health, but for the non-skeletal effects as well (6, 83). Others suggest that serum 25(OH)D concentrations of 50nmol/l are sufficient (84).

Metabolic Syndrome (MetS) is defined as a cluster of risk factors for cardiovascular disease (CVD) and type 2 diabetes mellitus (DM) (85). Several commonly used definitions of MetS have been proposed and, (85)although these definitions differ, they generally agree on the following criteria for diagnosis of MetS: central obesity, dyslipidemia (elevated triglycerides and reduced HDL cholesterol), hypertension and hyperglycemia (85).

The association between vitamin D status and MetS has previously been studied in different populations (86, 87). Animal models and in vitro studies provide an insight into mechanisms (88, 89) of vitamin D metabolic actions, while meta-analyses of epidemiological studies confirm inverse associations between serum vitamin D status and MetS (86, 87). However, the most of the studies published on this topic were conducted in younger populations (90) and subgroup analyses of data from the elderly (aged 65 years and older) are not conclusive and two of the studies focusing on this age group yielded contradictory results (91, 92). Additionally, there has not been sufficient attention paid to finding the specific groups among the elderly who may

benefit the most from improved vitamin D status (e.g. gender or those with obesity or impaired kidney function).

Thus, our study aimed to evaluate whether vitamin D status is associated with risk of metabolic syndrome in the elderly. Furthermore, we aimed to evaluate the association of vitamin D status with individual components of the metabolic syndrome to assess whether the association differed by age, gender, BMI, and kidney function. Finally, we assessed which, if any, of the associations between vitamin D status and individual components of MetS were the strongest.

3.1.2. Methods

3.1.2.1.Study design

This study was embedded in the Rotterdam Study, a large prospective population-based cohort study conducted among residents in of Ommoord, a district of Rotterdam, the Netherlands (15). These participants were aged 55 years or older at baseline. Of 10,275 eligible subjects, 7,983 (78%) participated in the baseline examinations between 1990 and 1993. All participants were interviewed at home and visited the research center for further examinations. Additional follow-up visits were conducted every 3-4 years.

The study was approved by the medical ethics committee at Erasmus University Rotterdam, The Netherlands and written informed consent was obtained from all participants.

3.1.2.2. 25-hydroxyvitamin D

Between 1997 and 1999, the third survey of the cohort described above, serum 25hydroxivitamin D concentrations were assessed using electrochemiluminescence immunoassay (COBAS, Roche Diagnostics GmbH, Germany). The sensitivity of the test was 10nmol/L and serum 25(OH)D concentrations detected were within range of 7.5nmol/l and 175nmol/l. Intra-assay precision was <6.5% and the inter-assay precision was <11.5%. Concentrations of serum 25(OH)D under 50nmol/l were categorized as "deficient", and concentrations of serum 25(OH)D from 50nmol/l to 75nmol/l as "insufficient," according to cutoffs described by Holick (6).

3.1.2.3. Metabolic syndrome and its components

To define MetS, we used the joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; the National Heart, Lung, and Blood Institute; the American Heart Association; the World Heart Federation; the International Atherosclerosis Society; and the International Association for the Study of Obesity (85). According to this definition, an individual had MetS if three out of five of the following conditions are met: (i) waist circumference ≥102 cm for men and ≥88 cm for women; (ii) elevated triglycerides (≥ 1.7mmol/l) or alternatively drug treatment for elevated triglycerides; (iii) reduced concentration of HDL cholesterol (≤ 1.0mmol/l in males and ≤1.3mmol/l in females) or, alternatively, active drug treatment for reduced HDL cholesterol; (iv) elevated blood pressure (systolic ≥130mmHg and/or diastolic ≥85mmHg) or, alternatively, active antihypertensive drug treatment in a patient with a history of hypertension; (v) elevated fasting glucose (≥100mg/dl) or active drug treatment of hyperglycemia(85). All components of metabolic syndrome were assessed during the third survey of the Rotterdam Study cohort (1997-1999).

Waist circumference was measured half way between the lower rib margin and the iliac crest and presented in centimeters. During measurement participants were standing, wearing only light clothes, breathing gently.

Serum glucose was measured by hexokinase enzymatic method after overnight fasting. Fasting serum glucose was not assessed in subjects with diagnosis of type 2 diabetes mellitus. Hence, these subjects were excluded from the analysis (N=588).

Triglycerides were measured in fasting serum using enzymatic method. HDL cholesterol was measured by automatic enzymatic method from fasting serum after precipitation of non-HDL fraction. Blood pressure was measured at the right brachial artery using random-zero sphygmomanometer after 5 minutes of rest with the participants in sitting position. Mean of the two consecutive measurements was used.

3.1.2.4. Confounders and effect modifiers

At the baseline cohort visit (1989-1991), information on multiple factors was collected. Trained interviewers conducted home interviews, after which participants were invited to the research centers for the clinical examination

and laboratory tests. In our analyses, we used the following information collected at that time point: diet, family history of diseases, educational level and household income.

Family history of diseases included following diseases in siblings, children, or parents: diabetes mellitus, myocardial infarction, and cerebrovascular accident. Level of education was split into low (primary education or less) and high (more than primary education). The cutoff value of 2,699 euro per month (35,999 euro per year) was used to assess household income. Income below cutoff was considered as low household income, and everything above the cutoff as high household income. In order to adjust for overall dietary quality, the Dutch Healthy Diet (DHD) Index was used. DHD is a representative continuous score of dietary compliance to the Dutch Guidelines for a Healthy Diet, which has been assessed from the FFQ assessed at baseline and has been described in detail elsewhere (93).

Information on following factors we used in our analyses was collected during the third visit of the cohort participants (1997-1999) to the research center: weight, height, prevalent cardio-metabolic diseases, serum creatinine, smoking, and physical activity.

Body mass index (BMI) was calculated by dividing body weight (kilograms) by height (meters) squared. Serum creatinine was measured using an enzymatic assay method and used to calculate estimated glomerular rate (eGFR) with simplified Modification of Diet in Renal Disease (MDRD) formula. Chronic kidney disease was defined as eGFR< 60ml/min per $1.73m^2$. Cardio-metabolic baseline diseases (values: 0=no and 1=yes, subjects with diabetes mellitus were excluded) were identified on the basis of the presence of at least one of the following: heart failure, coronary heart disease, atrial fibrillation, cerebrovascular accident, and chronic kidney disease. Smoking status was assessed on the basis of three categories: non-smoker, former smoker, and current smoker. Physical activity levels (minutes per week) were assessed using a validated questionnaire of the Zutphen Study(94). Originally, the questionnaire provided information about walking, cycling, gardening, diverse sports and hobbies hence information about outdoors activities. Later, questions about housekeeping activities were also included.

3.1.2.5. Population for analysis

The initial study population consisted of 7,983 participants. We used the third visit of the cohort (1997-1999) in our analyses. A subsample of the initial population had serum 25(OH)D measured at the third visit (N=3,828). We excluded participants with diabetes mellitus which had been ascertained at the first visit or had been diagnosed between the first (1989-1993) and the third visit of the cohort (1997-1999). These participants had no fasting blood samples drawn at the third visit due to risk of hypoglycemia. After exclusion of participants with previously identified type 2 diabetes mellitus diagnosis, 3,240 participants with serum 25(OH)D measurements were available for analysis, including those with pre-diabetes and insulin resistance syndrome.

3.1.2.6. Statistical analysis

To determine the association between vitamin D status and prevalence of MetS (and each of its components) we used logistic regression models.

Firstly, a crude model was built adjusted for age and sex. Secondly, a multivariate model was built, further adjusted for potential confounders. The confounders were selected based on previously published literature, univariate inspection based on 10% change in the effect estimate, and association with the outcome and/or the exposure: dietary quality score, baseline diseases, family history of diseases, physical activity, season when blood was drawn, level of education and household income(95, 96). Serum 25(OH)D concentration was used as continuous variable and also as categorical (cutoff points described by Holick (6)).

We further tested whether the associations differed by age, gender, eGFR, and BMI. In case of significant effect modification, stratified analyses were performed ($P_{interaction}$ <0.05).

Additionally, we tested which of the associations between vitamin D status and individual components of MetS were independent from the rest of the MetS components. For this purpose we used multivariate model further adjusted for the rest of the components of MetS, tested individually.

Finally, to assess the effect of BMI on the association between vitamin D status and MetS, and the effect of BMI on the association between vitamin D status and individual components of MetS, we further adjusted the multivariate model to include BMI.

To reduce the potential for any biases associated with missing data, a multiple imputation procedure was performed, N=10 imputations. The multiple imputation procedure is based on prediction of missing data based on correlation with other observed data. The missing data are imputed with randomly selected values from the predicted distribution. In the next step, 10 different copies of the original data set with missing data imputed by this random selection of predicted values are created. The analyses are performed separately in all of the 10 datasets and pooled results from these 10 imputed datasets are reported. Final results are presented after the multiple imputation procedure.

Main results are presented as odds ratios (ORs) and 95% confidence intervals (CIs) for metabolic syndrome and for components of metabolic syndrome. A P- value < 0.05 was considered as statistically significant.

All analyses were performed with IBM SPSS Statistics version 20 (SPSS Inc., Chicago, Illinois).

Sensitivity analyses were performed using different cutoff points for serum 25(OH)D concentrations as suggested by the Institute of Medicine (97). According to these cutoffs, 25(OH)D concentrations less than 40nmol/l were defined as covering the needs of half of the population, while concentrations of 50nmol/l were defined as covering the needs of 97.5% of the population(97). Additional sensitivity analysis included use of alternative cut points for waist circumference, 94cm for men and 80cm for women (85).

3.1.3. Results

Table 3.1.1 presents the characteristics of our study population by vitamin D status. Only sixteen percent of our study participants had adequate vitamin D status (serum 25(OH)D concentrations of 75nmol/ and higher), twenty seven percent had a vitamin D insufficiency (serum 25(OH)D concentrations between 50nmol/l and 75nmol/l), and fifty six percent had a vitamin D deficiency(serum 25(OH)D concentrations lower than 50nmol/l). Participants with adequate vitamin D status were younger and more likely to be men (Table 3.1.1). Also, among participants who were vitamin D deficient, there was a greater prevalence of MetS and these participants also were likelier to have high blood pressure. However there were no differences in mean waist

circumference, serum glucose, HDL cholesterol, and total cholesterol across serum 25(OH)D strata (Table 3.1.1).

3.1.3.1. Vitamin D status and prevalence of metabolic syndrome and its components

Every 10nmol/l increase in serum 25(OH)D was significantly associated with a lower prevalence of MetS (OR=0.91, 95%CI 0.88, 0.94 and OR=0.90, 95%CI 0.87, 0.93 for crude model and multivariate model, respectively) among our study participants. Both insufficient and adequate categories of vitamin D status were associated with lower prevalence of MetS when compared to those with a vitamin D deficiency (Table 3.1.2).

Regarding the individual components of the MetS, vitamin D status was significantly associated with lower prevalence of elevated WC (multivariate adjusted OR=0.93, 95%CI 0.90, 0.96), lower prevalence of elevated triglycerides (multivariate adjusted OR=0.94, 95%CI 0.90, 0.97), lower prevalence of reduced HDL cholesterol (multivariate adjusted OR=0.94, 95%CI 0.90, 0.97), and lower prevalence of elevated fasting glucose (multivariate adjusted OR=0.96, 95%CI 0.93, 0.99) (Table 3.1.3). We also found an association between vitamin D status and lower prevalence of elevated blood pressure (multivariate adjusted OR=0.96, 95%CI 0.92, 0.99), however this finding was no longer significant when assessing the cutoffs (Table 3.1.3).

Furthermore, we found WC to be significantly associated with serum 25(OH)D independently of the other MetS components whereas no independent association was found for the other MetS components after mutual adjustment (Table 3.1.4). After additional adjustment for BMI, higher serum 25(OH)D concentrations were still significantly associated with lower odds of MetS.

Table 3.1.1. Baseline [RS-1-3] characteristics of the study population according to serum 25(OH)D.

	Deficient (<50nmol/l)	Insufficient (50-75nmol/l)	Adequate (≥75nmol/l)	P-value
	N= 1833 (56.6%)	N=874 (27.0%)	N=533 (16.4%)	
Serum 25(OH)D* [nmol/l]	31.3 (10.8)	61.2 (7.0)	93.3 (15.4)	<0.001
Metabolic syndrome (yes) N(%)	766 (41.8)	286 (32.7)	158 (29.6)	<0.001
Age* [years]	74.1 (7.6)	70.3 (5.8)	69.5 (5.9)	<0.001
Gender (female) N (%)	1244 (67.9)	459 (52.5)	226 (42.4)	<0.001
Glucose in serum* [mmol/l]	5.54 (0.60)	5.52 (0.56)	5.50 (0.54)	0.273
HDL cholesterol* [mmol/l]	1.42 (0.41)	1.43 (0.39)	1.39 (0.38)	0.082
Total Cholesterol* [mmol/l]	5.85 (1.02)	5.87 (0.93)	5.83 (0.99)	0.699
Triglycerides* [mmol/l]	1.49 (0.70)	1.41 (0.62)	1.42 (0.67)	0.005
Lipid lowering medication (yes) N(%)	254 (13.9)	110 (12.6)	68 (12.8)	0.902
Systolic blood pressure* [mmHg]	144 (21)	141 (20)	141 (21)	0.001
Diastolic blood pressure* [mmHg]	75 (12)	75 (11)	76 (10)	0.107
Blood pressure lowering medication (yes) N(%)	428 (23.3)	184 (21.0)	89 (16.7)	0.001
Cardiovascular diseases (yes) N(%)	378 (20.6)	138 (15.8)	78 (14.6)	0.001
Waist circumference* [cm]	93.1 (11.8)	92.3 (10.7)	92.4 (10.6)	0.204
BMI* [kg/m2]	26.9 (4.1)	26.3 (3.4)	26.1 (3.4)	<0.001
Physical activity* [min/week]	2558 (1150)	2699 (1094)	2753 (1084)	<0.001
DHDI-score*/‡	49.0 (10.1)	48.8 (10.2)	47.9 (10.0)	0.094
Smoking (current) N(%)	403 (22.0)	187 (21.4)	111 (20.8)	<0.001
Family history of diseases (yes)N(%)	1212 (66.1)	565 (64.6)	343 (64.4)	0.831
Education (low)‡ N(%)	618 (33.7)	229 (26.2)	124 (23.3)	<0.001
Income (below the average)‡ N(%)	1035 (56.5)	398 (45.5)	233 (43.7)	<0.001

*Mean (SD); ‡ Assessed at the previous visit to the research center (1989-1993); Prevalent diabetes cases were excluded in the analyses; Data presented after multiple imputation procedure.

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Table 3.1.2. Vitamin D status and prevalence of metabolic syndrome.

	N	Crude model OR (CI 95%)	Multivariate model OR (CI 95%)
25(OH)D continuous	3240	0.91(0.88,0.94)*	0.90(0.87,0.93)*
25(OH)D cut-offs			
<50nmol/l	1833	reference	Reference
50-75nmol/l	874	0.72(0.60,0.86)*	0.70(0.58,0.84)*
≥75nmol/l	533	0.65(0.52,0.81)*	0.61(0.49,0.77)*

*Crude model adjusted for age and sex only; Multivariate model is adjusted for age, sex, physical activity, diet quality score, family history of cardio-metabolic diseases, baseline cardio-metabolic diseases, smoking, education, income, season of blood draw, and year or blood draw; Prevalent diabetes cases were excluded in the analyses; Data presented after multiple imputation procedure; *p value <0.001; OR- odds ratio (for continuous serum 25(OH)D per 10 unit serum 25(OH)D increase); CI- confidence interval.*

Table 3.1.3. Vitamin D status and individual components of metabolic syndrome.

		Multivariate model OR (CI 95%)				
	N	Elevated waist circumference	Elevated triglycerides	Reduced HDL cholesterol	Elevated glucose	Elevated blood pressure
25(OH)D continuous	3240	0.92 (0.89,0.95)*	0.93 (0.89,0.96)*	0.93 (0.90,0.97)*	0.96 (0.93,0.99)	0.96 (0.93,1.00)
25(OH)D cut-offs						
<50nmol/l	1833	reference	reference	reference	reference	reference
50-75 nmol/l	574	0.76 (0.63,0.91)	0.74 (0.61,0.90)	0.76 (0.62,0.93)	0.94 (0.79,1.12)	0.98 (0.80,1.20)
≥75 nmol/l	533	0.66 (0.53,0.83)*	0.69 (0.54,0.88)	0.67 (0.52,0.86)	0.80 (0.65,0.99)	0.82 (0.65,1.03)

Multivariate model is adjusted for age, sex, physical activity, diet quality score, family history of cardio-metabolic diseases,
*baseline cardio-metabolic diseases, smoking, education, income, season of blood draw, and year of blood draw; Prevalent diabetes cases were excluded in the analyses; Data presented after multiple imputation procedure; *p value <0.001; OR- odds ratio (for continuous serum 25(OH)D per 10 unit serum 25(OH)D increase); CI- confidence interval.*

Furthermore, WC was significantly associated with serum 25(OH)D only when serum 25(OH)D was assessed continuously. The effect estimates for triglycerides and HDL cholesterol were slightly attenuated however, direction of the effects remained the same (data not shown).

3.1.3.2. Subgroup analysis

We found significant effect modification by gender ($P_{\text{interaction}}$ <0.05). In women, higher serum 25(OH)D concentrations were significantly associated with lower prevalence of elevated TG (data not shown), while lower prevalence of MetS was significantly associated with higher serum 25(OH)D in both, men and women, though results were stronger among women (data not shown).

Table 3.1.4. Vitamin D status and individual components of metabolic syndrome after mutual adjustment of the other MetS components.

		Multivariate model OR (CI 95%)				
	N	**Elevated waist circumference**	**Elevated triglycerides**	**Reduced HDL cholesterol**	**Elevated glucose**	**Elevated blood pressure**
25(OH)D continuous	3240	**0.93 (0.90,0.97)***	**0.96 (0.92,1.00)**	0.97 (0.93,1.01)	0.99 (0.96,1.02)	0.98 (0.94,1.01)
25(OH)D cut-offs						
<50nmol/l	1833	reference	reference	reference	reference	reference
50-75nmol/l	874	**0.82 (0.68,0.99)**	0.90 (0.72,1.12)	0.89 (0.71,1.11)	1.05 (0.88,1.26)	1.04 (0.85,1.27)
≥75nmol/l	533	**0.71 (0.57,0.90)**	0.82 (0.63,1.07)	0.78 (0.59,1.04)	0.91 (0.73,1.13)	0.89 (0.70,1.12)

*Multivariate model is adjusted for age, sex, physical activity, diet quality score, family history of cardio-metabolic diseases, baseline cardio-metabolic diseases, smoking, education, income, season of blood draw, year of blood draw plus additionally adjusted for the rest of the individual components of the MetS; for example: for WC as an outcome model was adjusted for TG, HDL, glucose and BP; Prevalent diabetes cases were excluded in the analyses; Data presented after multiple imputation procedure; *p value <0.001; OR- odds ratio (for continuous serum 25(OH)D per 10 unit serum 25(OH)D increase); CI- confidence interval; WC-waist circumference; TG-triglycerides; HDL-C-HDL cholesterol; BP-blood pressure.*

3.1.3.3. Sensitivity analysis

We conducted sensitivity analyses using alternative cutoff points for WC as suggested by Alberti et al. (85). For this analysis we considered a WC value of equal to or greater than 94cm to be elevated for men, and a WC value of equal to or greater than 80cm as elevated for women. The effect estimates of this analysis were similar for both groups (data not shown).We also evaluated whether the use of different cutoff points for serum 25(OH)D, as suggested by the Institute of Medicine would have any impact on our findings (2, 98), but

the use of the IoM cutoff points yielded similar effect estimates (data not shown).

