The relationship of vitamin D status to risk of cardiovascular disease and mortality

Tea Skaaby

This review has been accepted as a thesis together with five previously published papers by University of Copenhagen 9 October 2014 and defended on 24 November 2014.

Tutors: Allan Linneberg, Lise Lotte Nystrup Husemoen & Charlotta Pisinger.

Official opponents: Simon Francis Thomsen, Kaare Christensen & Ulf Lindblad.

Correspondence: Research Centre for Prevention and Health, Glostrup University Hospital, Nordre Ringvej 57, Building 84/85, 2600 Glostrup, Denmark.

E-mail: tea.skaaby.01@regionh.dk

Dan Med J 2015;62(2):B5008

LIST OF PAPERS

1. Skaaby T, Husemoen LL, Pisinger C, Jorgensen T, Thuesen BH, Fenger M, Linneberg A. Vitamin D status and changes in cardiovascular risk factors: a prospective study of a general population. Cardiology 2012;123(1):62-70.

2. Skaaby T, Husemoen LL, Martinussen T, Thyssen JP, Melgaard M, Thuesen BH, Pisinger C, Jorgensen T, Johansen JD, Menne T, Carlsen B, Szecsi PB, Stender S, Fenger RV, Fenger M, Linneberg A. Vitamin D status, filaggrin genotype, and cardiovascular risk factors: a Mendelian randomization approach. PLoS One 2013;8(2):e57647.

3. Skaaby,T, Husemoen LL, Pisinger C, Jorgensen T, Thuesen BH, Rasmussen K, Fenger M, Rossing P, Linneberg A. Vitamin D status and 5-year changes in urine albumin creatinine ratio and parathyroid hormone in a general population. Endocrine 2013 Oct;44(2):473-80.

4. Skaaby T, Husemoen LL, Pisinger C, Jorgensen T, Thuesen BH, Fenger M, Linneberg A. Vitamin D status and incident cardiovascular disease and all-cause mortality: a general population study. Endocrine 2012 Jun;43(3):618-25.

5. Skaaby T, Husemoen LL, Pisinger C, Jorgensen T, Thuesen BH, Fenger M, Linneberg A. Vitamin D status and cause-specific mortality: a general population study. PLoS One 2012;7(12):e52423.

INTRODUCTION

VITAMIN D

Vitamin D is a fat soluble vitamin ingested from the diet and dietary supplements, but the main source of vitamin D is solar radiation of the skin which transforms 7-dehydrocholesterol to vitamin D (1). Whether ingested or synthesised, vitamin D is transported to the liver by a specific vitamin D binding protein (VDBP) and hydroxylated into the primary circulating form, 25-hydroxyvitamin D (25-OH-D) (2). It is then mainly metabolised to its active form, 1,25-dihydroxyvitamin D, in the kidneys, although many tissues can in fact convert the primary circulating form of vitamin D into its active form (1).

Vitamin D exists in two forms: Vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol). Vitamin D3 is of animal origin or produced in the skin, while vitamin D2 is of plant origin (1). Natural dietary sources of vitamin D are mostly D3 (e.g. from fish and egg yolk) with only very small amounts of D2 coming from plants. 25-OH-Ds are the intermediate metabolites of vitamin D2 and D3.

Vitamin D is an essential regulator of calcium homeostasis and bone mineralisation. It is known to play a key role in bone health, and deficiency causes rickets, osteomalacia and osteoporosis. Vitamin D controls the uptake of intestinal calcium, which in turn regulates the expression, production and secretion of parathyroid hormone (PTH) (3). PTH is an important hormone for bone health, maintaining normal serum concentrations of calcium and phosphate. Also, there is a long-recognised inter-relationship between bone and muscle tissue; decreased muscle mass leads to decreased bone mass due to decreased muscle loading applied on the bone (4). However, the question of whether vitamin D supplementation can prevent reduced muscle strength, falls and fractures in the elderly is still unresolved (1;4-6).

The vitamin D receptor is found in most body tissues, and the hormonally active form of vitamin D has a diverse array of biological functions ranging from anti-proliferative and anti-angiogenic effects to modulation of the immune system (1). Low vitamin D status has recently been found to be associated with a wide range of diseases including cardiovascular disease (CVD) and diabetes (1;7;8).

Serum 25-OH-vitamin D is generally accepted as first choice measurement of vitamin D status, and it is used to classify vitamin D status (9). Most assays use some form of antibody technology, although several reference and research laboratories use liquid chromatography tandem mass spectroscopy enabling a distinction between 25-OH-D2 and 25-OH-D3 (4). The optimal vitamin D level is still under debate, in part because of the difficulty in comparing assays – inter-assay and inter-laboratory comparisons show significant variability – and in part because of the different possible thresholds of adequacy for different diseases; classifications are primarily based on bone-related outcomes because existing evidence is insufficient to make recommendations regarding other diseases (4;8). Thus, the Endocrine Society Guidelines classify 25-OH-D levels below 50 nmol/L (divide by 2.496 to convert nmol/L to ng/mL) as deficient and levels of 75 nmol/L as sufficient, whereas the Institute of Medicine (IOM) suggests vitamin D deficiency to be indicated by 25-OH-D levels below 30 nmol/L and a level of 50 nmol/L to be sufficient (10). The classifications of vitamin D status according to the Danish Health and Medicines Authority are summarised in table 1 (11).

Table 1
Classification of vitamin D status according to the Danish Health and Medicines Authority

Vitamin D, nmol/l	
<12	Severely deficient
12-25	Deficient
25-50	Insufficient
>50	Sufficient
75-150	Optimal for patients with osteoporosis or kidney disease
>200	Risk of toxicity

Vitamin D deficiency and insufficiency are common worldwide (1). In Denmark, Thuesen et al. found the prevalence of vitamin D deficiency (<25nmol/l) and insufficiency (<50nmol/l) among adults to be 13.8% and 52.2%, respectively (12). A recent meta-analysis found that more than one-third of the studies included mean 25-OH-D values below 50 nmol/l. They also concluded that newborns and institutionalised elderly worldwide appeared to be at a higher risk of exhibiting lower 25-OH-D values (13). Other determinants of vitamin D include season of blood sampling, age, gender, skin pigmentation, sun exposure, body mass index (BMI), physical activity, smoking habits, and alcohol consumption (8;12;14).

GENETIC DETERMINANTS OF VITAMIN D

Recently, genetic markers of various exposures have become popular instruments for evaluating causal effects (15). The most frequently used genetic markers, single-nucleotide polymorphisms (SNPs), are DNA sequence variations with a single nucleotide in the genome differing between members of a biological species or paired chromosomes in a human. A number of known SNPs, including common variants at the DHCR7/NADSYN1 and CYP2R1 loci, have been shown to be associated with circulating 25-hydroxyvitamin D (16). Also, loss-of-function mutations in the filaggrin gene have been found to result in up to 10% higher serum vitamin D concentrations (17), supposedly due to a decreased UV-protection of the keratinocytes (18). Mutations affect almost 10% of Northern Europeans, of which 2282del4 and R501X account for about 80% of the known mutations (19;20). Filaggrin deficiency is known to cause ichtyosis vulgaris, which is characterised by xerosis, keratosis pilaris and palmar hyperlinearity, but also increases the risk of atopic dermatitis, allergic rhinitis, asthma, and food allergies (21;22).

Mendelian randomisation is a term describing the random allocation of alleles during meiosis (23). The allocation is expected to be independent of behavioural and environmental factors allowing the estimation of un-confounded risk associations that are not due to reverse causation (23). If there is a lack of evidence from randomised controlled trials (RCTs), a Mendelian randomisation approach may be used to strengthen causal inference. Mendelian randomisation uses genetic variants, i.e. as instrumental variables (IVs), to estimate the causal effect of phenotypes, such as vitamin D status, on disease-related outcomes, and is believed to overcome unmeasured confounding factors such as sun exposure or dietary habits (24;25).

The three main assumptions when using a genetic variant as an instrumental variable are that it is associated with phenotype, it is independent of unmeasured confounders, and it is independent of the outcome (here cardiovascular risk factors) given the phenotype (here vitamin D status) and the unmeasured confounders (see figure 1) (24).

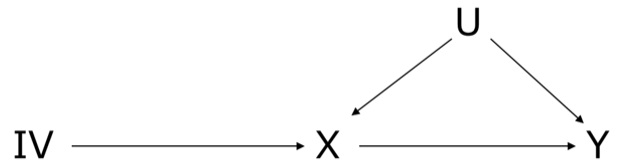

Figure 1
Directed acyclic graph (DAG) of an instrumental variable (IV). U refers to unmeasured confounders, X to the exposure and Y to the outcome.