3.1.4. Discussion
3.1.4.1. Main results
We found that vitamin D status, measured by serum 25(OH)D concentrations, was inversely associated with the prevalence of MetS in the elderly. This association was mainly driven by elevated waist circumference. In particular, we found that vitamin D status was inversely associated with the prevalence of elevated serum triglycerides, elevated waist circumference, reduced HDL cholesterol levels and elevated glucose levels. No significant association was found between serum 25(OH)D and blood pressure.

3.1.4.2. Comparison with other studies
Other previous studies have found an inverse association between vitamin D status and MetS (83, 86, 87, 90) and our estimates fall within the range reported. However, the majority of the associations reported elsewhere were observed in younger populations (86, 87), such as the findings from a longitudinal study conducted among Australian adults by Gagnon et al (11). They found that lower concentrations of serum vitamin D status were associated with increased risk of MetS (90). Within elderly populations, we identified just two cross-sectional studies which reported the association between vitamin D status and metabolic syndrome: the Rancho Bernardo Study (91) and the Longitudinal Ageing Study Amsterdam (LASA) (92). The LASA study found a significant association between higher serum 25(OH)D (>50nmol/L) and lower prevalence of MetS. Similar to our results, the LASA study also found a significant association with HDL-C and WC. The Rancho Bernardo study (mean age >75 years) found no significant association between serum 25(OH)D and MetS, but they did find a significant association between serum 25(OH)D and glucose levels, though that finding was in men only. The discrepancy between our findings and those of The Rancho Bernardo study may be explained by the relatively high serum 25(OH)D concentrations in the population of The Rancho Bernardo Study. In addition, this study took place in California where high exposure to UVB radiation caused serum 25(OH)D concentrations which were much higher than the

mean concentration in US elderly population in general (99) (in The Rancho Bernardo Study the mean serum 25(OH)D concentration was above 100nmol/l with the bottom quintile of 87.5nmol/l). It was hypothesized that there are different thresholds up to which vitamin D can have beneficial effects while raising serum 25(OH)D concentrations above the threshold produces small or no additional benefits (90, 92, 100-102).

The results we found are more closely aligned with those from LASA, where an association between vitamin D status and prevalence of MetS was found, odds ratio of 1.54 for serum 25(OH)D concentrations below 50nmol/l vs above 50nmol/l. These results were comparable to those we report here: multivariate adjusted odds ratio of 0.61 for serum 25(OH)D concentrations ≥75nmol/l vs <50nmol/l.

Based on results from these two studies and our results, we speculate that there might be a certain threshold effect of vitamin D status on MetS. Raising serum 25(OH)D concentrations above a certain threshold (eg >75nmol/l) adds little effect.

3.1.4.3. Potential mechanisms

The association between vitamin D status and MetS and its components can be explained in several ways. Firstly, vitamin D influences formation of HDL particles (103, 104) (105) (106) (107). Secondly, serum 25(OH)D inhibits adipocyte differentiation and may thereby influence the development of adiposity (88). Also, vitamin D deficiency causes an increase in parathyroid hormone (PTH), which is known to favor the process of lipid storage (108, 109). Thirdly, serum 25(OH)D regulates an enzyme directly involved in lipoprotein mechanism- lipoprotein lipase (LPL) (110). Fourthly, the active form, serum 1,25-dihydroxyvitamin D, acts as a suppressor of the renin-angiotensin system (RAAS) (111, 112) (113). Finally, angiotensin II has been shown to cause insulin resistance and, since vitamin D inhibits RAAS, it might indirectly improve insulin sensitivity (114, 115). Also, vitamin D helps insulin secretion from pancreatic beta cells because it enhances insulin sensitivity by stimulating the expression of insulin receptors, and by its participation in regulation of intracellular Ca^{2+} (116).

3.1.4.4. Subgroup analysis

We found an effect modification by gender. We found vitamin D status significantly associated with a lower prevalence of elevated triglycerides in women. However, vitamin D status was inversely associated with MetS in men and in women. The effect magnitude was slightly greater in women than in men (odds ratios 0.88 compared to 0.93 for women and men respectively). These results may be explained by the increased risk of both metabolic syndrome and vitamin D deficiency in women (6, 98).

Overall, the subgroup analysis suggests that the effect of serum 25(OH)D may differ in magnitude among risk groups for both metabolic syndrome and vitamin D deficiency. Also, it seems that women might benefit more from adequate vitamin D status. These findings warrant further study in the future.

3.1.4.5. Strengths and limitations

The main strengths of our study were its prospective design and the extensive records of population characteristics. However, some limitations need to be taken into account as well. Firstly, we did not assess PTH levels, which is an important factor related to serum 25(OH)D and calcium metabolism. Secondly, we were not able to exclude patients with MetS prior to serum 25(OH)D assessment since we had not measured triglyceride levels and we had no fasting blood samples at that time. Although we excluded subjects who had type 2 diabetes mellitus prior to serum 25(OH)D assessment, reverse causality cannot be fully ruled out (reverse causality occurs when the outcome is related to the exposure being studied. Specifically, the participant's ill health could cause low serum 25(OH)D concentrations rather than the other way around, which we investigated). Thirdly, some of the population characteristics we used in our analysis were recorded at the examination round of the cohort prior to serum 25(OH)D assessment, so some changes in serum concentration may have occurred within that period. However, only diet, family medical history, income, and education were assessed at that time, so the time delay may have influenced our results only in very specific circumstances. Specifically, this influence may have occurred if the particular change in the variables differently confounded the relationship between vitamin D status and metabolic syndrome at the moment of serum 25(OH)D assessment than prior to the serum 25(OH)D assessment, which is unlikely in

the elderly. Fourthly, although we adjusted for many potential confounders, this study is of observational design, so residual confounding may remain. For example, confounding may have occurred due to lack of data on time spent outdoors. Finally, we could not explore the relationship longitudinally, since not all components of metabolic syndrome were measured in the subsequent rounds of the Rotterdam Study.

3.1.4.6. Main conclusion and future directions

Higher serum 25(OH)D concentrations were associated with lower prevalence of MetS in the elderly. Moreover, vitamin D status was associated with lower prevalence of dyslipidemia, abdominal obesity and hyperglycemia. Additionally, vitamin D status was associated with abdominal obesity independently of other MetS components. The beneficial effects of vitamin D might differ in magnitude in different risk groups for the metabolic syndrome. Some effects of vitamin D were stronger in females. We conclude that the elderly might benefit from higher serum 25(OH)D, especially women. However, the causality between vitamin D status and MetS still needs to be investigated and, therefore, well designed supplementation trials are needed.

3.2. Vitamin D and body composition in the elderly

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Abstract

Objective: *To investigate the association between vitamin D status and body composition in the elderly.*

Methods: *This study was embedded in the Rotterdam Study, a population-based prospective study in Rotterdam, the Netherlands, including subjects aged 55 years and older. Serum 25-hydroxyvitamin D (25(OH)D) was measured between 1997 and 1999. Total body fat, android fat, gynoid fat and lean mass were assessed using dual-energy X-ray absorptiometry (DXA) during a follow-up visit after a median time of 5 years (2002-2004). We calculated body fat percentage, lean mass percentage, and android/gynoid fat ratio. We had 2,158 participants included in our analysis. We used multivariable linear regression models. Serum 25(OH)D was analyzed continuously and after categorization according to cut-offs.*

Results: *Mean (±SD) serum 25(OH)D concentration of the study population was 52.6 ± 25.4nmol/L. Compared to subjects with an adequate vitamin D status (25(OH)D ≥75nmol/L), vitamin D eficient participants (25(OH)D <50nmol/L) had a higher body fat percentage (β=1.29, 95%CI: 0.55, 2.04) whereas no association was found with lean mass (β=0.008, 95%CI: -0.333, 0.35). Lower 25(OH)D was associated with higher total body fat percentage specifically in participants without cardio-metabolic disease. Each 10 unit increase in serum 25(OH)D was associated with 0.03 unit decrease in android fat (β=-0.03, 95%CI: -0.06, -0.01); after adjustment for BMI the association was no longer significant. Serum 25(OH)D was associated with gynoid fat, and the android/gynoid fat ratio but this was mainly explained by BMI.*

Conclusion: *Lower serum 25(OH)D concentrations were associated with a higher fat mass percentage. The association between serum 25(OH)D and differential fat distribution in the elderly was mainly explained by BMI and deserves further study.*

3.2.1. Introduction

Low concentrations of Vitamin D (25(OH)D) have been associated with bone health and also with obesity and many obesity-related disorders such as metabolic syndrome, cardiovascular disease, diabetes mellitus, and mortality(6, 117).

The prevalence of obesity is common worldwide(118) and obesity is a well-known risk factor for diseases like type 2 diabetes mellitus and cardiovascular disease(119, 120). Fat tissue located in the abdominal region, especially around the internal organs, is considered to be hormonally active, causing low grade inflammation, which contributes to the development of insulin resistance and subsequently type 2 diabetes mellitus (121, 122) (123).

It is known that circulating concentrations of serum 25(OH)D are lower in obese individuals, most likely due to sequestration of serum 25(OH)D in the fat tissue and due to other lifestyle factors related with sun exposure (e.g. physical activity) (124). On the other hand, there are numerous mechanisms reported in the literature describing the involvement of vitamin D in adipose tissue metabolism (124) and also mechanisms describing the role of muscle cells in metabolism of vitamin D (125). For example, it has been suggested there might be increase in catabolism of vitamin D in the adipose tissue(126), while, on the other hand, active $1,25(OH)_2D$ might activate muscle tissue growth and improve muscle function(127-129). Observational studies found serum 25(OH)D associated with different anthropometric measures and body composition measures such as body mass index, body fat mass, but also visceral fat and lean mass (124, 130, 131). Conversely, a study using a bidirectional Mendelian randomization approach found higher body mass index causing lower serum 25(OH)D concentrations but not the other way around(132).

However, it is unclear whether vitamin D status is associated with fat mass stored in specific regions of the body and what is the direction of this association. Furthermore, since most of the research on this topic was performed in younger populations the association between vitamin D status and body composition in the elderly remains unexplored. Knowledge on potential preferential storage of vitamin D in specific fat depots is important to clarify because it might help further elucidate the mechanisms how vitamin D and obesity are associated and may have implications for supplementation

of specific populations. Therefore, we investigated whether there is an association between vitamin D status and body composition including measures of fat and lean mass and fat distribution by DXA in the elderly. Additionally, we evaluated whether this association differed by age, gender, presence of cardio-metabolic disease and metabolic syndrome since these are all factors that might relate to vitamin D status and also body composition.

3.2.2. Methods
3.2.2.1. The Rotterdam Study
The Rotterdam Study is an ongoing prospective population-based follow-up study comprising of people aged 45 years or older from a suburb of Rotterdam, the Netherlands(15). The study was approved by the Medical Ethics Committee of the Erasmus Medical Center and written informed consent was obtained from all participants. Baseline measurements were obtained between 1990 and 1993. During this phase, information on current health status, use of medication, medical history, lifestyle, and risk indicators for chronic diseases was collected. Subsequently, the participants visited the study center for detailed clinical examinations and assessment of diet. Follow-up visits were held every 2 to 3 years(15). The vital status of the participants was obtained regularly from the municipal population registry. Morbidity and mortality were assessed using information from the general practitioners or, in case of hospitalization, by discharge reports from the medical specialists. Furthermore, data were obtained using linkage with pathology registries.

The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC and by the Ministry of Health, Welfare and Sport of the Netherlands , implementing the Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study). All participants provided written informed consent to participate in the study and to obtain information from their treating physicians.

3.2.2.2. Serum 25-hydroxyvitamin D
At the third examination round of the cohort (1997-1999) serum 25(OH)D concentrations were measured using electrochemiluminescence immunoassay (COBAS< Roche Diagnostics GmbH, Germany). The range of the test for serum 25(OH)D concentrations was 7.5nmol/L to 175nmol/L. The sensitivity

of the test was 10nmol/L, within-run precision was <7.8%, and intermediate precision was <13.1%. We categorized serum 25(OH)D concentrations as follows: <50nmol/L as vitamin D deficiency, 50-75nmol/L as vitamin D insufficiency, and ≥75nmol/L as adequate vitamin D status (6).

3.2.2.3. Anthropometrics

Participants' height, weight, waist circumference, and hip circumference were measured at the third examination round of the cohort (1997-1999) and at the fourth examination round of the cohort (2002-2004), with the participants standing without shoes and heavy outer garments. Waist circumference was measured at the level midway between the lower rib margin and the iliac crest with participants in standing position without heavy outer garments and with emptied pockets, breathing out gently. Hip circumference was measured as the maximum circumference over the buttocks. Body mass index (BMI, kg/m^2) and waist-to-hip ratio were calculated.

3.2.2.4. Measures of body composition

Body composition was assessed using Dual-energy X-ray absorptiometry (DXA) during the fourth examination round of the Rotterdam Study (2002-2004). Whole body DXA scans were acquired using ProdigyTM total body-fan beam densitometer (GE Lunar Corp, Madison, WI, USA) (133). Total body weight (grams) was divided into bone mineral content, lean mass, and fat mass. In addition, fat mass of android and gynoid regions of the body was analyzed. Body fat percentage, android fat percentage, gynoid fat percentage and lean mass percentage where calculated by expressing these measures as percentage of total body weight. We calculated the ratio of android fat and gynoid fat, and a ratio of fat mass and lean mass as additional measures of body fat distribution.

3.2.2.5. Potential confounding variables

Information on current health status, medical history, smoking behavior, education level attained and socioeconomic status was obtained during home interviews at the first examination round of the cohort (baseline visit). Education was defined as low (primary education), intermediate (secondary general education or secondary vocational education), or high (higher

vocational education or university). Household income was categorized into low income, middle income or high income. Cardio-metabolic disease was defined as presence of cardiovascular disease and/or presence of type 2 diabetes mellitus. History of cardiovascular disease was defined as having a history of coronary heart diseases (myocardial infarction, revascularization, coronary artery bypass graft surgery or percutaneous coronary *intervention*) and was verified from the medical records of the general practitioner. Baseline diabetes mellitus was defined as having a serum glucose level ≥11mmol/L or use of glucose lowering drugs. Chronic kidney disease was defined as having an estimated glomerular filtration rate (eGFR) <60mL/min/1.73m^2. Metabolic syndrome was defined according to the interim definition proposed by Alberti and colleagues(85). Physical activity was assessed with an adapted version of the Zutphen Physical Activity Questionnaire(134). Every activity mentioned in the questionnaire was attributed a MET-value according to the 2011 Compendium of Physical Activities(135). The questionnaire contained questions on walking, cycling, gardening, diverse sports, hobbies and on housekeeping. Total time spend on physical activity was calculated as the sum of minutes per week for each type of activity. Food intake was assessed at baseline using a semi-quantitative food frequency questionnaire. To assess overall dietary quality, the Dutch Healthy Diet (DHD) index was used(93), which is a continuous score that represents compliance to the Dutch Guidelines for a Healthy Diet. The following DHD-index components were available and included in the DHD index in this study: intake of vegetables, fruits, polyunsaturated fatty acids, saturated fatty acids, trans fatty acids, fish, dietary fiber, alcohol and sodium. Dates when the blood was drawn were categorized into summer, autumn, winter and spring according to the Dutch standard seasons.

3.2.2.6. Study population

Participants from the third visit of the first cohort of the Rotterdam Study (1997-1999) were eligible for inclusion into this analysis (N=4,787). Serum 25(OH)D data were available for 3,828 of these participants. During 7 years of follow up 337 participants from the initial population died and 1,333 participants had no dual-energy X-ray absorptiometry (DXA) scan examination, leaving 2,158 participants for our final analysis.

3.2.2.7. Statistical analysis

Descriptives are presented as mean ± standard deviation (SD) unless indicated otherwise. Multivariable linear regression was used to examine whether vitamin D status was independently associated with body fat percentage, android fat percentage, gynoid fat percentage, android fat/gynoid fat ratio and lean mass percentage. In the first model, we calculated the age and gender adjusted regression coefficients and their 95% confidence interval (CI). Then the second model was built for all outcomes, which was further adjusted for season when the blood was drawn, total alcohol intake (continuous), total physical activity (continuous), smoking status, education, income, DHD-index (continuous), prevalent cardio-metabolic disease (cardiovascular diseases and diabetes mellitus) and presence of chronic kidney disease. Finally, the third model was built depending on the outcome of interest; for the analysis concerning total body fat and lean mass percentage as the outcomes, we additionally adjusted for height (continuous), weight change (continuous) during the follow up and lean mass or total fat mass in kilogram respectively; whereas for the analyses concerning android fat percentage and gynoid fat percentage, and android fat/gynoid fat ratio as outcomes, we additionally adjusted for BMI measured at the third visit (the baseline visit for this analysis). We chose confounders based on a 10% change in regression coefficient (95) and we consulted previously published literature on the topic. We tested for possible nonlinear effects by adding a quadratic term of 25(OH)D into the model. To test for effect modification by age, gender, cardio-metabolic diseases or metabolic syndrome, the product term of 25(OH)D with each one of the potential effect modifiers separately was added as independent variable to the models. Results were stratified if an interaction term was significant at a *P-value* lower than 0.05.

To examine the association between serum 25(OH)D and repeatedly measured anthropometric measurements (waist circumference, waist to hip ratio and BMI) we fitted linear regression models using generalized estimating equations (GEE) with exchangeable correlation structure adjusting for the within-subject correlations due to the repeated measurements of anthropometric measurements in the same individual(136). To account for the time difference in measurements at the three time points, a time variable was entered in the model and coded as 1, 6 and 13.

To adjust for potential bias associated with missing data, a multiple imputation procedure was performed (N= 5 imputations). For the pooled regression coefficients (β) and 95% CIs we used Rubin's method (137). All analyses were performed using IBM SPSS Statistics 20. A *P*-value lower than 0.05 was considered statistically significant.

3.2.3. Results

The mean (± SD) age of the participants was 70.5 ± 5.9 years; mean time difference between the 25(OH)D measurements and DXA measurements was 4.6 years; 19% of study participants had adequate vitamin D status (25(OH)D≥75 nmol/L), 29% had vitamin D insufficiency (50-75 nmol/L) and 52% had vitamin D deficiency (<50 nmol/L) (Table 3.2.1).

3.2.3.1. Vitamin D, total body fat and lean mass

In the age, gender and covariates adjusted model, subjects with vitamin D deficiency had a 1.29 unit higher body fat percentage than participants with adequate vitamin D status (95%CI=0.55, 2.04) (Table 3.2.2). When analyzed continuously an inverse association was observed between serum 25(OH)D and body fat percentage: a 10 nmol/L increase in serum 25(OH)D was associated with a 0.22 unit lower body fat percentage (95%CI= -0.33, -0.11) in model 2 (adjusted for confounders) (Table 3.2.2). In contrast, a 10 nmol/L increase in serum 25(OH)D was associated with a 0.23 higher lean mass percentage (95%CI=0.11, 0.35) in model 2 (Table 3.2.2), which did not remain significant after further adjustment for height, weight change and total fat mass in kilogram (model 3). A higher serum 25(OH)D was associated with a lower fat mass/lean mass ratio (Table 3.2.2).

Table 3.2.1. Selected characteristics of study population at baseline.