VITAMIN D AND MORTALITY

Vitamin D affects a large number of genes, including those responsible for the regulation of cellular differentiation, proliferation, apoptosis, and angiogenesis, giving it the potential to affect much more than the musculoskeletal system (1). Low vitamin D status has been associated with mortality in several studies (26;27). A meta-analysis of vitamin D supplementation reported a decrease in mortality, whereas a recent one found reduced mortality with vitamin D and calcium supplementation in the elderly, but no effect of vitamin D supplementation alone (28;29). A Cochrane review found that vitamin D3 seemed to decrease mortality in elderly people, whereas vitamin D2 had no statistically significant effect; the study concluded that more RCTs are needed (30).

The specific causes of death underlying the association with mortality are uncertain, but there is no shortage of possibilities: in observational studies, in addition to cardiovascular disease (CVD), low vitamin D status is associated with an increased risk of endocrine and metabolic diseases such as diabetes mellitus, obesity, and metabolic syndrome, cancers such as colorectal cancer (31), lung cancer (32), and breast cancer (33), respiratory disease such as chronic obstructive pulmonary disease (COPD) (34;35), and respiratory infections (36), diseases of the digestive system such as liver disease (37;38), inflammatory bowel disease (39), and coeliac disease (39), and mental disorders such as dementia (40;41), and depression (42), and kidney disease (1) such as albuminuria (43). Some of the possible extraskeletal effects are shown in table 2.

VITAMIN D AND CARDIOVASCULAR DISEASE

CVD is a major cause of mortality and morbidity with ischaemic heart disease (IHD) and stroke accounting for more deaths worldwide than any other disease and is the leading cause of death in the western world (57). Although IHD and stroke have several well-established risk factors in common, the reasons for the global variation in the relative burdens of these diseases are poorly understood (58). The pathogenesis involves the formation of atherosclerotic plaques, which eventually ruptures with superimposed thrombosis (59).

Atherosclerotic plaque formation in the vascular tree is a complex process, involving several pathological pathways. Hypertension, abnormal blood lipids, and the metabolic syndrome are three of the major modifiable risk factors for CVD (60). The metabolic syndrome is a cluster of cardiovascular and metabolic disorders with insulin resistance, central obesity, dyslipidaemia and hypertension being the major components (61;62). Hypercholesterolaemia results in an increased influx of low density lipoprotein (LDL) cholesterol, which is released from the LDL

Table 2
Proposed effects of low vitamin D status on different organ systems/diseases

Organ system or disease	Example	Possible pathway or mechanism
Liver disease	Cirrhosis Viral hepatitis Fatty liver disease	Inflammation and fibrosis (39) Reduced antiviral response (44) Fat accumulation in hepatocytes (45) Steatosis (46)
Kidney	Chronic kidney disease	Albuminuria (43) Reduced GFR (47) Increased PTH (3)
Mental disorders	Alzheimer's Depression	Neuronal degeneration (48) Vascular neuropathology (49)
Respiratory disease	COPD Infections (e.g. tuberculosis)	Fibroblast proliferation (50) Reduced tissue remodelling (50) Reduced innate immunity (51)
Neoplasms	Breast cancer Colorectal cancer Lung cancer	Reduced induction of apoptosis (1) Reduced prevention of angiogenesis in malignant cells (1)
Endocrine diseases	Diabetes mellitus	Reduced insulin production (52) Increased insulin resistance (52) Reduced insulin sensitivity due to a reduction of osteocalcin (8)
Other autoimmune disorders	Multiple sclerosis	Reduced restraining of the adaptive immune system (4)
Circulatory disease	Ischaemic heart disease Stroke	Hypercholesterolaemia (53) Hypertension (54) Obesity (55) Metabolic syndrome (56) Diabetes (7)

Abbreviations: COPD, chronic obstructive pulmonary disorder; GFR, glomerular filtration rate; PTH, parathyroid hormone

particles and oxidised, in turn attracting and stimulating macrophages – a key step in the inflammatory process (63). The process is increased in cases of insufficient HDL-cholesterol, which normally removes cholesterol from tissues and carries it back to the liver. Adipose tissue can give rise to cytokines that decrease insulin sensitivity and puts the system into a pro-inflammatory state (63). Hypertension puts added force on the artery walls. Over time, the extra pressure and the exertion of oxidative stress will damage the arteries and make them more vulnerable to the narrowing and formation of plaques associated with atherosclerosis (64).