	(N=2158)	% Missing
Age	70.51 ± 5.89	0
Female %(n)	56.9 (1227)	0
Smoking status (current smokers) %(n)	18.7 (338)	0.5
Physical activity (total MET hours)	87.67 ± 44.92	0.6
Alcohol intake (g/day)*	2.86 (12.43)	0.5
Education Level		0.9
Low % (n)	43.4 (937)	
Medium % (n)	44.8 (966)	
High % (n)	11.8 (255)	
Income		10.0
Low % (n)	16.2 (350)	
Medium % (n)	41.1 (886)	
High % (n)	42.7 (922)	
BMI 3rd visit (kg/m^2)	26.85 ± 3.83	0.4
BMI 4th visit (kg/m^2)	27.42 ± 4.02	1.8
BMI 5th visit	27.45 ± 4.22	42.8
WC 3rd visit	93.20 ± 11.38	0.37
WC 4th visit (kg/m^2)	93.55 ± 11.72	0.32
WC 5th visit	92.31 ± 12.40	43.0
WHR 3rd visit (kg/m^2)	0.92 ± 0.1	0.37
WHR 4th visit (kg/m^2)	0.91 ± 0.09	0.32
WHR 5th visit	0.90 ± 0.09	43.0
Weight change (%)	-1.46 ± 5.71	2.0
DHDI	48.86 ± 10.21	11.8
Cardio-metabolic disease %(n)	24,8 (535)	0
Metabolic Syndrome %(n)	40.0 (864)	1.8
Chronic Kidney Disease %(n)	11.7 (253)	0.2
Vitamin D (nmol/L)	52.63 ± 25.39	0
Deficient %(n)	52.3 (1129)	
Insufficient %(n)	28.8 (621)	
Adequate %(n)	18.9 (408)	

DHDI, Dutch Health Diet Index; BMI, Body mass index; WC, Waist circumference; WHR, Waist hip

Values presented are mean ± SD;*Median (interquartile range).

Table 3.2.2. Serum 25(OH)D and body composition (2158 subjects).

Vitamin D	Total fat (%) β (95% CI)			Lean mass (%) β (95% CI)			Total fat mass/ Lean mass ratio		
	MODEL 1	MODEL 2	MODEL 3	MODEL 1	MODEL 2	MODEL 3	MODEL 1	MODEL 2	MODEL 3
Deficient	1.56 (0.81, 2.31)	1.29 (0.55, 2.04)	1.35 (0.63, 2.06)	-1.55 (-2.33, -0.78)	-1.30 (-2.07, -0.53)	0.008 (-0.33, 0.35)	0.04 (0.02, 0.06)	0.03 (0.02, 0.05)	0.04 (0.02, 0.06)
Insufficient	0.62 (-0.18, 1.42)	0.35 (-0.44, 1.14)	0.44 (-0.32, 1.20)	-0.42 (-1.25, 0.40)	-0.17 (-0.99, 0.65)	0.03 (-0.33, 0.39)	0.01 (-0.01, 0.03)	0.01 (-0.01, 0.03)	0.01 (-0.01, 0.03)
Adequate	reference	reference	reference	reference	reference	reference	reference	reference	reference
P-trend	<0.001	<0.001	<0.001	<0.001	<0.001	0.999	<0.001	<0.001	<0.001
Continuous (per 10 unit increase)	-0.26 (-0.37, -0.15)	-0.22 (-0.33, -0.11)	-0.24 (-0.35, -0.13)	0.27 (0.15, 0.38)	0.23 (0.11, 0.35)	-0.01 (-0.06, 0.04)	-0.01 (-0.01, -0.00)	-0.01 (-0.01, -0.00)	-0.01 (-0.01, -0.00)

Model 1: adjusted for age and gender; Model 2: model 1 additionally adjusted for season when the blood was drawn, chronic kidney disease, smoking status, alcohol intake, physical activity, highest education attained, household income, Dutch Healthy Diet-index, prevalent cardio-metabolic diseases; Model 3: model 2 additionally adjusted for height, weight change measured at the third visit and lean mass or total body fat in kilogram according to the analysis.

Additionally we performed the same analysis using absolute total body fat and absolute lean mass (data not shown). The results remained unchanged.

Furthermore, we observed an interaction between 25(OH)D and presence of cardio-metabolic diseases on total body fat percentage (P *interaction*=0.03). The inverse association between 25(OH)D and body fat percentage was present among subjects without cardio-metabolic diseases (β=1.70, 95%CI=0.87, 2.53 for deficiency vs. adequate vitamin D status, *P* for trend < 0.001), but not among participants with cardio-metabolic disease (Figure 3.2.1). No effect modification by age, gender and metabolic syndrome was present (P *interaction*>0.05).

3.2.3.2. Vitamin D and regional body fat distribution

There was no consistent association between vitamin D status and android fat percentage, gynoid fat percentage or android/gynoid fat ratio after adjustment for confounders (Table 3.2.3). Continuous analyses showed a weak inverse association between 25(OH)D and android fat percentage and android/gynoid ratio, but this was not independent of BMI.

Figure 3.2.1. Serum 25(OH)D and total body fat percentage stratified by presence of cardio-metabolic disease*.
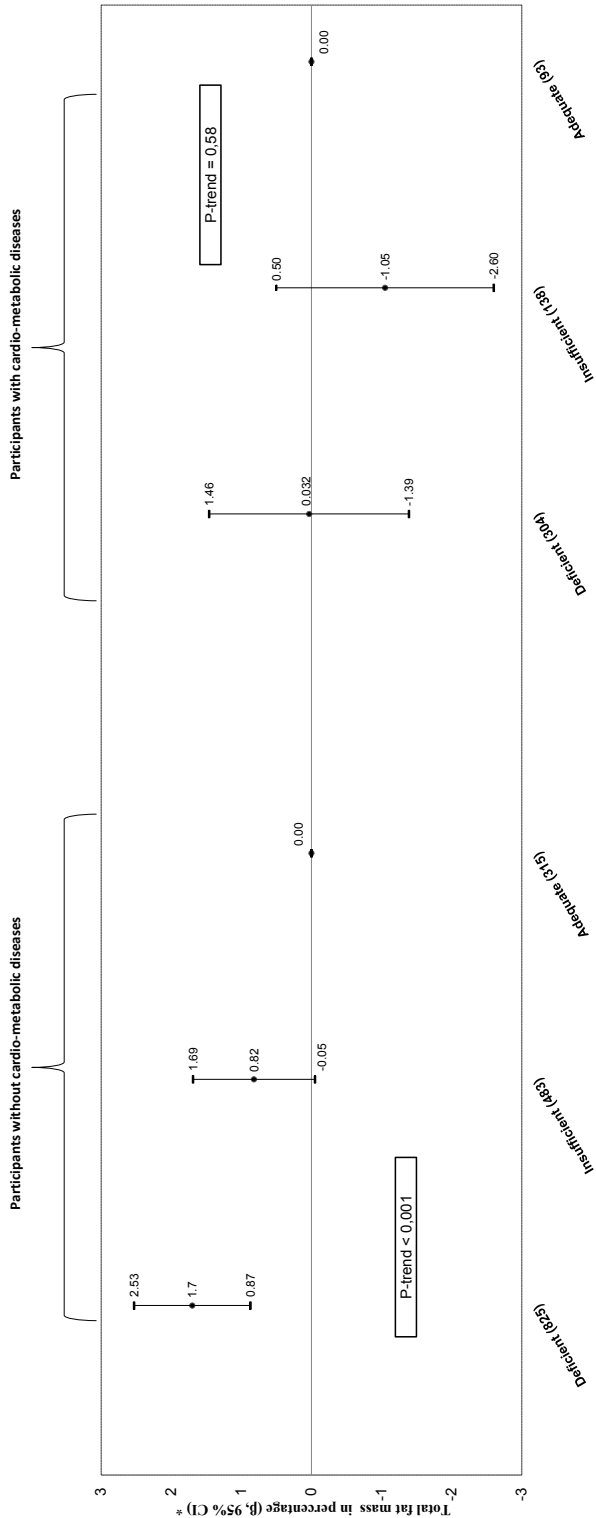
*P Interaction=0.03 for serum 25(OH)D X cardio-metabolic disease.

Also, a potential quadratic relation was observed in the association between serum 25(OH)D and android fat and android/gynoid ratio suggesting a non-linear relation (Table 3.2.3).

Table 3.2.3. Serum 25(OH)D and regional fat distribution (2158 subjects).

Vitamin D	Android fat (%) β (95% CI)			Gynoid fat (%) β (95% CI)			Android fat/Gynoid fat ratio β (95% CI)		
	MODEL 1	MODEL 2	MODEL 3	MODEL 1	MODEL 2	MODEL 3	MODEL 1	MODEL 2	MODEL 3
Deficient	0.18 (0.01, 0.35)	0.17 (-0.00, 0.34)	0.08 (-0.08, 0.24)	-0.08 (-0.29, 0.14)	-0.05 (-0.26, 0.17)	0.09 (-0.11, 0.30)	0.02 (-0.00, 0.03)	0.01 (-0.01, 0.03)	0.002 (-0.02, 0.02)
Insufficient	0.10 (-0.08, 0.28)	0.12 (-0.06, 0.29)	0.11 (-0.06, 0.28)	-0.02 (-0.25, 0.21)	0.00 (-0.23, 0.23)	0.01 (-0.20, 0.22)	0.01 (-0.01, 0.03)	0.01 (-0.01, 0.03)	0.005 (-0.01, 0.02)
Adequate	reference	reference	reference	reference	reference	reference	reference	reference	reference
P-trend	0.04	0.06	0.47	0.43	0.54	0.30	0.08	0.15	0.91
Continuous (per 10 unit increase)	-0.03 (-0.06, -0.01)	-0.03 (-0.06, -0.01)	-0.01 (-0.04, 0.01)*	0.02 (-0.02, 0.05)	0.01 (-0.02, 0.05)	-0.01 (-0.04, 0.02)	0.00 (-0.01, -0.00)	0.00 (-0.01, -0.00)	-0.00 (-0.00, 0.00)*

Model 1: Adjusted for age and gender; Model 2: Model 1 additionally adjusted for season when the blood was drawn, chronic kidney disease, smoking status, alcohol intake, physical activity, highest education attained, household income, Dutch Healthy Diet-index, prevalent cardio-metabolic diseases, weight change; Model 3: Model 2 additionally adjusted for body mass index measured at the third visit;*Quadratic term significant, $P<0.05$.

3.2.3.3. Additional analysis

We observed an inverse association between vitamin D status and BMI; in the fully adjusted model, a 10 nmol/L increase in serum 25(OH)D was associated with a 0.16 unit decrease in BMI (data not shown). When stratified by metabolic syndrome status (P *interaction*=0.001) a decrease in BMI with increasing concentrations of serum 25(OH)D was observed only among subjects with metabolic syndrome (P for trend=0.001), but not in those without (data not shown). The analysis of serum 25(OH)D and waist circumference showed a statistically significant inverse association while in the case of serum 25(OH)D and waist to hip ratio this inverse association was weaker and not independent of BMI (data not shown).

3.2.4. Discussion

We found adequate vitamin D status to be inversely associated with total body fat mass percentage. Our results suggest a BMI dependent role for vitamin D status in the regional distribution of body fat in the elderly.

3.2.4.1. Comparison with other studies

In our study, it was observed that low concentrations of serum 25(OH)D are associated with a higher body fat percentage. Several previous studies also investigated this association and found similar results, however, most of them were of cross-sectional design. For example, a cross-sectional study by *Kremer et al. (2008)*(138) found that vitamin D insufficiency was associated with increased body fat in young women. Similarly, a study by *Snijder et al. (2005)*(139) found an inverse association between total body fat and serum 25(OH)D concentrations in older men and women. This latter study was performed in a population similar to ours (mean age ±75 years), but with only 453 participants included in the analysis. However, the conclusions coming from intervention studies are inconsistent. For example, *Salehpour et al. (2012)*(140) found that supplementation with vitamin D3 for 12 weeks in 77 overweight and obese healthy subjects led to a statistically significant decrease in body fat in the intervention group compared to placebo group. Conversely, *Wamberg et al. (2013)* (141) found that increasing serum 25(OH)D concentrations with cholecalciferol treatment during 26 weeks in 52 obese subjects had neither an effect on body fat, nor specifically on subcutaneous or visceral adipose tissue. However, not many studies investigated the association between vitamin D status and regional fat distribution specifically in the elderly. We found a cross-sectional study by *Moschonis et al. (2009)* reporting on vitamin D status as an outcome and body composition, including regional fat mass (measured by calibrated Lange skinfold caliper) as an exposure. This study was performed in 112 non-osteoporotic, overweight, postmenopausal women (mean age 60 years)(130) and showed inverse associations of vitamin D status with total body fat and with all measures of regional body fat mass (assessed as arms fat mass percentage, legs fat mass percentage and trunk fat mass percentage) and a positive association with fat-free mass. Compared to this study by Moschonis and colleagues(130) we had a bigger sample but we did not find a consistent association between vitamin D status and gynoid fat but we did find an association between vitamin D status and android fat even though it was not independent of BMI. However, our findings of an inverse association between vitamin D status and total body fat are in line with the results reported by *Moschonis et al.*(130).

3.2.4.2. Potential mechanisms

Even though many studies report associations between low vitamin D status and obesity, the underlying mechanisms and the direction of this association are not fully understood. There are several pathways by which vitamin D and body composition may be connected in both directions. Firstly, obese people have commonly lower serum 25(OH)D concentrations compared to lean people(124). This might be a result of decreased bioavailability of vitamin D because of sequestration of vitamin D by the adipose tissue(126); dilution of vitamin D in the large fat mass of obese people (142); increased catabolism of vitamin D in the adipose tissue(143); decreased synthesis of 25(OH)D in the liver(144); reduced sun-exposure (145); or decreased hepatic synthesis of 25(OH)D caused by a negative feedback loop of $1,25(OH)_2D$ and parathyroid hormone (PTH)(146)**.** Also, clearance of vitamin D might be increased by inflammation related to obesity(147). In addition, vitamin D might have anti-inflammatory effects(148) and low vitamin D has been associated with visceral adiposity that is associated with inflammation (149). Furthermore, a study using bidirectional Mendelian randomization approach found higher BMI leading to lower 25(OH)D but not the other way around(132). Secondly, it has been suggested that low serum 25(OH)D concentrations lead to increase in PTH levels as a normal physiological response, which in turn may favor the lipid storage metabolism (150, 151). In vitro studies found that receptors for vitamin D (VDRs) and the enzyme 1-α-hydroxylase needed for production of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), are both present in adipocytes(110, 143, 152) suggesting a role of $1,25(OH)_2D$ in metabolism of the adipose tissue. Also, $1,25(OH)_2D$ was found to regulate expression of some genes involved in genesis of adipose tissue(88). Thirdly, it was found that skeletal muscles act as a functional store of 25(OH)D - a proportion of circulating 25(OH)D binds to vitamin D binding protein in muscle cells and can be released back to the circulation. This retention by muscle cells protects the 25(OH)D from degradation by the liver(125). Additionally, active $1,25(OH)_2D$ bound to VDR receptor in muscle tissue activates muscle growth and improves muscle function and may thereby influence overall body composition(127-129). With this in mind, we speculate that with the increase in body fat percentage the concentration of 25(OH)D in the circulation decreases, subsequently leading to a decrease in the functional store of 25(OH)D in the skeletal muscles. This decrease in the functional store of

25(OH)D might in turn lower serum 25(OH)D concentration that might contribute to more unfavorable body composition.

3.2.4.3. Interaction with cardio-metabolic diseases

We found an interaction between 25(OH)D, body composition and cardio-metabolic diseases. Several studies have shown vitamin D status to be associated with metabolic syndrome, cardiovascular disease and type 2 diabetes mellitus(6, 153). Also, body fat and BMI are associated with an increased risk of cardiovascular disease, metabolic syndrome and type 2 diabetes mellitus(122).

We found an association between 25(OH)D and total fat percentage only in subjects free of cardio-metabolic disease. This finding can be explained in several ways. Firstly, it might be possible that people with cardio-metabolic disease at baseline changed their life-style after diagnosis(154). This change might have resulted in for example weight loss or improvement of vitamin D status and diluting any association between 25(OH)D and body composition. Secondly, it might be that due to inflammatory processes that may come along with cardiovascular disease(155) and type 2 diabetes mellitus(156) the clearance of vitamin D is increased (147) diluting the association with 25(OH)D in individuals with cardiovascular disease and type 2 diabetes mellitus. In contrast, we only found a significant association between 25(OH)D and BMI in those with a metabolic syndrome suggesting that a complex interaction between 25(OH)D, body composition and cardio-metabolic diseases may exist and it needs further elucidation.

3.2.4.4. Strengths and limitations

Main strengths of our study are the prospective, population-based design, large sample size, use of accurate measures of body fat, as well as collection of numerous population characteristics. However, it is also important to mention the limitations of our study. There is a time difference between measurements of 25(OH)D and measurements of body composition, and changes in serum 25(OH)D could have occurred during this time. Another limiting factor to the current investigation is that body fat was measured only once. However, where appropriate we adjusted our analyses for baseline BMI, which correlates well with total fat (partial Pearson correlation coefficient 0.87) and our results were also confirmed by BMI that was repeatedly

measured. Another limitation is the fact that we did not have PTH levels measured and therefore could not test if the observed association between 25(OH)D and body fat might be explained by PTH.

3.2.4.5. Main conclusion and future directions

Lower serum 25(OH)D concentrations were associated with higher total body fat percentage, which was independent of BMI. However, the association between 25(OH)D and fat distribution in this population was mainly explained by BMI. The association between 25(OH)D and body composition might be modified by cardio-metabolic disease. Further studies are needed to confirm our findings and to clarify the underlying mechanisms.

Chapter 4
Vitamin D and Cardio-vascular Health

4.1. Vitamin D Status and Atrial Fibrillation in the Elderly: The Rotterdam Study

Anna Vitezova, Natasha S. Cartolano, Jan Heeringa, M. Carola Zillikens, Albert Hofman, Oscar H. Franco, Jessica C. Kiefte-de Jong

Abstract

Objective: Atrial fibrillation (AF) is the most common chronic arrhythmia and it increases the risk of cardiovascular morbidity and mortality. Still there is not a complete understanding of its etiology and underlying pathways. Vitamin D might regulate renin-angiotensin-aldosterone system and might be involved in inflammation, both implicated in the pathophysiology of AF. The objective of this work was to investigate the association between vitamin D status with the risk of AF in the elderly.

Methods: This study was conducted within the Rotterdam Study, a community-based cohort of middle-aged and elderly participants in Rotterdam, The Netherlands. We had 3,395 participants who were free of AF diagnosis at the start of our study and who had vitamin D data available. We analyzed the association between serum 25-hydroxivitamin D (25(OH)D) and incidence of AF using

Cox regression models. Vitamin D deficiency was defined as serum 25(OH)D concentrations <50nmol/l, insufficiency between 50nmol/l and 75nmol/l, while serum 25(OH)D concentrations equal to and above 75nmol/l were considered as adequate.

Results: After mean follow-up of 12.0 years 263 (7.7%) participants were diagnosed with incident AF. Vitamin D status was not associated with AF in any of the 3 multivariate models tested (model adjusted for socio-demographic factors and life-style factors: HR per 10 unit increment in serum 25(OH)D 0.96, 95% CI: 0.91-1.02; HR for insufficiency: 0.82, 95%CI: 0.60-1.11,and HR for adequate status: 0.76, 95%CI: 0.52-1.12 compared to deficiency).

Conclusion: This prospective cohort study does not support the hypothesis that vitamin D status is associated with AF.

4.1.1. Introduction

Atrial fibrillation (AF) is the most common chronic arrhythmia and it has a significant effect on morbidity and mortality (157-160). Since AF is mainly a disease of the elderly, the prevalence of this arrhythmia is expected to increase due to aging populations (161), which has a major impact on healthcare expenditure (162, 163).

Despite extensive research on AF, there is still not a complete understanding of its etiology and underlying pathways. Risk factors for AF include older age, male sex, hypertension, valvular heart disease, congestive heart failure, ischemic heart disease and hyperthyroidism (164-166).

Vitamin D is associated with calcium metabolism and bone health. However, vitamin D receptors (VDRs) have been found in cells throughout the body, such as cardiomyocytes and endothelial cells (167), suggesting this hormone has additional functions in the human body. Furthermore, vitamin D deficiency is highly prevalent in Western populations and has been considered a global health issue, especially in the elderly (117, 168-172).

Amongst different mechanisms through which vitamin D is involved in human health, vitamin D can regulate the renin-angiotensin-aldosterone system (RAAS) activity and has also been involved in the inflammatory processes, both implicated in the pathophysiology of AF, therefore suggesting a potential role of vitamin D in the etiology of AF. Nevertheless, only a few studies have analyzed the possible association between vitamin D deficiency and AF. Two cohorts investigating the association between vitamin D status and atrial fibrillation reported opposite results (173, 174). Thus, it remains unknown whether there is an association between AF and vitamin D status.

The aim of our study was to investigate the association between serum levels of 25-hydroxyvitamin D (25(OH)D) with the risk of AF using data from a community-based cohort study of middle aged and elderly participants.

4.1.2. Methods
4.1.2.1. Study Design

This study was conducted among individuals from The Rotterdam Study, a population based prospective cohort study investigating frequency and determinants of disease in the middle aged and the elderly. The Rotterdam

Study started in 1990 when 10,275 inhabitants of the Ommoord district of Rotterdam, The Netherlands, aged 55 years and older, were invited to participate in the study. Of these 7,983 (78%) provided written consent to participate. They were interviewed at home and subsequently examined in the research center from 1990 to 1993. The examinations were repeated every 3-4 years. For this study, the third examination round (1997-1999) was considered as baseline when 25-hydroxivitamin D (25(OH)D) levels were assessed. All participants gave informed consent and the study was approved by the medical ethics committee of the Erasmus Medical Center, Rotterdam. The study is described in more details elsewhere (15).

4.1.2.2. Study Population

From 7,983 participants enrolled to the first examination round of The Rotterdam Study, 3,828 had data on serum 25(OH)D available at the examination round between 1997-1999. Of these, 434 participants were excluded because they had no data on AF, had prevalent AF or did not have follow-up information recorded. The remaining study population consisted of 3,395 participants of The Rotterdam Study.

4.1.2.3. Assessment of 25(OH)D

Serum 25(OH)D concentrations were assessed in 3,828 participants of the Rotterdam Study. The measurements were performed with a electrochemiluminescence immunoassay (COBAS, Roche Diagnostics GmbH, Germany). Test sensitivity was 10nmol/L, serum 25(OH)D concentrations ranged from 7.5nmol/L to 175nmol/L, the within-run accuracy was less than 7.8% and intermediate precision accuracy was less than 13.1%. Serum 25(OH)D concentrations were analyzed both as a continuous variable and as a categorical variable according to cut-offs proposed by M. Holick (6). Participants were categorized as being vitamin D deficient (<50 nmol/L), insufficient (50-75 nmol/L) or having adequate vitamin D status (≥75 nmol/L) according to their serum 25(OH)D concentrations.

4.1.2.4. Assessment of Atrial Fibrillation

Between 1997-1999, AF was collected using 10-second, 12-lead electrocardiography (ECGs) recorded with an ACTA electrocardiograph (ESaOte, Florence, Italy) and by screening of general practitioners (GPs) records from Ommoord region. During follow-up, ECGs were performed during the re-examinations every 3-4 years. In addition, GPs weekly updated information on AF based on their own records and hospital discharge letters. Information on hospital discharge was also collected from a national registration system (Landelijke Medische Registratie system). A diagnosis of AF was only accepted when it was supported by a ECG diagnosis. The ECGs done at baseline and during follow-up were stored digitally, and analyzed by the modular ECG analysis system (MEANS), which has high specificity (99.5%) and high sensibility (96.6%) for detection of arrhythmias (175, 176). Two research physicians, who were blinded for the MEANS result, verified all ECGs with a diagnosis of atrial fibrillation, atrial flutter or any other arrhythmia done by the computer system (166). In case of disagreement, diagnosis of a cardiologist was considered as decisive. In this study no distinction was made between atrial fibrillation and atrial flutter (177, 178). A participant was not considered to have AF if a transient AF occurred during myocardial infarction or during cardiac operative procedures. If AF occurred during the process of dying and was not the cause of death, the person was not considered as a case and was censored on the date of AF diagnosis.

4.1.2.5. Assessment of Confounders

Socio-demographic, lifestyle and medical information was assessed during a home interview.

Education was assessed using the highest level attained. Low education level was considered as primary education only or primary education with uncompleted higher education. Income was assessed as net income per year. Income was categorized as high or low income, low income was considered as less than 35.999 euros per year and high income above or equal to 35.999 euros per year. Smoking was assessed by using questions on current and past smoking of cigarettes, cigars, or pipe. The information on medication use included information on lipid lowering and blood pressure lowering drugs. Information on diet were obtained through a 170-item validated

semiquantitative food frequency questionnaire (SFFQ)(179). From that an overall healthy diet score representing adherence to the Dutch dietary guidelines was calculated as described previously by van Lee et al (93). Physical activity was assessed using a validated adapted version of Zutphen Physical Activity Questionnaire(134). Questions on housekeeping activities were added to the original questionnaire that already included questions on walking, cycling, gardening, hobbies, and diverse sports.

During the examination visit (1997-1999) physiological measurements were performed as well as blood collection. Blood pressure (BP) was measured twice on the right arm in the sitting position with a random zero sphygmomanometer. The average of two consecutive measurements was used. Body mass index (BMI) was calculated by dividing weight in kilograms by height in meters squared. Waist circumference was measured at the level midway between the lower rib margin and the iliac crest with the participant in a standing position. Diabetes mellitus was considered to be present if fasting (8–14 h) glucose value was 7.0 mmol/l or higher or a random or postload glucose value was 11.1 mmol/l or higher in any of the examination rounds up to 1997-1999 or if a participant used anti-diabetic medication or diet treatment and was registered by a general practitioner as having diabetes. The estimated glomerular filtration rate (eGFR) was computed according to the simplified Modification of Diet in Renal Disease (MDRD) formula (180), chronic kidney disease was defined as a eGFR below 60 mL/min/1.73 m^2. A history of stroke at study entrance was defined as a self-reported stroke that was confirmed by medical records. Subsequently up to 1997-1999 stroke was assessed from the follow up information obtained from the GP's files. At study entrance history of myocardial infarction (MI) was positive if MI was reported during baseline interview and confirmed by hospital admission and/or MI was present on the ECG. During follow up until 1997-1999 MI was confirmed using medical records (181)**.** At the entrance to the Rotterdam Study cases of heart failure were classified according to definition by the European Society of Cardiology based on following: 1) at least two symptoms of heart failure present (shortness of breath, swelling of ankles, or pulmonary crepitation), 2) use of medication prescribed for heart failure (diuretics, glycosides, or ACE inhibitors) together with cardiovascular disease (182-184). During follow up until 1997-1999 the information on heart failure was

obtained from general practitioner's records (184). To identify patients potentially eligible for use of vitamin D medication we used diagnosis of osteoporosis assessed by dual-energy x-ray absorptiometry as a proxy.

4.1.2.6. Statistical analyses

Cox proportional hazards regression was used to relate serum 25(OH)D concentrations with incident atrial fibrillation. Proportional hazard assumption was tested including time variable x serum 25(OH)D and time variable x cut-offs of serum 25(OH)D into the model. Follow-up duration in years was used as time variable. The participants were followed from date of the third visit to the research center of the Rotterdam Study (1997-1999) to the end of follow up (March 22, 2010). Subjects were censored when they died or were lost to follow up. Prevalent cases of atrial fibrillation between study entrance and 1999 were excluded from the analyses. Multiple imputation procedure was performed to reduce bias from missing data (0 – 22%). Ten imputations were done using Markov chain Monte Carlo method (S1 Table). Potential confounders were chosen based on change in the effect estimates and/or based on the literature (95, 185, 186). Three multivariate models were created for Cox regression analyses. The first model was the crude model, adjusted for age and gender. The second model was adjusted additionally for the following confounders: net household income, highest education level attended, BMI, physical activity, diet quality score, current smoking, and season and year when the blood was drawn. The third model was additionally adjusted for potential mediators: use of serum lipid lowering drugs, use of BP lowering drugs, systolic BP, and prevalent diseases (cardiovascular diseases including coronary heart disease, heart failure, and stroke; chronic kidney disease; diabetes mellitus). Stratification was performed by age, gender, smoking, and use of BP lowering drugs. Also, interaction terms of covariate x serum 25(OH)D and covariate x dummy variables of serum 25(OH)D cut-offs were created for assessing possible effect modification by these variables. Additional analyses included testing for potential confounding or modification by serum calcium; firstly the models were adjusted for serum calcium concentrations; secondly, the interaction between serum calcium and serum 25(OH)D was tested; thirdly the analyses were performed excluding participants with hypercalcaemia. Sensitivity analyses were performed by

censoring cases of coronary heart disease (CHD) that occurred before the onset of AF and excluding participants potentially eligible for receiving vitamin D supplementation (i.e. subjects with osteoporosis). Data analyses were performed using SPSS version 21.0 (SPSS IBM, New York, USA).

4.1.3. Results

Baseline characteristics of the study population are summarized in Table 4.1.1. The population for analysis consisted of 3,395 participants. After a mean follow-up of 12.0 years 263 (7.7%) participants were diagnosed with incident atrial fibrillation. The median (range) age of the study population was 71(44) years and 2,007 (59.1%) participants were female. The mean (SD) 25(OH)D concentration was 49.3 (25.4) nmol/l. According to cutoffs, 57% of the study population had deficiency (<50nmol/l), 27% had insufficiency (50-75nmol/l) and 16% had adequate vitamin D status (≥75nmol/l). Individuals with vitamin D deficiency or insufficiency were more often female, more likely to be older, to have lower education degree, lower income, higher BMI, and more often had diabetes mellitus and cardiovascular disease (CVD) (Table 4.1.1). The percentage of missing data varied from 0 percent for variables like age and gender up to 12.9 percent for data on physical activity (data not shown).

After adjustment for age and gender no significant association was found between vitamin D status and atrial fibrillation, both when serum 25(OH)D was analyzed continuously (HR per 10 unit increment in serum 25(OH)D: 0.95, 95% CI: 0.90-1.01) or by cutoffs (HR for insufficient levels: 0.79, 95%CI: 0.58-1.07 and HR for adequate levels: 0.74, 95%CI: 0.50 – 1.08, compared to deficient levels). The results remained non-significant after further adjustments in model 2 (HR per 10 unit increment in serum 25(OH)D: 0.96, 95% CI: 0.91-

Table 4.1.1. Baseline characteristics of study population according to Vitamin D status.

	Total	Deficient <50nmol/l	Insufficient 50-75nmol/l	Adequate ≥75nmol/l
Individuals N(%)	3395 (100)	1939 (57.1)	909 (26.8)	547 (16.1)
Age (years)**	71.0 (44.4)	72.8 (44.3)	69.3 (29.2)	68.3 (27.1)
Females N(%)	2007 (59.1)	1314 (67.8)	464 (51)	229 (41.9)
Serum 25(OH)D (nmol/l) *	49.3 (25.4)	31.4 (10.7)	61.1 (7)	93.1 (15.4)
Follow up time (years) **	12 (16)	11 (16)	12 (16)	13 (16)
AF incidence N(%)	263 (7.7)	167 (8.6)	61 (6.7)	35 (6.4)
Low education [#] N(%)	1006 (29.6)	646 (33.3)	232 (25.5)	128 (23.4)
Diet quality score (DHDI) *[#]	48.7 (9.6)	48.9 (9.5)	48.8 (9.6)	47.9 (9.6)
Current smokers N(%)	557 (16.4)	333 (17.2)	128 (14.1)	86 (15.7)
BMI (kg/m²)*	26.9 (3.9)	27.2 (4.2)	26.4 (3.4)	26.3 (3.4)
Waist circumference (cm)*	93.4 (11.4)	93.9 (11.9)	92.7 (10.6)	92.8 (10.6)
Systolic BP (mmHg)*	143 (21)	145 (21)	142 (20)	141 (20)
Diastolic BP (mmHg)*	75 (11)	75 (11)	75 (11)	76 (10)
Use of BP lowering drugs N(%)	940 (27.7)	600 (30.9)	227 (25)	113 (20.7)
Presence of DM N(%)	487 (14.3)	334 (17.2)	105 (11.6)	48 (8.8)
Presence of CVD N(%)	495 (14.6)	315 (16.2)	116 (12.8)	64 (11.7)
Presence of CKD N(%)	479 (14.1)	302 (15.6)	101 (11.1)	77 (14.1)
Lipid lowering drugs N(%)	512 (15.1)	305 (15.7)	127 (14)	80 (14.6)
Hypercalcaemia N(%)	61 (1.8)	44 (1.1)	11 (1.2)	6 (2.3)
Hypocalcaemia N(%)	21 (0.6)	18 (0.9)	2 (0.2)	1 (0.2)

BMI, body mass index; BP, blood pressure; DM, diabetes mellitus; CVD, cardiovascular disease (considered as the presence of coronary artery disease, heart failure or stroke); CKD, chronic kidney disease; *Mean (Standard Deviation); **Median (Range); #Data collected prior to serum 25(OH)D assessment; Notes: prevalent cases of AF were excluded from the analysis; Imputed data are shown.

1.02, HR for insufficient levels: 0.82, 95%CI: 0.60-1.11, HR for adequate levels: 0.76, 95%CI: 0.52 – 1.12) (Table 4.2.2). Additional adjustment for potential mediators did not change the results (Table 4.2.2).

Table 4.2.2. Serum 25(OH)D and incidence of atrial fibrillation.

25(OH)D cutoffs	Model 1 HR (95% CI)	Model 2 HR (95% CI)	Model 3 HR (95% CI)
Deficient	reference	reference	Reference
Insufficient	0.79 (0.58, 1.07)	0.82 (0.60, 1.11)	0.85 (0.62, 1.15)
Adequate	0.74 (0.50, 1.08)	0.76 (0.52, 1.12)	0.81 (0.55, 1.20)
25(OH)D continuous±	0.96 (0.90, 1.01)	0.96 (0.91, 1.02)	0.97 (0.92, 1.03)

Model 1 adjusted for age and gender; Model 2 adjusted for age, gender, income#, education#, BMI, physical activity, diet quality score#, smoking status and season and year when the blood was drawn; Model 3 adjusted for all covariates in Model 2 plus use of serum lipid lowering drugs, use of blood pressure lowering drugs, systolic blood pressure and baseline diseases: cardiovascular diseases (coronary heart disease, heart failure, stroke), chronic kidney disease and diabetes mellitus; ±Results shown per 10 units of serum 25(OH)D (nmol/l); #Data collected prior to serum 25(OH)D assessment; Notes: Prevalent cases of AF were excluded from the analysis. Imputed data are shown.

We tested the interaction of serum 25(OH)D with gender, age, current smoking and use of BP lowering drugs. None of the interaction terms entered in the model were statistically significant. We also performed stratifications based on these covariates, but no significant associations were seen in these strata (data not shown). Additional adjustment for serum calcium did not change the results (data not shown). We also tested for the interaction between serum calcium and serum 25(OH)D but this was also not significant (data not shown). Excluding participants with hypercalcaemia did not change the results significantly (data not shown).

Furthermore, we did sensitivity analyses. Firstly, we censored cases of CHD that occurred before the incidence of AF. Secondly, we excluded participants on therapy with vitamin D. Neither of the two sensitivity analyses markedly changed the results (data not shown).

4.1.4. Discussion

Overall, vitamin D status was not associated with the incidence of AF in this prospective cohort study. Besides, no association was found after censoring of CHD cases that occurred before AF. Stratification according to potential effect modifiers also did not change the results.

Vitamin D has attracted much attention for its potential relation with non-skeletal disorders. Experimental research has supported the role of vitamin D deficiency in several cardiovascular diseases (187). The role of vitamin D deficiency in the onset of AF was suggested because of numerous potential mechanisms described previously. Vitamin D regulates inflammatory responses and up-regulates the expression of anti-inflammatory cytokines as IL-10 according to in-vitro experiments (188). Also, vitamin D regulates RAAS activity. Activated RAAS can lead to oxidative stress and inflammation both of which can culminate in AF (189, 190). It is hypothesized that tissue angiotensin II can induce apoptosis of cardiomyocytes and this way can contribute to changes in atrial structure. (191). Also, angiotensin II can modulate the expression of several ion channels in cardiomyocytes (192). Another way how RAAS might be involved in pathophysiology of AF is the altered atrial expression of angiotensin receptors found in patients with AF (193). Moreover, angiotensin antagonists prevent the electrical atrial remodeling seen in AF (194). Finally, experiments with mice show higher serum angiotensin II and renin activity in mice knocked-out for VDR (195). In addition, mice unable to synthesize $1,25(OH)_2D$ due to 1-alpha–hydroxylase deficiency have elevated RAAS activity, hypertension and cardiac hypertrophy (196).

Until now not much research has been done on the association of vitamin D deficiency and the risk of AF. We identified two cohorts reporting on this topic. The first was Framingham Heart Study and analyses were conducted by Rienstra and colleagues (174). The study population counted 2930 participants out of which 15 percent developed AF during follow-up. The mean age was 66 years. Authors report results similar to ours, HR per SD increment in 25(OH)D fully adjusted model: 0.99, 95% CI: 0.88-1.10 (174). The second cohort coming from Kansas, USA was used to investigate the association between vitamin D deficiency, vitamin D supplementation and numerous outcomes, including AF (173). This was a large cohort counting 10,899 participants with mean age 58 years. Only 5 percent of these participants developed AF. Even though the authors reported vitamin D deficiency significantly associated with several cardiovascular diseases and found vitamin D deficiency to be a strong predictor of all-cause mortality they also found vitamin D deficiency negatively associated with risk of AF (OR 0.83, 95% CI 0.693, 0.984). However, the authors obtained these results using

univariate analysis. It might be very likely that these results were confounded and thus not reflecting the true nature of the relation between vitamin D status and AF. In their case analysis Qayyum and colleagues found no association between vitamin D status and AF regardless of valvular disease (197).

Finally, two cross-sectional studies reporting on vitamin D deficiency and AF (198, 199) found an inverse association between vitamin D status and AF not related to valvular heart disease. Valvular AF and non-valvular AF is an important classification from therapeutic perspective. There is not much literature supporting a difference in the mechanisms underlying these two conditions (200). Unfortunately, we had no information on valvular heart disease to reproduce these latter findings. However, our study may mainly reflect the relation between vitamin D status and AF not related to valvular heart disease, since it has been estimated that only about 10% of the AF cases are due to valvular heart disease in The Netherlands (201).

In our study we decided to stratify analyses by gender, age, current smoking and use of BP lowering drugs because of the interaction they could play with the incidence of AF. Older age is a major risk factor for AF. In our cohort it was already shown that the incidence rate of AF increased from 1.1 cases at ages 55-60 to 20.7 cases at ages 80-85 per 1000 person-years (166). Similar increases were reported in several studies. The aging process is related to changes in atrial structure that favor AF development (185, 202). Men and women differ in incidence and prognostic related to AF (166, 203, 204). The mechanisms behind these findings are not totally clear. However it was shown that men and women have a different expression of ion channels in atria and cardiac myocytes. Also, fibroblasts express functional estrogen receptors. Moreover, genes related to atrial remodeling can be regulated by estrogens (205-207). We hypothesized that different mechanisms could be related to development of AF in men and women and vitamin D deficiency could interfere differently according to gender. Smoking was shown to alter the effect of vitamin D in specific diseases; low concentrations of vitamin D were a risk factor for tobacco related cancers (208). In addition, smoking is a risk factor for development of AF in the Rotterdam Study (209). Some classes of blood pressure lowering drugs were also shown to prevent atrial remodeling in animal models (210). Although the findings in humans are controversial (210), we hypothesized that some classes of antihypertensive drugs could interfere with effects of vitamin D status on the atria. In our

analyses, there was no differential association between vitamin D status and AF by strata of age, gender, smoking and hypertension however our results may have been limited due to small sample size to detect any potential effect modification. Furthermore, we expected that CHD could be a potential mediator in the association between vitamin D status and incidence of AF since several studies has shown an association between vitamin D status and CHD (211) but also association between CHD and AF (212). However, no changes were seen in the HRs when we censored participants with any CHD prior to AF.