Microalbuminuria refers to a situation where the kidney leaks small amounts of albumin into the urine. As a marker of chronic kidney disease, it is associated with a higher risk of cardiovascular disease (65-67) and can be diagnosed from the albumin to creatinine ratio in the urine. A decrease in UACR is associated with a lower renal and cardiovascular risk, making UACR an important therapeutic target (68;69). Previous studies have implied an effect of vitamin D on microalbuminuria (43;69). PTH, on the other hand, is inversely associated with glomerular filtration rate (GFR), is known to be closely related to vitamin D status (3), and diseases with increased PTH are associated with a higher risk of cardiovascular disease and death (70): Hagstrom et al. found elevated plasma PTH to account for 20% of the population-attributable risk proportion of cardiovascular mortality (70).

The presence of vitamin D receptors in tissues like vascular smooth muscle, endothelium and cardiomyocytes and an increasing amount of epidemiological evidence suggest that vitamin D deficiency can adversely affect the cardiovascular system; as a result, vitamin D has been proposed as a modifiable risk factor for cardiovascular disease (1;71). Vitamin D has many proposed effects that could affect the cardiovascular system. A selection of these is summarised in table 3.

A meta-analysis of prospective studies reported an inverse association between vitamin D status and cardiovascular risk. RCTs of the effect of vitamin D supplementation or UV radiation (to improve the vitamin D status) on the lipid profile and blood pressure have been divergent, but two meta-analyses of vitamin D supplementation found weak evidence to support a small effect of vitamin D on blood pressure (79;80).

Table 3
Possible vitamin D mediated effects on different cardiovascular risk factors

Cardiovascular risk factors	Possible vitamin D mediated effect
Hypercholesterolaemia /dyslipidaemia	Suppression of PTH (3) (PTH can reduce lipolysis) Increased calcium levels causing a reduction of hepatic triglyceride formation and secretion (72)
Hypertension	Inhibition of the renin-angiotensin system (73;74) Reduction of vascular calcification (75)
Obesity	Mobilisation of free fatty acids from adipose tissue (76) Increase in energy expenditure due to uncoupling of oxidative phosphorylation in adipose tissue (77)
Albuminuria	Prevention of podocyte loss (43) Prevention of glomerulosclerosis (43)
Diabetes	Insulin production increase (78) Insulin resistance decrease (78)
Metabolic syndrome	Please see under the individual components*

* The individual components of metabolic syndrome are insulin resistance, central obesity, dyslipidaemia and hypertension.
Abbreviations: PTH, parathyroid hormone

OBJECTIVES

The aims were to investigate

1. the association of vitamin D status to 5-year changes in cardiovascular risk factors such as blood pressure, lipid profile, the metabolic syndrome and UACR
2. the causal effect of vitamin D status on cardiovascular risk factors using a known genetic determinant of vitamin D status and a Mendelian randomisation approach
3. the association of vitamin D status to the incidence of cardiovascular disease and all-cause mortality
4. the association of vitamin D status to cause-specific mortality

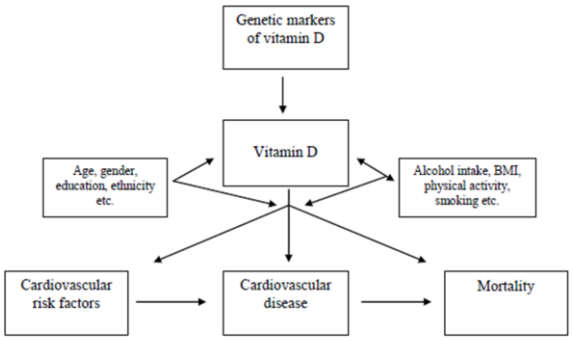

Figure 2
Simplified diagram of determinants of vitamin D status and cardiovascular outcomes and mortality.

MATERIAL AND METHODS

STUDY POPULATIONS

Data from the 3 population based studies Monica10, Inter99 and Health2006 conducted at the Research Centre for Prevention and Health were used (81). The three populations were recruited from the Danish Central Personal Register as random samples of the same background population at different time points. The studies included questionnaires, physical examinations, and blood tests. Characteristics of the included studies and main exposures are summarised in table 4. For a more comprehensive description, please see the article in question.

Serum samples from the participants in the Monica10 and the Inter99 studies were stored at -20°C, while the samples from the Health2006 study were stored at -80°C, until the analyses of 25-OH-D in 2009, 2010, and 2011, respectively (see table 4).