The strengths of this study are the prospective cohort design and the extensive information on covariates. There are also some limitations that need to be addressed. Serum 25(OH)D was measured only once and may not reflect the values during the onset of AF. Also, the lack of data on valvular heart disease and parathyroid hormone levels limited replication of previous studies on this topic. We diagnosed AF with ECG or from medical records. However, many of the AF cases are asymptomatic, which may have underestimated the true prevalence of AF in our study population. However, this probably did not affect the direction of our results since this misclassification likely happened independently of vitamin D status. Furthermore, in our study the participants were Caucasians, therefore our findings must be interpreted with caution for other ethnic populations.

4.1.5. Conclusion

In conclusion, our prospective cohort study does not support the hypothesis that vitamin D status may play a role in the etiology of AF in the elderly. Further studies using repeated measurements of serum 25(OH)D as well as performing analysis in other populations may shed further light on whether the role of vitamin D status in the etiology of AF is justified.

Chapter 5
Pathways of Vitamin D and Health

5.1. The interplay between magnesium and vitamin D status on incidence of type 2 diabetes mellitus: The Rotterdam Study

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Abstract

Objective: to investigate whether the association between 25-hydroxyvitamin D (25(OH)D) and type two diabetes mellitus is modified by serum magnesium status.

Methods: The study was embedded in The Rotterdam Study, a population-based cohort among middle aged and the elderly free of type 2 diabetes mellitus at the baseline examination (N=8,481). Serum 25(OH)D and serum magnesium were assessed at the baseline examination rounds of the study and diabetes cases were monitored until 2012.

Results: Compared to adequate vitamin D levels, vitamin D deficiency was significantly associated with the incidence of type 2 diabetes mellitus

(multivariate adjusted HR=1.25, 95%CI 1.02, 1.52). In stratified analysis we found vitamin D deficiency to be significantly associated with a higher incidence of type 2 diabetes mellitus (multivariate adjusted HR=1.59, 95%CI 1.12, 2.26, for 10 units of 25(OH)D) in the middle tertile of serum magnesium distribution (representing the normal serum magnesium levels) but not in the bottom or the top tertile.

Conclusion: The association between 25(OH)D and type 2 diabetes mellitus may depend on adequate magnesium levels. These results imply that it may be important in clinical practice to assess serum 25(OH)D and magnesium levels simultaneously to identify people at risk.

5.1.1. Introduction

The prevalence of type 2 diabetes mellitus is estimated to be 6.4% among the adults in the year 2010 and it has been predicted to increase up to 7.7% by the year 2030(213). Multiple environmental and genetic risk factors can contribute to this increase of type 2 diabetes mellitus including vitamin D deficiency(214, 215). Low vitamin D status has been associated with higher incidence of type 2 diabetes mellitus in different populations(86, 215-218). Even though meta-analyses of observational studies confirm these associations, the results are inconsistent(217).

These inconsistencies may be related to the potential interaction between vitamin D and magnesium. Back in 1974 Reddy and Sivakumar described two cases of rickets resistant to vitamin D therapy(219). They demonstrated that additional supplementation with magnesium reversed the resistance to vitamin D supplementation(219). Hence they proposed that hypomagnesaemia might alter vitamin D metabolism by decreasing conversion of the 25-hydroxyvitamin D to1,25-dihydroxyvitamin D, the main active vitamin D metabolite(220, 221).

Magnesium (Mg) is the fourth most common mineral in the human body and functions as cofactor in many different metabolic processes (222). Various studies have suggested a relation between Mg deficiency and type 2 diabetes mellitus(223) .

Recently *Deng et al.* suggested that magnesium might contribute to vitamin D status and that it might modify the association between 25(OH)D and mortality(224). Since then, another study confirmed the existence of interaction between 25(OH)D and magnesium on mortality(225).

 Up to date, few studies have shown that 25(OH)D levels might depend on serum magnesium levels (226-228). More specifically, the enzymes involved in multiple steps of vitamin D metabolism such as 25-hydroxylase in the liver or 1-α hydroxylase in the kidneys, are magnesium dependent(224, 226, 227). Also, the process of vitamin D binding to the vitamin D binding protein seems to be depend on magnesium(224).

Based on these findings, we hypothesize that any association between vitamin D and type 2 diabetes mellitus might be modified by magnesium status. Hence, the aim of the current study is to investigate whether any association

between 25(OH)D and type 2 diabetes mellitus is modified by serum magnesium.

5.1.2. Methods
5.1.2.1. Study design
This study was embedded in the Rotterdam Study. The Rotterdam Study is a large prospective population-based cohort study conducted among inhabitants of Ommoord, a district of Rotterdam, the Netherlands(15). The start of the study was in 1990 when all inhabitants of Ommoord district aged 55 years and older were invited to participate (cohort RS-1). Subsequently, the study was expanded with additional cohorts in 2000 (cohort RS-2) and in 2006 (cohort RS-3). In 2006 all inhabitants aged 45 years and older were invited to participate in the study. Since 1990 14,926 people have been enrolled in to the Rotterdam Study. At baseline visit all participants were interviewed at home and invited for the detailed examinations at the research center. The follow-up visits were conducted every three to four years. All participants provided informed consent and the study was approved by the medical ethics committee of the Erasmus Medical Center, Rotterdam.

5.1.2.2. 25-hydroxyvitamin D
Serum 25(OH)D was assessed using electrochemiluminescence immunoassay (COBAS, Roche Diagnostics GmbH, Germany). The test range was between 7.5nmol/l and 175nmol/l with sensitivity of 10nmol/l. Intermediate precision of the test was <13.1% and within run precision was <7.8%. Vitamin D deficiency was defined as 25(OH)D concentrations lower than 50nmol/l, insufficiency was defined as 25(OH)D concentrations from 50nmol/l to 75nmol/l, while adequate vitamin D status was defined as concentrations equal to or higher than 75nmol/l(6). For the first cohort we used the 25(OH)D measurements performed at the third visit to the research center (1997-1999) while for the second and the third cohort we used measurements done at the first visit to the research center of each cohort (2000-2001 for RS-2 and 2006-2008 for RS-3).

5.1.2.3. Serum magnesium

Serum magnesium levels were assessed at the same time points as 25(OH)D using Roche/Hitachi Cobas c501 analyser. The analyses were performed at the Erasmus Medical Center Department of Clinical Chemistry. The cutoff value for clinical hypomagnesaemia was 0.72mmol/l and it was calculated as mean value minus 1.96 standard deviations as described earlier (***Kieboom et al., 2015***).

5.1.2.4. Type 2 diabetes mellitus

The participants were followed from the date of baseline center visit onwards. At baseline and during follow-up, cases of type 2 diabetes mellitus were ascertained through active follow-up using general practitioners' records, hospital discharge letters and glucose measurements from Rotterdam Study visits which take place approximately every 4 years (181). Diabetes, prediabetes and normoglycemia were defined according to recent WHO guidelines (World Health Organization. Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia: report of a WHO/IDF consultation. *Geneva: World Health Organization* 2006). Normoglycemia was defined as a fasting blood glucose level lower than 6.0 mmol/L; prediabetes was defined as a fasting blood glucose between 6.0 mmol/L and 7.0 mmol/L or a non-fasting blood glucose between 7.7 mmol/L and 11.1 mmol/L (when fasting samples were absent); type 2 diabetes mellitus was defined as a fasting blood glucose $\geq$7.0 mmol/L, a non-fasting blood glucose $\geq$ 11.1 mmol/L (when fasting samples were absent), or the use of blood glucose lowering medication. Information regarding the use of blood glucose lowering medication was derived from both structured home interviews and linkage to pharmacy records (181). At baseline, more than 95% of the Rotterdam Study population was covered by the pharmacies in the study area. All potential events of type 2 diabetes mellitus were independently adjudicated by two study physicians. In case of disagreement, consensus was sought with an endocrinologist. Follow-up data was complete until January 1st 2012.

5.1.2.5. Population for analysis

Serum 25(OH)D was measured at the third examination round of the first cohort (this time point was considered as a baseline for the first cohort), and at

the first examination round of the second and the third cohort. Out of 14,926 Rotterdam study participants 9,746 had their serum 25(OH)D measured. After exclusion of those with prevalent type 2 diabetes mellitus we had 8,481 participants from all three cohorts included in to our analyses.

5.1.2.6. Covariates

At first examination rounds of all three cohorts home interviews were conducted and numerous individual characteristics were collected such as demographics, lifestyle and medical factors. Smoking status was categorized into current smoker, former smoker and never smoker. Also, information on family history of cardio-metabolic diseases was collected at this time point. Family history of diseases (yes, no, not known) was defined on the basis of history of stroke, myocardial infarction or type 2 diabetes mellitus in parents. Further, variable baseline cardio-vascular diseases was created based on presence of myocardial infarction or stroke. At the same time point the information on highest education attained was collected and categorized as follows: 0- primary education; 1- lower/intermediate general education or lower vocational education; 2- intermediate vocational education or higher general education; 3- higher vocational education or university. Information on diet quality were collected at the first examination round of the first cohort, at the third examination round of the second cohort and at the first examination round for the third cohort. The Dutch Healthy Diet (DHD) index was then computed as described earlier (93). At the baseline visit to the research center participants had their weight and height measured. These were used to calculate body mass index (BMI) by dividing body weight (kilograms) by height (meters) squared. The adapted version of the Zutphen Physical Activity Questionnaire was used to assess physical activity (134). Every form of physical activity was attributed a MET-value according to the 2011 Compendium of Physical Activities (135). Questions on walking, cycling, gardening, diverse sports, hobbies and housekeeping were included in the questionnaire. Total time spend on physical activity was calculated as the sum of minutes per week for all types of activities mentioned. Dates when the blood was drawn were categorized into spring, summer, autumn and winter.

5.1.2.7. Statistical analysis

We used Cox proportional hazard model to determine the association between 25(OH)D and the risk of developing type 2 diabetes mellitus. The crude model was built after adjustment for age, gender and the Rotterdam Study cohort. Additionally a multivariate model was built adjusted for age, gender, body mass index (BMI), smoking status, season when the blood was drawn and the Rotterdam Study cohort. Variables such as highest education attained, prevalent baseline diseases, family history of diseases and diet quality score were also tested as potential confounders.

To test for interaction between serum 25(OH)D and magnesium an interaction term 25(OH)D x magnesium was entered into the model. To facilitate comparison across the three cohorts, the stratified analyses by magnesium levels were performed according tertiles of magnesium (the middle tertile representing adequate magnesium levels).

Additionally, sensitivity analyses were performed to adjust for physical activity levels since these were not available for the Rotterdam Study 3. We run the analysis in the Rotterdam Study cohort 1 and 2 with additional adjustment for physical activity. Furthermore, we run the analysis according to magnesium strata based on clinical cutoffs for hypomagnesaemia in The Rotterdam Study, as described earlier by *Kieboom et al.*(229). To test if the vitamin and mineral supplementation might have affected our results we have performed additional sensitivity analysis excluding participants who reported supplement use.

To reduce the bias associated with missing data a multiple imputation procedure was performed (N=10 imputations). Final results that are presented are after the multiple imputation procedure.

All analyses were performed with IBM SPSS Statistics version 21 (SPSS Inc., Chicago, Illinois).

A P-value of less than 0.05 was considered as statistically significant.

5.1.3. Results

During median follow-up of 6 years (range 14 years), 801 participants developed type 2 diabetes mellitus. Baseline characteristics of our study population are presented in Table 5.1.1. Briefly, participants with vitamin D

deficiency were older and more often women compared to the other two groups.

Table 5.1.1. Study population baseline characteristics according to vitamin D status.

	Total	Deficiency	Insufficiency	Adequate
	N=8481	N=3838	N=2503	N=2140
Age* (years)	62,0 (8,2)	63,6 (9,1)	60,92 (7,3)	60,40 (6,8)
Gender (female) N (%)	4961 (58,5)	2446 (63,7)	1367 (54,6)	1103 (51,5)
25(OH)D* (mmlo/l)	57,3 (27,9)	33,04 (10,4)	61,8 (7,1)	95,44 (17,1)
Magnesium* (mmol/l)	0,85 (0,06)	0,85 (0,06)	0,85 (0,06)	0,84 (0,06)
BMI* (kg/m2)	27,0 (4,1)	27,5 (4,4)	26,8 (3,8)	26,33 (3,5)
Smoking (current) N (%)	1693 (20,0)	820 (21,4)	461 (18,4)	411 (19,2)
Prevalent CVD N(%)	4028 (47,5)	2034 (53,0)	1131 (45,2)	863 (40,3)
Education (primary) N (%)	1012 (11,9)	550 (14,3)	253 (10,1)	209 (9,8)
Season when the blood was drawn (winter) N (%)	1852 (21,8)	937 (24,4)	536 (21,4)	379 (17,7)

mean (SD)

On average they had a lower educational level and smoked more often (Table 5.1.1).

After adjustment for confounders, vitamin D deficiency was significantly associated with a higher incidence of type 2 diabetes mellitus (HR for deficiency compared to adequate vitamin D status 1.25, 95%CI 1.02, 1.52) (Table 5.1.2). Further, serum magnesium levels were associated with serum 25(OH)D concentrations (β=-33.2, 95%CI -44.4, -23.1) (data not shown).

5.1.3.1. Stratified analysis according to serum magnesium

We found a significant interaction between tertiles of serum magnesium and vitamin D status on the risk of type 2 diabetes mellitus (Tables 5.1.3 and 5.1.4). In crude analyses, we found that higher serum 25(OH)D was significantly associated with a lower incidence of type 2 diabetes mellitus in bottom tertile of magnesium distribution (HR=0.94, 95%CI 0.89, 0.98 per 10 unit increase in 25(OH)D) and in the middle tertile of magnesium distribution (HR=0.91, 95%CI 0.86, 0.95 per 10 unit increase in 25(OH)D), but not in the top tertile (Table 5.1.3).

Table 5.1.2. Serum 25(OH)D and incidence of type 2 diabetes mellitus.

25(OH)D	Model 1 N=8481/797 HR (95%CI)	Model 2 N=8481/797 HR (95%CI)
continuous*	0,94 (0,91 0,96)	0,96 (0,94 0,99)
Deficiency	1,46 (1,20 1,76)	1,25 (1,02 1,52)
Insufficiency	1,20 (0,98 1,48)	1,16 (0,95 1,43)
Adequate	reference	reference

*Model 1 adjusted for age, gender and Rotterdam study cohort; Model 2: model 1 additionally adjusted for BMI, smoking status, and season when the blood was drawn; *For continuous 25(OH)D per 10 units increase in 25(OH)D; Imputed data are shown.*

In the multivariate analysis we found serum 25(OH)D inversely associated with incidence of diabetes mellitus only in middle tertile of magnesium distribution (Table 5.1.4). Similarly, we found that vitamin D deficiency was associated with increased risk of diabetes (HR=1.59, 95%CI 1.12, 2.26; *Pinteraction* =0.05) only in the middle magnesium tertile (Table 5.1.4).

5.1.3.2. Sensitivity analysis

Sensitivity analysis excluding the third Rotterdam Study cohort yield similar results (for middle tertile of magnesium distribution HR=0.94, 95%CI 0.89, 0.99 per 10 unit increase in 25(OH)D) (data not shown). Additional adjustment for physical activity also did not change the results markedly (for middle tertile of magnesium distribution HR=0.94, 95%CI 0.89, 1.00 per 10 unit increase in 25(OH)D) (data not shown). Additional sensitivity analysis performed excluding vitamin and mineral supplement users did not change the results (for middle tertile of magnesium distribution HR=0.91, 95%CI 0.86, 0.96 per 10 unit increase in 25(OH)D) (data not shown).

Table 5.1.3. Serum 25(OH)D and incidence of type 2 diabetes mellitus according to magnesium strata (model 1).

25(OH)D	Bottom magnesium tertile median Mg=0,80 mmol/l N=2876/286 HR (95%CI)	Middle magnesium tertile median Mg=0,85 mmol/l N=2981/287 HR (95%CI)	Top magnesium tertile median Mg=0,90 mmol/l N=2624/228 HR (95%CI)	p interaction bottom vs top Mg tertile/ middle vs top Mg tertile
continuous*	0,94 (0,89 0,98)	0,91 (0,86 0,95)	0,97 (0,92 1,02)	0,58/0,13
Deficiency	1,46 (1,08 1,97)	1,93 (1,38 2,72)	1,08 (0,75 1,56)	0,41/0,05
Insufficiency	0,98 (0,70 1,38)	1,43 (1,00 2,06)	1,30 (0,88 1,89)	0,24/0,77
Adequate	Reference	reference	Reference	

*Model 1 adjusted for age, gender and Rotterdam Study cohort; *For continuous 25(OH)D per 10 units increase in 25(OH)D. Imputed data are shown.*

Table 5.1.4. Serum 25(OH)D and incidence of type 2 diabetes mellitus according to magnesium strata (model 2).

25(OH)D	Bottom magnesium tertile median Mg=0,80 mmol/l N=2876/286 HR (95%CI)	Middle magnesium tertile median Mg=0,85 mmol/l N=2981/287 HR (95%CI)	Top magnesium tertile median Mg=0,90 mmol/l N=2624/228 HR (95%CI)	p interaction bottom vs top Mg tertile/ middle vs top Mg tertile
continuous*	0,96 (0,92 1,01)	0,94 (0,89 0,99)	0,99 (0,94 1,05)	0,63/13
Deficiency	1,26 (0,93 1,71)	1,56 (1,10 2,23)	0,94 (0,64 1,37)	0,47/0,05
Insufficiency	0,96 (0,68 1,35)	1,36 (0,94 1,96)	1,24 (0,84 1,83)	0,27/0,71
Adequate	Reference	reference	reference	

*Model 2 adjusted for age, gender, BMI, smoking status, season when the blood was drawn and Rotterdam Study cohort; *For continuous 25(OH)D per 10 units increase in 25(OH)D. Imputed data are shown.*

5.1.4. Discussion

This study demonstrated that the association between 25(OH)D deficiency and incidence of type 2 diabetes mellitus may be modified by magnesium status. Moreover, the inverse association between 25(OH)D and incidence of type 2 diabetes mellitus was present in only those participants in the normal range of serum magnesium status.

As expected, we found an inverse association between serum 25(OH)D and incidence of type 2 diabetes mellitus. This association has been already established (86) and the mechanism behind it goes most likely via influence of vitamin D on insulin metabolism, more specifically insulin secretion and insulin activity (230).

As to our knowledge, this is the first study to explore the interaction between 25(OH)D and serum magnesium levels on incidence of type 2 diabetes mellitus. Up to date, we have identified two other studies focusing on this interaction, however, both of the studies used magnesium data based on dietary intake. The first study, by *Deng et al.(224)*, performed in the National Health and Nutrition Examination Survey (NHANES) found higher 25(OH)D concentrations to be associated with a lower risk of total mortality in participants who had higher intake of magnesium. The median magnesium intake in this population (264mg/day) was used as a cutoff point for high and low magnesium intake. Even though we have focused on a different health outcome, our results are in line with the study by *Deng et al.* since we also found the association between 25(OH)D and risk of type 2 diabetes mellitus to be significant in the middle tertile of serum magnesium distribution representing normal magnesium status. Unexpectedly, the second study by *Mursu* and colleagues found an association between 25(OH)D and risk of mortality only in the lower magnesium intake group, but not in the group with higher intake of magnesium(225). In this study median intake of magnesium that was used as a cutoff point for high and low magnesium group was 414mg/day which was much higher compared to the US study but also higher than recommended daily intake which is 330mg/day for magnesium (222)**.** It may be argued that the results from these two studies contradict each other. However, the intervals of magnesium intake in which the associations between 25(OH)D and risk of mortality was found, overlap (264mg/day-414mg/day). Nonetheless, there are also some inconsistencies between these

two studies which are worth of further exploration: mainly, as mentioned before, the intake of magnesium was higher in the Finnish study population compared to the one in the US (224). In contrast, mean 25(OH)D concentrations were lower in this population. These discrepancies may be explained by the fact that both studies used dietary magnesium data, which cannot depict the true magnesium status as serum magnesium concentrations can reflect.

The potential mechanisms behind the potential interaction between 25(OH)D and magnesium levels are not yet fully understood. It has been suggested that serum magnesium is needed as a key co-factor in multiple steps of vitamin D metabolism; more specifically enzymes 1-α-hydroxylase, and 25-hydroxylase, which are all crucial in determining 25(OH)D concentrations, as well as vitamin D binding protein are all magnesium dependent (220, 226-228).

Our findings imply that in the future it might be useful for clinicians to assess serum magnesium together with serum 25(OH)D concentrations in order to identify people at higher risk of developing type 2 diabetes mellitus, especially having in mind that the loss of magnesium is increased in type 2 diabetes mellitus(231, 232).

Main strengths of our study are its prospective design and large study size (8,481 participants included). Also, we used serum magnesium concentrations instead of dietary magnesium intake that was used in previous studies. However, the limitations of our study should be mentioned as well. Firstly, since we combined three cohorts within The Rotterdam Study we did not have all the covariates assessed all three time points, which limited our ability to adjust for all possible confounders such as physical activity data. Further, when stratified by magnesium status according to hypomagnesaemia cutoffs (229) we had a very small group of participants with actual hypomagnesaemia which limited our ability to perform all the analyses in this specific subgroup. Since magnesium is a co-factor in many enzymatic systems in the human body (222) it is possible that there are other factors influencing magnesium metabolism but also vitamin D metabolism which might have affected our results.

To conclude, we found the inverse association between 25(OH)D and incidence of type 2 diabetes mellitus to be modified by serum magnesium status suggesting magnesium might play a role in determination of vitamin D

status. This finding implies that besides vitamin D monitoring patients magnesium status might be important in future clinical practice. Since this is the first study to use serum magnesium data and also the first one focusing on diabetes mellitus as an outcome we believe further studies on this topic are warranted.

5.2. Bidirectional associations between circulating vitamin D and cholesterol levels: The Rotterdam Study

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Abstract

Objectives: Higher levels of vitamin D have been associated with lower rates of cardiovascular disease perhaps through improved lipid profiles. However, results are inconsistent and the direction of the association between vitamin D and lipid levels remains unknown. We examined bidirectional associations between serum 25-hydroxyvitamin D (25(OH)D) and cholesterol concentrations.

Study design: We used data from 1165 participants aged 55 to 88 years from the Rotterdam Study, a population-based prospective cohort study.

Main outcome measures: Serum concentrations of 25(OH)D, total cholesterol (TC) and HDL cholesterol (HDL-C) were measured at two time points with a median time difference of 6 years. Bidirectional associations between 25(OH)D and each of the blood lipids was examined with path analyses in cross-lagged models. All models were adjusted for baseline age, sex, BMI, smoking status, and diet quality.

Results: The best-fit model for 25(OH)D and TC indicated that higher baseline TC concentrations were associated with lower 25(OH)D concentrations (standardized regression coefficient -0.05 (SE 0.02)), but 25(OH)D at baseline did not predict TC. For HDL-C, the best-fit model suggested a bidirectional inverse association between HDL-C and 25(OH)D (standardized regression coefficients of -0.03 (SE 0.02)) for both directions.

Conclusions: Our results from path analyses on repeatedly measured 25(OH)D and lipid levels suggest that total cholesterol may be associated with decreased in 25(OH)D concentrations, but not the other way around, whereas the observed inverse association between HDL-C and 25(OH)D may be bidirectional.

5.2.1. Introduction

Vitamin D deficiency is a highly prevalent condition associated with multiple health outcomes including diabetes mellitus, cancer, and cardiovascular disease (CVD) (6, 86).Furthermore, repletion of vitamin D status might reduce the risk of death from CVD and several other diseases(117). It is known that dyslipidemia is a major risk factor for CVD and it has been hypothesized that vitamin D deficiency is linked to higher CVD risk via different pathways including lipid levels (233). Studies showing that heterozygotes with familiar hypercholesterolemia suffer more often from vitamin D deficiency support this hypothesis (234). Additionally, an association between vitamin D deficiency and dyslipidemia has been reported in several populations (235-237).

Nevertheless, the direction of the association between vitamin D status and blood lipid levels remains unclear. Previous observational studies all had a cross-sectional design and could therefore not study the directionality of the association (238). Vitamin D might affect dyslipidemia via increased intestinal calcium absorption, suppression of secretion of parathyroid hormone (239), or through effects on insulin secretion (240). On the other hand cholesterol is a precursor of 25(OH)D and may thereby affect circulating 25(OH)D concentrations (241). Finally, adequate vitamin D status may also just be a mere reflection of overall health status and may not be causally associated with blood lipids. A recent Mendelian Randomization Study by *Ooi et al.* suggested that increased total cholesterol levels lead to lower vitamin D levels, but these results were not found for high-density lipoprotein (HDL)cholesterol (242). Intervention studies may provide answers on potential causality. Some intervention studies have indeed shown improvement of lipid levels after vitamin D supplementation (243), but mostly with joint calcium supplementation (244-247) whereas others did not find any effect of single vitamin D supplementation (248-250).

The aim of our study was to examine the bi-directionality of the associations between repeatedly measured serum vitamin D and lipids (total cholesterol and HDL-cholesterol), in a population-based prospective cohort study, using a cross-lagged modeling approach.

5.2.2. Methods

5.2.2.1. Study design

The current study was embedded in the first cohort of The Rotterdam Study (237). The Rotterdam Study is a prospective population-based cohort carried out in the Ommoord district, in the city of Rotterdam. All 10,275 inhabitants of the Ommoord district who were aged 55 years and older were invited to participate in the study. Baseline examinations (RS-I-1) were conducted between 1989 and 1993 with 7,983 subjects participating (98% Caucasian). The participants were interviewed at home and later visited the research center for additional examinations. Every 3-4 years the participants were invited for follow-up visits. The study was approved by the medical ethics committee at Erasmus University Rotterdam, the Netherlands and all participants gave written consent to participate in the study.

5.2.2.2. 25-hydroxyvitamin D

At the baseline examination round of the cohort (1989-1993) serum 25(OH)D concentrations were assessed using radioimmunoassays (IDS Ltd, Boldon, UK, available at www.idsltd.com). The test sensitivity was 3 nmol/L, the range of the test was 4 to 400 nmol/L, intra-assay accuracy was <8%, and inter-assay accuracy was <12%. Between 1997 and 1999, during the third examination round, serum 25(OH)D concentrations were re-assessed using electrochemiluminescence immunoassay (COBAS, Roche Diagnostics GmbH, Germany). For this analysis, test sensitivity was 10 nmol/L, and the test range for 25(OH)D 7.5 nmol/L to 175 nmol/L. Within-run precision of the test was <7.8% and intermediate precision was <13.1%.

5.2.2.3. Serum lipids

At the first examination round of the cohort (1989-1993) total cholesterol and HDL-C were measured in non-fasting serum samples according to the CHOD-PAP method (Monotest Cholesterol kit, Boehringer Mannheim Diagnostica). At the third examination round (1997-1999), the lipids were measured in fasting serum using the same enzymatic method. HDL-C was measured in serum after precipitation of non-HDL fraction.

5.2.2.4. Population for analysis

At the first examination round of the cohort (1989-1993), most of the participants had cholesterol levels analyzed, but only a subgroup of 1,437 participants had serum 25(OH)D measured. This subgroup was selected based on availability of knee radiographs as described by *Bergink et al.*(251) The differences between this subsample and the complete Rotterdam Study are described elsewhere(252). At the third examination round (1997-1999), a subgroup of 3,828 participants had serum 25(OH)D measured. Altogether 1,333 participants had their serum 25(OH)D and cholesterol levels measured at both time points. We excluded those who used lipid lowering medication at any of the two examination rounds leaving 1,165 participants for final analysis.

5.2.2.5. Covariates

At the baseline examination round of the cohort (1989-1993), information on numerous population characteristics was collected using home interviews. Smoking status was categorized as following: current smoker, former smoker and non-smoker. We used the Dutch Healthy Diet (DHD) index to assess overall diet quality (93) based on a validated food frequency questionnaire at baseline (179). The DHD index initially consisted of ten components: physical activity, vegetable, fruit, dietary fiber, fish, saturated fat, transfat, consumption of acidic drinks and foods, sodium and alcohol described previously (93). Since physical activity data were not assessed at baseline but only during the third examination round (1997-1999), we excluded physical activity from the DHD-index and adjusted for it in sensitivity analyses. Also, consumption of acidic drinks and foods was omitted from the DHD-index since the food frequency questionnaire did not include specific questions on the frequency of acidic food intake.

Height and weight were measured at the research center, with participants wearing only light clothes and no shoes. Body mass index (BMI) was calculated as weight divided by height in meters squared. We had information available on the date and year when the blood was drawn. The dates were categorized into summer, fall, winter, and spring based on the Dutch standard seasons.

5.2.2.6. Statistical analysis

To account for missing data, we multiple imputed (n=10 imputations) missing values of HDL-C at baseline (<0.01% missing), HDL-C at follow-up (1.46% missing), smoking (1.03% missing), BMI (0.60% missing), and diet quality (13.05% missing) using the Markov chain Monte Carlo method (253). Concentrations of 25(OH)D were adjusted for season and year of blood draw using the residual method.

In order to assess the direction of the association between serum 25(OH)D and serum lipids we used a cross-lagged modelling approach. In these path analyses all associations are adjusted for each other (eg. analyses are adjusted for the underlying association between 25(OH)D at two time points (stability paths), lipid levels at the two time points (stability paths) and the cross sectional, and the mutual prospective association between 25(OH)D and lipids). These path analyses generates standardized coefficients that can be directly compared to assess which direction of the association between 25(OH)D and serum lipids is stronger.

These path analyses were all adjusted for age, sex, smoking status, BMI, and diet quality at the first visit (Supplemental figure S1). We built four different models, for which schematic examples are provided in Supplemental figure S1. The first was baseline model which did not include any cross-lagged associations (model 1). In models 2 through 4 we entered different jcross-lagged associations and for these models we assessed goodness-of-fit and improvement of model-fit over the baseline model (Supplemental figure S1). The model fit was considered to be good with a comparative fit index > 0.90 and a root mean squared error of approximation < 0.08. Model improvement was assessed using the Satorra-Bentler X2-difference test for maximum likelihood estimation (254).

Since physical activity data was only available at the 3rd visit, we additionally adjusted the analyses for physical activity (minutes/week) in sensitivity analyses to assess whether physical activity levels had an impact on the analyses.

Statistical analyses were performed using SPSS version 21.0 and Mplus version 6.0.

5.2.3. Results

5.2.3.1. Subject characteristics

Characteristics of our study population are shown in Table 5.2.1. Mean age at baseline examination (1989-1993) was 67 years and 60 percent of participants were females. Mean serum 25(OH)D concentration at the first examination round was 65.7 nmol/L while at the third examination round it was 46.8 nmol/L. The prevalence of vitamin D insufficiency was 68% at the first visit, which increased to a prevalence of 75% at the second visit.

Table 5.2.1. Selected characteristics of study population for 25(OH)D and serum lipids path analysis.

	First visit of the cohort (1989-1993)	Third visit of the cohort (1997-1999)
No of participants	1165	1165
Age (years)*	66.6 (6.9)	73.2 (6.9)
Gender %(N) female	60 (699)	60 (699)
Serum 25(OH)D (nmol/L)*	65.7 (27.3)	46.8 (25.4)
Total cholesterol (mmol/L)*	6.6 (1.1)	5.9 (1.0)
HDL cholesterol (mmol/L)*	1.4 (0.4)	1.4 (0.4)
BMI (kg/m2)*	26.3 (3.6)	26.7 (4.0)
Physical activity (min/week)*	NA	2693 (1170)
Diet quality index*	50.3 (10.5)	NA
Smoking %(N) current	21.2 (247)	15.4 (179)

mean (standard deviation)

Serum TC concentrations also decreased between the two time points; the mean TC concentration at the first examination round was 6.6 mmol/L, while at the third round it was 5.8 mmol/L. HDL-C and BMI did not differ between the two examination rounds. Median time difference between the two time points was 6 years (range 6-9 years).

Cross sectional analyses showed that 25(OH)D was negatively associated with TC but not with HDL-C (data not shown).

Additional adjustment for physical activity levels did slightly alter the analysis on HDL cholesterol (β=1.007E-005 per mmol/L; 95%CI: -0.001-0.001, P=0.098 and -4.539E-005 per mmol/L; -0.001-0.001, P=0.929).

5.2.3.2. Correlations between measurements at two time points

The correlation between 25(OH)D levels between the first and the third examination round was 0.575 (p<0.001) while the correlations between lipid levels between the first and the third examination round were 0.745 (p<0.001) for TC and 0.751 (p<0.001) for HDL-C.

5.2.3.3. Bidirectional analysis of 25(OH)D and total cholesterol levels

The best-fit model for circulating 25(OH)D and TC levels was model 3 (Table 5.2.2). This model significantly improved the basic model fit. This model included the cross-lagged association from TC to 25(OH)D.

Table 5.2.2. Model Fit of Different Cross-lagged Models With TC.

	Model 1: Stability	Model 2: 25(OH)D to TC	Model 3: TC to 25(OH)D	Model 4: Bidirectional
CFI	0.914	0.913	0.917	0.917
RMSEA	0.101	0.106	0.104	0.109
X^2 (df)	155.020 (12)	155.137 (11)	148.598 (11)	148.609 (10)
ΔX^2 (df)	-	0.117 (1)	6.422 (1)	6.411 (2)
p value ΔX^2	-	0.72	0.0113	0.0405

Model 1 (stability) includes the association between 25(OH)D levels at visit 1 and visit 2 as well as the association between TC at visit 1 and visit 2; Model 2 (25(OHD) to TC) includes the association between 25(OH)D at visit 1 and TC at visit 2 after adjustment for model 1; Model 3 (TC to 25(OH)D) includes the association between TC at visit 1 and 25(OH)D at visit 2 after adjustment for model 1; Model 4 (bidirectional) reflects all the associations mutually adjusted for model 1, 2, and 3;
The comparative fit index (CFI) indicates improvement in fit of the model relative to the baseline model (model 1) where a value of 1 indicates perfect fit and lower values indicates a worse fit. The root mean squared error of approximation (RMSEA) indicates the overall model fit where a value of 0 indicates a perfect fit and a higher values indicates a worse fit.

Baseline TC was significantly inversely associated with later serum 25(OH)D (β=-0.053 per SD, SE=0.023, p=0.019) (Figure 5.2.1). Model 4, including both cross-lagged associations, also improved the model fit of the basic model however it did not improve the model fit of model 3. Additional adjustment for physical activity levels did not alter these results (data not shown).

Figure 5.2.1. Best fitted model (model3) + effect estimates (TC).
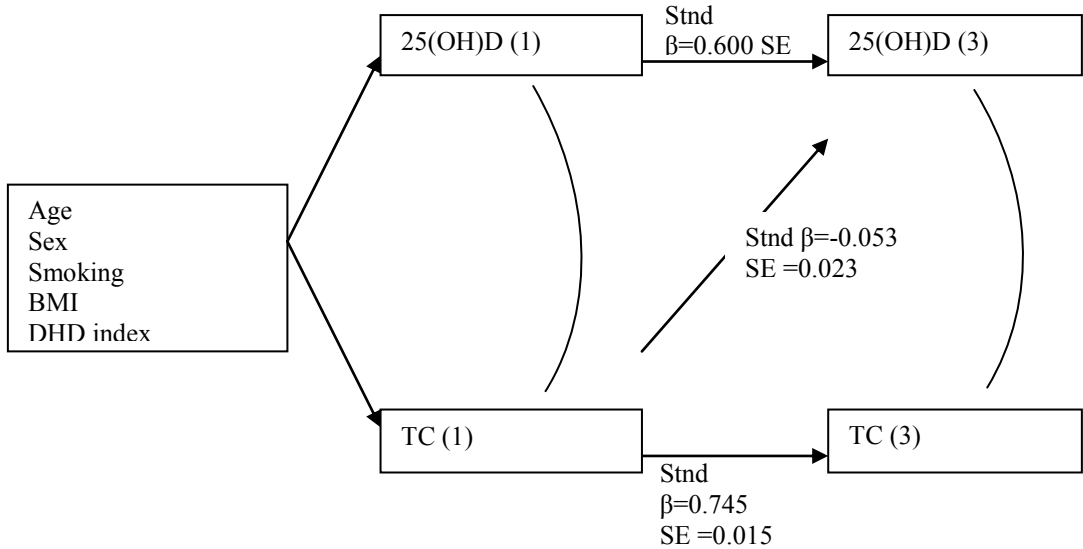
5.2.3.4. Bidirectional analysis of 25(OH)D and HDL cholesterol levels

For 25(OH)D and HDL-C levels, both the second and the third cross-lagged models improved the model fit of the first basic model (Table 5.2.3). The best model fit was achieved with model 4, which included both the associations from HDL-C to 25(OH)D and from 25(OH)D to HDL-C.

Table 5.2.3. Model Fit of Different Cross-lagged Models With HDL-C.

	Model 1: Stability	Model 2: 25(OH)D to HDL-C	Model 3: HDL-C to 25(OH)D	Model 4: Bidirectional
CFI	0.894	0.898	0.896	0.900
RMSEA	0.091	0.093	0.094	0.097
X^2 (df)	128.324 (12)	122.978 (11)	125.420 (11)	119.817 (10)
ΔX^2 (df)	-	5.340 (1)	2.904 (1)	8.507 (2)
p value ΔX^2	-	0.0208	0.088	0.014

Model 1 (stability) includes the association between 25(OH)D levels at visit 1 and visit 2 as well as the association between HDL-C at visit 1 and visit 2; Model 2 (25(OHD) to HDL-C) includes the association between 25(OH)D at visit 1 and HDL-C at visit 2 after adjustment for model 1; Model 3 (HDL-C to 25(OH)D) includes the association between HDL-C at visit 1 and 25(OH)D at visit 2 after adjustment for model 1. Model 4 (bidirectional) reflects all the associations mutually adjusted for model 1, 2, and 3. The comparative fit index (CFI) indicates improvement in fit of the model relative to the baseline model (model 1) where a value of 1 indicates perfect fit and lower values indicates a worse fit. The root mean squared error of approximation (RMSEA) indicates the overall model fit where a value of 0 indicates a perfect fit and a higher values indicates a worse fit.

However, none of the inverse associations between 25(OH)D and HDL-C were statistically significant (β=-0.033 per SD, SE=0.024, p=0.162 for the association from HDL-C to 25(OH)D; and β=-0.031 per SD, SE=0.016, p=0.054 for the association from 25(OH)D to HDL-C) (Figure 5.2.2).

Figure 5.2.2. Best fitted model (model4) + effect estimates (HDL-C).
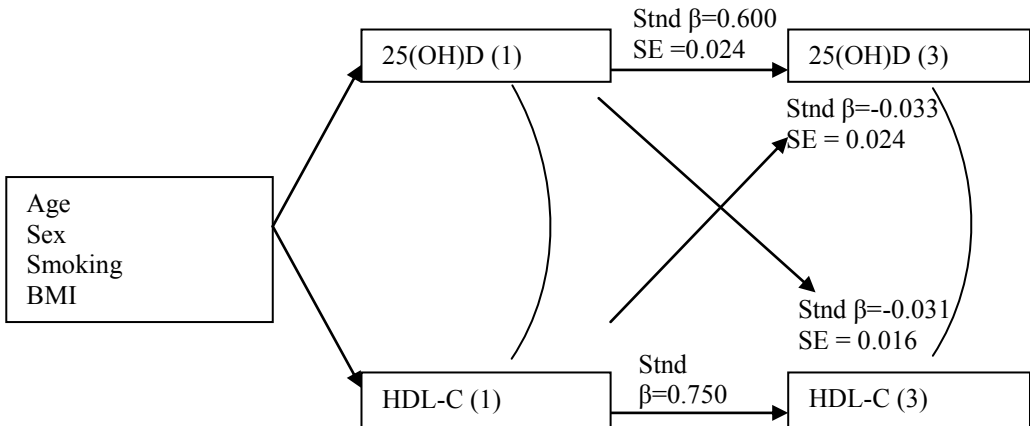
Additional adjustment for physical activity levels did not alter the results from the pathway analyses (data not shown).

5.2.4. Discussion

According to our knowledge this is the first study to examine the directionality of the association between 25(OH)D and serum lipids. Using a cross-lagged modeling approach on repeated measurements of 25(OH)D and cholesterol levels, we observed that the direction of the inverse association between total cholesterol and 25(OH)D levels is likely to follow the direction from cholesterol to 25(OH)D, therefore cholesterol levels might actively contribute in the determination of future Vitamin D levels. In contrast, we found both- 25(OH)D to be associated with lower HDL as well as HDL itself to be associated with lower vitamin D suggesting that the relation might be bidirectional.

5.2.4.1. Other studies

The results of our study are in line with findings by *Ooi et al.*, who performed a bidirectional Mendelian randomization study on the relation between 25(OH)D and blood lipids(242). This study found that genetically elevated total cholesterol was associated with low 25(OH)D levels, but that genetically elevated vitamin D levels were not related to lipids, suggesting a causal effect of cholesterol on decreased 25(OH)D, but not vice versa. The results from our path analysis of 25(OH)D and total cholesterol point in the same direction. Furthermore, also in line with our results, Ooi and colleagues unexpectedly found an inverse association between HDL cholesterol levels and 25(OH)D levels, which was bidirectional: genetically elevated HDL-C was associated with lower 25(OH)D and genetically higher 25(OH)D was associated with lower HDL-C.

To date several studies have been published on vitamin D and its potential effect on blood lipids. They are summarized in two recent reviews by *Jorde et al.* (238) and *Zittermann et al.* (255). Briefly, cross-sectional analyses within observational studies report an inverse association between serum 25(OH)D and serum total cholesterol and a positive association between serum 25(OH)D and serum HDL cholesterol. Randomized control trials (RCTs) report contradictory results and therefore cannot confirm the reports coming from observational studies. A meta-analysis published in 2012 on effects of vitamin D supplementation on serum lipids found no beneficial effect (256). However, most of the vitamin D RCTs were heterogeneous; for example they used a wide range of vitamin D dosages, they combined vitamin D with other nutrients in one supplement, or they were performed in different patient groups. Furthermore, many of the RCTs were not specifically designed to answer this question (238, 255) and therefore could not shed additional light on the direction of the association between 25(OH)D and blood lipids.

5.2.4.2. Potential mechanisms

The effects of vitamin D on serum lipids might not be that straight forward since vitamin D metabolism and lipids metabolism are connected in through multiple pathways. Firstly, we found that TC is associated with lower 25(OH)D but not the other way around. From genome-wide association studies it is known that some genetic variants share activities related to

cholesterol metabolism but also in vitamin D transport, such as DHDR7, CYP24A1, CYP2R1, which implies common pathways (257). For example, 25(OH)D and cholesterol have the same precursors: 7-dehydrocholesterol (7DHC). 7DHC is being transformed into pre-vitamin D under the effects of UVB radiation but 7DHC is also a substrate for the enzyme 7-dehydrocholesterol reductase (DHCR7) which catalyzes the production of cholesterol. Although the regulation of 7DHC has not been completely understood, it has been shown that for example statin treatment decreases cholesterol but surprisingly increases 25(OH)D (255) whereas several intervention studies did not show an effect of single vitamin D supplementation on lipid levels(248-250). This may confirm the hypothesis that change in cholesterol levels may be related to a change in 25(OH)D but not the other way around.

Secondly, vitamin D and HDL-C are related as well. For example, a recent study by Schuch et al. found an interaction between vitamin D receptor polymorphisms and HDL-C (258). A study by Shirts et al., investigating vitamin D dependent effects of APOA5 polymorphisms on HDL-C, found APOA promoter polymorphism (which is modified by VDR) to be associated with lower HDL-C (259). Conversely, the Mendelian randomization study mentioned earlier found an inverse bidirectional association between HDL-C and 25OHD levels (242). Based on research of statin use and its effect on 25(OH)D levels(260, 261) Ooi and colleagues speculate that up-regulation in cholesterol biosynthesis leads to down-regulation of 25(OH)D synthesis. Subsequently, the unexpected association between 25(OH)D and HDL cholesterol according to their hypothesis may be related to already established inverse association between HDL cholesterol and total cholesterol(242). However, since for example Schwartz and colleagues found that even supplementing with vitamin D can lower total cholesterol levels it seems that a more complex mechanisms might be involved in the association between 25(OH)D and serum lipids(262).

All the potential mechanisms mentioned above illustrate that the association between 25(OH)D and blood lipids is not that straight forward and the specific mechanisms need further study and clarification.

5.2.4.3. Strengths and limitations

The main strengths of our study include its longitudinal design with measurements of both serum 25(OH)D and serum cholesterol at different time points. Furthermore, an important strength is that we used these repeated measurements in a cross-lagged modeling approach to assess the direction of the associations. To our knowledge, our study is one of the first studies with longitudinal data on both 25(OH)D and cholesterol levels, and the first to use cross-lagged modeling to assess bi-directionality between serum 25(OH)D and serum lipids.

There were some differences in our assessment procedures during the first and second biomarker measurement (fasting vs. non-fasting and assay methods). It has been shown that lipid levels may be subject to changes in fasting status (263). However, it is unlikely that these different procedures affect the relations under study (264). In addition, 25(OH)D levels are not dependent on short-term fasting status since 25(OH)D levels has a half-life of 10-40 days (265). We used different assay methods for the two measurements of 25(OH)D (IDS RIA vs. Roche Elecsys). Nevertheless the within-batch and between-batch CVs are fairly similar and both have a specificity of 100% to detect 25(OH)D. Detection limits are slightly different between the essays: 3-300 nmol/L for ISD RIA and 10-250 nmol/L for Roche Elecsys. Nonetheless, it has been shown previously that 25(OH)D results from Roche Elecsys are in good overall agreement with those measured with RIA (difference only 0.7 nmol/L on a group level) (266). Since we also adjusted the pathway analyses for the correlation between 25(OH)D between the two visits, it is unlikely that the different assay methods have majorly influenced our results.

Our models fit indicated a RMSEA >0.08 whereas the CFI were all lower or around 0.90 indicating reasonable fit. This may be explained by the different approximations that these measures reflect (267). RMSEA is a measure of approximation and may be subject to sampling error since it takes sample size into account whereas the CFI represents the difference of the model relative to the baseline/simplified model. In addition, it has been discussed that the RMSEA tend to over reject good population models if compared to the CFI when sample sizes are small (268), which may have been the case in our study.

A limitation of our study is that we had no information on triglyceride levels to discuss the complete lipid profile. Furthermore, serum 25(OH)D concentrations were available only in a subgroup of the cohort, which reduced the power of our analyses. It can be expected that directionality of results does not differ between the group included in to our study and the total study population(269).

5.2.4.4. Conclusion

Using cross-lagged modeling of repeated measurements, our results suggest that total cholesterol levels may be associated with decreased vitamin D levels but not the other way around, whereas the inverse association between HDL cholesterol and vitamin D levels may be bidirectional.

These results imply that the direction of the relation between vitamin D and lipid metabolism may not be that straightforward and may depend on the type of lipids. Further studies to clarify the complex interplay between serum lipids and 25(OH)D pathways are warranted.

Chapter 6
General Discussion

6.1. Summary of results and main conclusions
6.1.1. Vitamin D and mortality- systematic review and meta-analysis

As mentioned earlier in the introduction, the discovery of extra-skeletal effects of vitamin D has attracted a lot of attention in research in the past decades but also in public health policy makers. Based on the hypothesis of extra-skeletal effects of vitamin D but also having in mind sometimes opposite results reported on this topic, we systematically studied if there is an effect of vitamin D on risk of death (chapter 2). For this purpose we performed a systematic review and meta-analysis of reports from observational cohort studies and also from intervention studies. To study the sole effect of vitamin D we only included intervention studies using vitamin D supplementation only. With this inclusion criterion we wanted to elucidate further the effect of vitamin D supplementation, since up to date many intervention studies have been performed but quite large portion of them used combined supplementation, usually vitamin D in combination with calcium, which subsequently prevented interpretation of the results in the light of vitamin D effects only (246).

With the results of our analysis confirmed that with higher concentrations of 25-hydroxyvitamin D was associated with a decreased risk of all-cause mortality. Further, we found that supplementation with vitamin D3 but not D2 decreased the risk of death.

6.1.2. Metabolic syndrome

Incidence of cardiovascular diseases and diabetes mellitus type 2 has increased markedly worldwide in the last decades (270). The association of vitamin D with these conditions has been well established in epidemiological studies (chapter 3.1). With our research presented in this chapter, we studied the association of vitamin D with metabolic syndrome. Metabolic syndrome is a risk-factor for diabetes mellitus type 2 and cardiovascular disease. It is already known that the prevalence of these diseases and vitamin D deficiency is especially present in the elderly, which is why we decided to test this association in the sample of this population.

In our analyses we found that higher 25-hydroxyvitamin D concentrations were associated with a lower prevalence of metabolic syndrome. Participants with adequate vitamin D status had a 39 percent lower odds of having metabolic syndrome compared to those with vitamin D deficiency. Further we

found higher 25(OH)D concentrations in the elderly to be associated with higher HDL cholesterol and lower triglycerides levels, waist circumference and serum glucose levels.

According to a recent review, findings from epidemiological and intervention studies so far are not consistent to conclude that there is an effect of vitamin D on metabolic syndrome and its components(271). These inconsistencies mainly originate from different quality of the studies reviewed such as adjustment for confounders in observational studies. On the other hand, intervention studies may not be able to assess the true effect of vitamin D because of low supplementation dosage or because of short duration of supplementation.

6.1.3. Body composition

Obesity correlates with increased waist circumference, as one of the components of the metabolic syndrome. Several studies have shown an association between vitamin D levels and obesity (chapter 3.2). We extended the current body of evidence by additionally analyzing vitamin D and its association with body fat distribution. While obesity is a well-known risk factor for cardiovascular disease as well as for diabetes mellitus type 2, the role of vitamin D in the development of abdominal obesity is still unclear. Some observational studies found significant associations of vitamin D and certain body composition measures (138, 139) however, the direction of this association remains under investigation. The question of causality and the effect direction has been further analyzed in the recent Mendelian randomization study using genetic variants related to both BMI and vitamin D, which has found that higher body mass index causes lower levels of vitamin D(132). Our research mirrors these findings well since we have found significant negative association between vitamin D and fat mass percentage. As opposed to most of the empirical findings so far, we confirmed this association is also present in the elderly population. Additionally, we found the association of vitamin D and differential fat distribution to be mainly explained by body mass index.

6.1.4. Atrial fibrillation

Another condition that is likely to occur more frequently in the elderly is atrial fibrillation (chapter 4.1). It is also one of the most common chronic

arrhythmias related to morbidity and mortality (157), and therefore its impact on public health is large. While its etiology and underlying pathways of this disease are not completely known yet, there is evidence suggesting vitamin D may play a significant role in atrial fibrillation at a cellular level. It has been suggested that this mechanism mainly goes through the proposed role of vitamin D in regulation of renin angiotensin aldosterone system (RAAS) activity which might be related to incidence of atrial fibrillation (189-191).

We tested the association between vitamin D and the incidence of atrial fibrillation and we did not confirm the hypothesis that vitamin D status was associated with the incidence of atrial fibrillation. Therefore, based on the evidence of this thesis we conclude vitamin D may not have a role in atrial fibrillation in the elderly.

Up to date only two cohort studies investigated the possible association between vitamin D status and incidence of atrial fibrillation- one of them reported statistically non-significant results, very similar to ours(174). The other study found a significant inverse association between vitamin D deficiency and the risk of atrial fibrillation; however these results were obtained using univariate analysis(173). Further, two cross-sectional studies found an inverse association between vitamin D status and atrial fibrillation while a case analysis study did not find any association(272, 273).

Since the existing research on this association between vitamin D and atrial fibrillation is scarce, we can draw limited conclusions on the consistency of our findings with other literature on this topic.

6.1.5. Serum magnesium- vitamin D interaction

The association between vitamin D and diabetes mellitus type 2 has been well studied in the literature (chapter 5.1). However, some studies show inconsistent results(217). We hypothesized that these inconsistencies may be due to the potential role that magnesium may have in the vitamin D metabolism and subsequently the possible effect magnesium might have on the association between vitamin D and diabetes mellitus type 2. Our research has addressed this possibility as one of the first ones in the research literature. As to our knowledge only two more studies investigated this possible interaction but focusing on mortality as an outcome(224, 225). Further, both of the studies used dietary magnesium data while we are the first to use serum

magnesium levels in our analysis which depict the true magnesium status more precisely.

We have found that the association between vitamin D and type 2 diabetes mellitus might be modified by the magnesium status. In particular, the reverse association between serum 25-hydroxyvitamin D and incidence of type 2 diabetes mellitus was found to be present only in the case of the participants with adequate magnesium levels. Further studies to elucidate the physiological interaction of serum magnesium and vitamin D are warranted.

6.1.6. Bidirectional associations between vitamin D and serum lipids

Dyslipidemia is also a well-known risk-factor for cardiovascular disease. The association between vitamin D and dyslipidemia is hypothesized to be inverse but the direction of the effect has not been firmly established (chapter 5.1). We used a unique approach by examining the directionality of the association by employing cross-legged modeling approach as well as longitudinal data. Using the novel cross-legged modeling approach on repeated measurements of vitamin D and cholesterol levels we have found that total cholesterol levels might lead to lower vitamin D levels, but not the other way around. Also, we found an inverse association between HDL cholesterol and vitamin D which might be bidirectional. This finding is consistent with the finding reported by Ooi et al. from 2014 who found genetically elevated total cholesterol to be associated with low vitamin D using a Mendelian randomization approach for this analysis(242). This study also found a bidirectional inverse association between genetically elevated HDL cholesterol and low vitamin D. Based on the evidence collected so far we conclude that the relationship between vitamin D and lipid metabolism is not straightforward and may be bidirectional for specific lipids but warrants further investigation.

6.2. Methodological considerations
6.2.1. Residual confounding

In observational studies there remains a possibility of residual confounding(274). Our work presented in this thesis uses mainly an observational design. Even though we used vitamin D status measured as serum 25-hydroxivitamin D which captures deficiency more precisely than it could be done with dietary data, the relations between this variable and outcomes studied in this thesis are still influenced by a number of lifestyle and

socio-demographic factors. For example time spent on physical activity might influence the vitamin D status, as suggested in a study by Wanner et al., published in 2015(275). In The Rotterdam Study we have many of such factors collected, also at different time points, and we have taken into account many of them in our analyses. However, there is also a possibility that some of these factors have not been measured precisely, which is especially the case with data derived from interviews and questionnaires. As a result these imprecisions might contribute further to residual confounding. Further, the interplay between lifestyle and socio-demographics and vitamin D status is still under investigation and thus we cannot state with total certainty that we have managed to take all relevant factors into consideration. Hence, residual confounding may have influenced the observed associations in this thesis.

6.2.2. Reverse causality
Reverse causality is another factor that needs to be taken into account when interpreting the results from observational studies(274). Reverse causality occurs when the outcome of the analysis is related to the exposure being studied. This is especially important when interpreting results from cross-sectional observational studies because there we cannot follow certain characteristics and their changes over time. Our analysis on vitamin D status and metabolic syndrome can serve as an example: for this analysis we used vitamin D status assessed at the same time point as the components of metabolic syndrome, making it more complicated to determine if ill health, thus metabolic syndrome, caused low levels of vitamin D or the other way around (chapter 3.1).

6.2.3. Information bias
One other important factor that might have affected our analyses is information bias(274). This type of bias might cause misclassification of the outcome or of the exposure. The exposure in all of our analyses is serum 25-hydroxyvitamin D (25(OH)D). Even though serum levels might be considered more precise than dietary data when it comes to assessment of vitamin D status, a possibility of misclassification remains. For example, the season when the blood was drawn might cause a person to be misclassified with regard to vitamin D status. This can easily happen if we compare vitamin D levels which were obtained during winter or early spring when the 25(OH)D

concentrations are at their lowest to 25(OH)D concentrations measured at the end of the summer when they peak. We adjusted the analyses for season to overcome this potential misclassification to certain extent.

Also, although 25(OH)D levels is a reliable biomarker for vitamin D status, variability in assay's for vitamin D assessment exist. At the time of data collected we used radioimmunoassays (IDS-RIA) and electrocemiluminescence immunoassays (EIA) but not liquid chromatography-tandem mass spectrometry (LC-MS/MS). Although 25(OH)D assessment from IDS-RIA and EIA are comparable, they differ from assessment by LC-MS/MS (276). As a result 25(OH)D may be over- or underestimated using IDS-RIA or EIA. Nonetheless, it may be likely that the accuracy of these essays may not be related to the health outcomes of our study and therefore not affect the direction of our observed associations.

Further, when it comes to certain health outcomes such as for example atrial fibrillation, the possibility of misclassification is present since atrial fibrillation might occur silently without recognizable symptoms (chapter 4.1). In this case the patient with atrial fibrillation would be categorized as not having an event- thus dilution of the effect might occur. Another example where misclassification might have occurred is the use of lipid-lowering medication in analysis of serum lipid levels (chapter 3.1). Even though we have excluded participants using lipid-lowering medication from this analysis if there was a case when the use of lipid-lowering medication was not documented properly the patient might have been classified as having normolipaemia which would also lead to dilution of the effect under investigation. Finally, some confounders that we used in our analyses might have been misclassified, for example history of diseases such as stroke or myocardial infarction. Although seldom, it might be the case that these conditions, if not severe, remain undetected. The main reason why we take into account prevalent diseases of the participants is to avoid potential reverse causality or recall bias in which subjects with prevalent disease may change their lifestyle which in turn might influence the association between vitamin D and the health outcome studied.. Dietary data collected based on food frequency questionnaires in subgroup of obese subjects might serve as good example of differential misclassification. It is possible that subjects with overweight or obesity might underreport their true daily food intake which in

turn might lead to underestimation or overestimation of the association studied.

6.2.4. Missing data and attrition bias

Another important issue that is often present in observational epidemiological studies is the issue of missing data(274, 277). There might be several reasons for occurrence of missing data. Firstly, missing data might occur as a result of a random mistake- for example the interviewer makes a mistake during the data entry or a laboratory technician makes a mistake while analyzing certain blood sample. Secondly, the missing data might occur in a specific case of non-response, for example, when subjects from certain socio-economic class refuse to answer the question about their income or for example in the case when obese people refuse to answer a question regarding their eating habits.

Further on, missing data might occur due to loss-to-follow-up, which in the case of Rotterdam Study means that the subjects did not return to the research center for the subsequent examinations rounds. Loss-to-follow-up is also known as an attrition bias.

In order to deal with the issue of missing data we used multiple imputation procedure which reduced the potential bias associated with missing data related to participant characteristics(277).

Also, it may be argued whether our results may be influenced by selection or attrition bias. Nonetheless, according to recent study by Lacey et al. (2013), attrition in cohort studies does not necessarily indicate presence of bias(278). They found there was very little evidence for differential associations between those with or without follow-up(278).

6.3. Public health implications

In last couple of years vitamin D deficiency has been reported word wide, affecting both genders and all age categories. Based on the research conducted so far it seems that optimal vitamin D status might have beneficial effects on certain aspects of human health, even though the final conclusions regarding causality on cardio-metabolic outcomes cannot bemade yet. Since repletion of vitamin D status by supplementation or just mere sun exposure is quite inexpensive it might be considered as cost-effective public health measure in prevention of certain conditions and improving some aspects of overall human health.

Before discussing the potential implications of adequate vitamin D status it is important to acknowledge the controversy behind the assessment of adequate vitamin D status and recommended daily allowance (RDA) of vitamin D intake. In 2011 the Institute of Medicine (IoM) published a report declaring 600IU daily intake of vitamin D to be enough to meet the requirements of 97.5% of the population regarding bone health. This recommendation caused many vitamin D experts to object(11). Very soon after The Endocrine Society published a clinical practice guideline for evaluation, treatment and prevention of vitamin D deficiency where they recommended a much higher RDA for vitamin D; for example while the IoM RDA for the elderly (over 70 years) was 800IU of vitamin D the Endocrine Society recommended daily requirement from 1500IU to 2000IU(4). Further, the IoM report concluded the recommended 600IU to be enough to raise serum concentrations of 25-hydroxyvitamin D above 50nmol/l which they defined as adequate for ensuring bone health. Although cautiously, the Endocrine Society panel suggested that this concentration of serum 25-hydroxyvitamin D might not be enough to ensure optimal health. They suggested the concentrations of 75nmol/l and higher to be adequate. Since then, the debate on this topic had been ongoing but providing no definite answers. Finally, last year a report by Veugelers and Ekwaru shed some light on this issue(279). They found the calculations behind the IoM recommendations to be incorrect. According to calculations of Veugelers and Ekwaru using the IoM data they found the recommended 600IU of vitamin D intake to be sufficient to raise the 25-hydroxyvitamin D concentrations only up to 26.8nmol/l instead of 50nmol/l as suggested by IoM. Moreover, they calculated that 8895IU per day of vitamin D to be needed to raise serum 25-hydroxyvitamin D concentrations above 50nmol/l in 97.5% of the population. Of note is that this dosage is much higher than 4000IU which are defined as tolerable upper intake for vitamin D according to IoM. Recently, another confirmation of calculations of Veugelers and Ekwaru was published. Earlier this year Heaney and colleagues also confirmed the findings of these investigators (280). As a result RDAs for vitamin D and also cutoff levels for adequate vitamin D status are being analyzed by many investigators from the vitamin D field. Until final consensus is reached, it is important to note that the relation between vitamin D and health outcomes may be highly dependent on the dosage of vitamin D supplementation as well as how optimal levels of vitamin D are defined.

6.3.1. Screening for vitamin D deficiency

Many general practitioners have started testing for vitamin D deficiency on regular basis. The potential of having such a straightforward, cost-effective and easy to implement measure which would help prevent many diseases and contribute to increase in life expectancy together with the increase in overall quality of life raised enthusiasm among many.

However, according to a joint statement by a group of leading scientists researching on vitamin D, screening for vitamin D deficiency is not recommended for the general population but only for the specific groups at high risk of vitamin D deficiency, such as the elderly or pregnant women(4).

In the future, if causality is proven for other specific health outcomes including cardiometabolic diseases, screening for vitamin D deficiency in the general population could be a powerful tool in the prevention of different highly prevalent diseases since the costs of these measurements may be outweighed by the benefits of replete vitamin D status(281, 282).

6.3.2. Potential strategies to improve vitamin D status

Food fortification might serve as one of the tools in fighting the global challenge of vitamin D deficiency. Countries like the United States already fortify some of the basic food items like milk, margarine, orange juice or breakfast cereals. In Europe this is still not a common practice even though it is possible to buy for example margarine fortified with vitamin D(4).

Perhaps the most straightforward way to tackle the issue of vitamin D deficiency is vitamin D supplementation. Even though according to the Endocrine Society guidelines the tolerable upper intake level of vitamin D for adults considered to be safe is up to 10,000IU per day, some issues regarding this strategy still need to be clarified. As mentioned before, there is still no universal agreement on the right dosage of vitamin D needed for achieving the adequate vitamin D status and which concentrations of 25-hydroxyvitamin D represent the adequate vitamin D status. Also, it seems that vitamin D3 supplementation should be encouraged at the expense of supplementation with vitamin D2(9). Finally, the most natural way of improving ones vitamin D status is exposure to sunlight. To avoid the excessive sun exposure leading to sun burns and possibly to subsequent skin cancer it is important to educate the public on exposing safely to sunlight. For example, according to the recent recommendation by Vitamin D society of Canada short but frequent bouts of

sun exposure is the best way to make enough vitamin D. However, sometimes it is not wise to use general recommendations for all, since the duration of safe sun exposure also depends on skin pigmentation, geographical latitude, and time of the day. The education of public could be conducted via health promotion programs promoting activity outside which could offer an approach to eliminating vitamin D deficiency based on advice regarding safe sun exposure, adequate diet and supplement use.

6.4. Future perspectives

Large part of the literature published on vitamin D and its effects on human health still comes from observational studies. Observational studies might reveal associations between vitamin D status and different diseases however they can never be used to imply causality of these associations. To prove causality studies by randomized control trials (RCTs) are needed. Even though some RCTs have already been conducted most of them did not supplement vitamin D in dosages sufficient to adequately raise serum concentrations of 25-hydroxyvitamin D or were of very short duration- not sufficient to see the potential effects of the vitamin D supplementation. Now, the public but also vitamin D research society awaits the results from an ongoing vitamin D supplementation trial such as the VITAL study(283), which should shed some additional light on true effects of vitamin D on numerous aspects of human health. Briefly, VITAL is an ongoing study in 25,875 men and women in the United States. It investigates whether taking daily dietary supplements of vitamin D3 or omega-3 fatty acids reduces the risk for developing different diseases. The first results are expected to be published in 2017.

In conclusion, vitamin D seems to be a promising factor in prevention of premature death and probably of many prevalent diseases nowadays. However, the latter can be confirmed only after properly designed high quality supplementation trials publish their results.

Chapter 7
Summary

Summary

Chapter 1 of this thesis introduces the reader to the background of the main topic – vitamin D, its metabolism and physiological effects. Since its discovery vitamin D has been recognized as an important factor in calcium homeostasis and bone metabolism. Recently, there has been extensive research performed on possible extra-skeletal effects of vitamin D. Nowadays vitamin D deficiency has been linked to a higher risk of many prevalent diseases such as cardio-vascular disease or type 2 diabetes mellitus. Based on this new research it is considered that improving vitamin D status may be a cost-effective tool to improve public health. The main objective of this thesis was to investigate the association of vitamin D and cardio-metabolic health.

Chapter 2 presents a systematic review and meta-analysis of observational studies and randomised controlled trials (RCTs) on vitamin D and the risk of mortality. The objective was to evaluate the extent to which circulating biomarker and supplements of vitamin D are associated with mortality under various circumstances. With this meta-analysis we found that the evidence from observational studies shows inverse associations of circulating 25-hydroxyvitamin D with risks of vascular, cancer and nonvascular-noncancer deaths. Further we found that supplementation with vitamin D_3 reduces overall mortality among older adults significantly; however, further investigations are required to establish the optimal dose and duration, and whether vitamin D_3 affects mortality risk differently than vitamin D_2.

In chapter 3 we investigated the associations between vitamin D and metabolic outcomes. More specifically, in chapter 3.1 we focused on possible association between vitamin D status and prevalence of metabolic syndrome in the elderly since the findings on this topic in the elderly are inconsistent. The aim was to evaluate the association between vitamin D status and the metabolic syndrome in the elderly, as well as between vitamin D status and the components of metabolic syndrome (i.e. serum glucose, triglycerides (TG), HDL cholesterol (HDL-C), waist circumference (WC), and blood pressure (BP)). We found higher 25-hydroxyvitamin D concentrations in the elderly to be associated with a lower prevalence of metabolic syndrome and, in particular, with more beneficial HDL-C, TG, WC and serum glucose.

Further in this chapter (3.2) we examined the association between vitamin D and body composition in the elderly. We found lower serum 25-hydroxyvitamin D concentrations to be associated with a higher fat mass

percentage. We also demonstrated that the association between serum 25-hydroxyvitamin D and differential fat distribution in the elderly was mainly explained by BMI, which deserves further study.

The focus of chapter 4 was on the association of vitamin D and cardio-vascular health. Here we investigated the possible association between vitamin D status and the incidence of atrial fibrillation in the elderly. We found vitamin D status not to be associated with atrial fibrillation in any of the 3 multivariate models tested and concluded that this prospective cohort study did not support the hypothesis that vitamin D status is associated with atrial fibrillation.

Chapter 5 introduces new ideas in the vitamin D research. Firstly, we have investigated the possible interaction between serum magnesium levels and vitamin D in relation to incidence of type 2 diabetes mellitus (5.1). We found that the association between 25-hydroxyvitamin D and type 2 diabetes mellitus may depend on adequate serum magnesium levels. These results imply that it may be important in clinical practice to assess serum 25-hydroxyvitamin D and magnesium levels simultaneously to identify people at risk of type 2 diabetes mellitus. In the second part of chapter 5 (5.2), we explored the association between vitamin D and serum lipids. Higher levels of vitamin D have been associated with lower rates of cardiovascular disease perhaps through improved lipid profiles. However, results reported were inconsistent and the direction of the association between vitamin D and lipid levels remained unknown. For that reason the aim of this chapter was to assess the potential bidirectional associations between 25-hydroxyvitamin D and blood lipids using path analyses in cross-lagged models a novel approach in analyzing this kind of data. Our results from path analyses on repeatedly measured 25-hydroxyvitamin D and lipid levels suggest that total cholesterol might be related with a decrease in 25-hydroxyvitamin D concentrations, but not the other way around, whereas the observed inverse association between HDL cholesterol and 25-hydroxyvitamin D may be bidirectional.

Chapter 6 discusses the main findings of this thesis as well as methodological issues arising from the design of our study, public health implications of our findings and future directions in vitamin D research. Repletion of vitamin D status might be considered as a cost-effective public health measure in prevention of certain conditions and improving some aspects of overall human health. However, to prove the causality of the association of vitamin D

and different diseases with the certainty, properly designed randomized controlled trials are needed.

Samenvatting

Hoofdstuk 1 is beschrijft de algemene introductie van vitamine D, en het metabolisme en fysiologische effecten. Vanaf de ontdekking van vitamine D werd deze vitamine gezien als een belangrijke factor in de stofwisseling van calcium en de aanmaak van bot. Recent is er uitgebreid onderzoek verricht naar mogelijke effecten van vitamine D buitende effecten op botgezondheid. Vandaag de dag laten steeds meer studies zien dat vitamine D deficiëntie ook gerelateerd aan een hoger risico op veelvoorkomende aandoeningen zoals cardiovasculaire ziekten en type 2 diabetes mellitus. Gebaseerd op deze bevindingen, wordt verondersteld dat het verbeteren van de vitamine D status van de bevolking wellicht een kosten-effectieve methode is om de volksgezondheid te verbeteren. Het primaire doel van dit proefschrift was om de relatie te onderzoeken tussen vitamine D en cardiometabole gezondheid.

Hoofdstuk 2 beschrijft een systematisch literatuurreview met een meta-analyse van observationele onderzoeken en gerandomiseerde interventie studies die de relatie bestudeerden van vitamine D en het risico op vroegtijdige sterfte. Het doel van deze review was om te onderzoeken in welke mate vitamine D bloedwaardes of vitamine D supplementen gerelateerd waren aan hetrisico op sterfte . De meta-analyse liet zien dat er in observationele onderzoeken een relatie is tussen lagere bloedwaarden van vitamine D en een hoger risico op sterfte aan hart-en-vaatziekten, sterfte aan aan kanker, en sterfte door andere oorzaken. Ook werd gevonden dat vitamine D_3 supplementen het risico op vroegtijdige sterfte verlagen. Echter, vervolgonderzoek moet uitwijzen wat de optimale dosis en duur van supplementatie is, en of vitamine D_3 een ander effect heeft op overlijdensrisico dan vitamine D_2.

In hoofdstuk 3 is de relatie tussen vitamine D en metabole uitkomsten bestudeerd. In het eerste gedeelte, hoofdstuk 3.1., lag de focus op mogelijke associaties tussen vitamine D bloedwaarden en metabool syndroom bij ouderen, aangezien bevindingen van eerder onderzoek bij ouderen inconsistent waren. Het doel was daarom om bij ouderen te onderzoeken of er een relatie was tussen vitamine D bloedwaarden en metabool syndroom, en de individuele componenten van het metabool syndroom; bloedwaarden van

glucose, triglycerides en HDL cholesterol, en buikomtrek en bloeddruk. Mensen met hogere bloedwaarden van 25-hydroxyvitamine D hadden minder vaak metabool syndroom, en hadden met name gunstigere waardes van HDL-cholesterol, triglycerides en glucose en een lagere buikomvang.

In het tweede gedeelte van dit hoofdstuk, hoodstuk 3.2, lag de focus op de relatie van vitamine D met lichaamssamenstelling van ouderen. Lagere bloedwaardes van 25-hydroxyvitamine D waren gerelateerd aan een lager vetpercentage. Daarnaast werd gezien dat de associatie tussen bloedwaardes van 25-hydroxyvitamine D en vetverdeling van het lichaam voornamelijk werd verklaard door BMI.

De focus van hoofdstuk 4 lag op de relatie tussen vitamine D en cardiovasculaire gezondheid. Hierin werd onderzocht wat de relatie is tussen bloedwaarden van vitamine D en de aanwezigheid van boezemfibrilleren bij ouderen. Er werd geen relatie gevonden tussen vitamine D bloedwaarden en boezemfibrilleren in de verschillende multivariate modellen die geanalyseerd waren. Geconcludeerd kan worden dat er op basis van deze studie geen bewijs is voor de hypothese dat vitamine D gerelateerd is aan boezemfibrilleren.

Hoofdstuk 5 beschrijft nieuwe hypotheses in vitamine D onderzoek. In hoofdstuk 5.1. is de mogelijke interactie van vitamine D en magnesium bestudeerd, in relatie tot type 2 diabetes mellitus. Er werd gezien dat de relatie tussen 25-hydroxyvitamine D bloedwaarden en type 2 diabetes mellitus mogelijk afhangt van bloedwaarden van magnesium. Deze resultaten impliceren dat het wellicht belangrijk is om in de kliniek zowel 25-hydroxyvitamine D als magnesium waarden in het bloed te bepalen, om mensen met een hoog risico op type 2 diabetes mellitus te identificeren.

In het tweede gedeelte van dit hoofdstuk, hoofdstuk 5.2, is de relatie tussen vitamine D en cholesterol bloedwaarden onderzocht. De relatie tussen vitamine D en risico op cardiovasculaire aandoeningen zou wellicht verklaard kunnen worden door gunstigere cholesterol waarden. Eerder onderzoek was echter niet consistent en de richting van de relatie tussen vitamine D en cholesterol was niet bekend. Daarom was het doel van het onderzoek in dit hoofdstuk om de mogelijke biderectionele relatie te analyseren tussen 25-hydroxyvitamine D en cholesterol waarden, met behulp van analyses met herhaaldelijk gemeten 25-hydroxyvitamine D en cholesterol waarden. De resultaten van deze analyses suggereren dat hoger totaal cholesterol wellicht

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gerelateerd is aan een afname van 25-hydroxyvitamine D bloedwaardes, maar niet andersom. Echter was de relatie tussen hoger HDL-cholesterol en lagere 25-hydroxyvitamine D mogelijk wel bidirectioneel.

Tot slot worden in hoofdstuk 6 de belangrijkste bevindingen van dit proefschrift bediscussieerd, evenals de methodologische problemen bij dit type onderzoek en de implicaties voor de maatschappelijke gezondheidszorg. Ook worden aanwijzingen gegeven voor vervolgonderzoek naar vitamine D. Het optimaliseren van vitamine D bloedwaardes is mogelijk een kosten-effectieve methode voor de maatschappij om sommige chronische aandoeningen te voorkómen en gezondheid te verbeteren. Echter is het noodzakelijk dat er meer interventie studies worden gedaan om de mogelijke causale gezondheidseffecten van vitamine D aan te tonen.

Chapter 8
Appendices

8.1. References

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8.3. PhD Portfolio

Summary of PhD training and teaching

Name of the PhD student: Anna Vitezova
Erasmus MC department: Department of Epidemiology
PhD period: August 2012 - July 2015
Promotors: Prof.dr. Oscar H. Franco
                                 Prof.dr. Andre G. Uitterlinden
Co-promotors: Dr. Jessica C. Kiefte - de Jong
                                 Dr. M. Carola Zillikens

Training

Master of Science in Health Sciences (NIHES)
Specialization: Epidemiology   (2012-2013)

Courses:
Principles of research in medicine
Methods of public health research
Topics in meta-analysis
Health economics
Introduction to global public health
Methods of health services research
Primary and secondary prevention research
Causal inference
History of epidemiologic ideas
Social epidemiology
Markers and prognostic research
Advances in genomics research
Advances in epidemiologic analysis
Study design

Biostatistical methods I: basic principles
Methodological topics in epidemiologic research
Biostatistical methods II: classical regression models
Public health research method
Women's health
Environmental epidemiology (Institute of Public Health, Cambridge, UK)
Public health in low and middle income countries
Introduction to medical writing
Courses of the quantitative researcher

Other courses and workshops

English biomedical writing, ErasmusMC	(2013)
Integrity in scientific research, ErasmusMC	(2014)
Literature search - Pubmed, Medical library, ErasmusMC	(2013)
Literature serach - other databases, Medical library, ErasmusMC	(2013)
Endnote course, Medical library, ErasmusMC	(2013)
Workshop systematic review and meta - analysis, ErasmusAGE	(2012)
Master classes in epidemiology, NIHES	(2012, 2013)
Advanced medical writing and editing, NIHES	(2014)
Introduction to R, MolMed	(2014)

Seminars and conferences

Seminars, Department of epidemiology	(2012-2015)
Research meetings, ErasmusAGE	(2012-2015)
Research meetings, CVD group	(2012-2015)
2020 meetings, Department of epidemiology	(2012-2015)
Research meetings, nutritional epidemiology (SIGN-E), Department of epidemiology	(2012-2015)
PhD day, Promeras, ErasmusMC	(2014)

Presentations

Poster presentation:

EUROPrevent, Amsterdam, the Netherlands	(2014)

Oral presentation:

Meeting of Vitamin D Society of Canada, Toronto, Canada	(2014)
ErasmusAGE research meeting, Department of epidemiology, ErasmusMC	(2014)
CVD group research meeting, Department of epidemiology, ErasmusMC	(2014)
2020 meeting, Department of epidemiology, ErasmusMC	(2014)

Teaching

Leiden University College, The Hague, the Netherlands   (2014)

Supervising students

Natasha Cartolano, student of medicine, Brazil
Tugce Aca, undergraduate student of nutrition, Turkey
Anna Popkova, student of medicine, Russia

Additional publications (not included into the thesis)

1.   Authors' reply to Grant and Garland and to Bolland and colleagues; coauthor; published (BMJ. 2014 Apr 29;348:g2931);
2.   Effects of protein intake on blood pressure, insulin sensitivity and blood lipids in children: a systematic review; second author; published (Br J Nutr. 2015 Feb;113(3):383-402);
3.   Gene environment interactions of circadian-related genes for cardio-metabolic traits; contributing author; accepted for publication (Diabetes Care);
4.   Vitamin D and C-Reactive Protein: A Mendelian Randomization Study; coauthor; published (PLoS One. 2015 Jul 6;10(7):e0131740.);
5.   The effects of lutein on cardiometabolic health across the life course: a systematic review and meta-analysis; coauthor; under review (BMJ).