REGISTRY-BASED DIAGNOSES

Denmark has several unique and comprehensive nationwide registries. The assignment of a unique civil registration number of all citizens enables the cross-linkage of different registers. Information on deaths and emigration status was obtained from the Danish Civil Registration System (82). The Danish National Patient Register holds information on all admissions to Danish hospitals since 1977 (83). Each admission is registered by one primary diagnosis and one or more optional secondary diagnoses according to the International Classification of Diseases (ICD). Likewise The Danish Registry of Causes of Death contains the date of death and up to three ICD-10 diagnoses suspected to be the cause of death (84). Denmark has never used the ICD-9, instead moving directly from the ICD-8 to the ICD-10 before the studies took place.

In paper 4, the included diagnoses were the ICD10 and ICD8 diagnoses involving IHD (ICD10; I20-25, and ICD8; 410-414) and stroke (ICD10; I60-69, and ICD8; 431, 433-434, and 436). We defined three end-points: A diagnosis of or death caused by IHD; a diagnosis of or death caused by stroke; or death by any cause. Participants were followed until 31 December 2008.

In paper 5, the major groups of diagnoses of death were (all ICD-10): neoplasms, C00–D48; endocrine, nutritional and metabolic diseases, E00–E90; mental and behavioural disorders, F00–F99; diseases of the nervous system, G00–G99; diseases of the circulatory system, I00–I99; diseases of the respiratory system, J00–J99; and diseases of the digestive system, K00–K93. The remaining deaths (n=96) consisted mainly of deaths caused by "Symptoms, signs and abnormal clinical and laboratory findings, not elsewhere classified" (R) and external causes of morbidity and mortality (X). Participants were followed until 31 December 2009.

CARDIOVASCULAR RISK FACTORS

Height and weight were measured with light clothes and without shoes. BMI was calculated as weight (kg) divided by height (m) squared. Waist circumference was measured between the lowest rib and the iliac crest. Blood pressure (mmHg) was calculated as the average of two measurements in the sitting position.

From fasting blood samples, the concentration of triglycerides, total cholesterol and high density lipoprotein (HDL) cholesterol was measured by enzymatic procedures (Boehringer Mannheim). The concentration of low density lipoprotein (LDL) cholesterol and very low density lipoprotein (VLDL) cholesterol was calculated by Friedewald's equation (85). The metabolic syndrome was defined according to the International Diabetes Foundation (86). PTH was measured as intact PTH (IMMULITE 2000 System, Siemens Healthcare Diagnostics, Deerfield, IL, USA). From spot urine samples, urine albumin and creatinine were analysed and the ratio calculated. Increased UACR was defined as UACR >4mg/g (the upper quartile at baseline). The classification of cardiovascular risk factors is summarised in table 5. For more detailed information, please see the article in question.

OTHER COVARIATES

The other covariates (and possible confounders) used for adjustments are summarised in table 6. For a more detailed description and definition, please see the relevant article.

STATISTICAL METHODS

Stata/IC 12.1 for Mac (StataCorp, College Station, Texas) was used for the IV analyses with dichotomous outcomes. All other analyses were performed with SAS, version 9.2 or 9.3 (SAS Institute Inc., Cary, N.C., USA). All p values were two-tailed, and statistical significance was defined as $p<0.05$. Descriptive characteristics of the participants were compared with non-parametric statistics (paper 4+1+5) or the chi-squared test (paper 2).

To take potential confounders into account, we used regression analyses (please see table 7 and the respective paper for more information). We used a complete case analysis in the regressions (only participants with complete information on all considered variables were included). In paper 2, the Bonferroni adjusted p-values were also given to adjust for multiple comparisons. Inter99 data were considered observational, and analyses were adjusted for randomisation status.

Table 4
Characteristics of the included studies and main exposures.

Characteristics	Monica10*	Inter99**	Health2006
Year of examination	1993-1994	1999-2001	2006-2008
Number	2656	6794	3471
Age, years	40-71	30-60	18-69
Participation rate	64.3%***	52.5%	43.8%
In paper	**2**, **4**, **5**	**1**, **2**, **3**, **4**, **5**	**2**
25-OH-D			
Method	IDS-SYS 25-Hydroxy Vitamin D method with the IDS-iSYS Multi-Discipline System (IDS Nordic A/S, Herlev, Denmark)	High performance liquid chromatography (HPLC)	Immunoassay using Cobas e411 (Roche Diagnostics, Mannheim, Germany)
Median (IQR), nmol/l	60.9 (44.7-80.8)	48.0 (33.0-65.0)	41.2 (29.2, 55.2)
Filaggrin genotype			
Genotyped mutations	R501X and 2282del4	R501X, 2282del4 and R2447X	R501X, 2282del4 and R2447X
Fractions genotyped	99.96%	99.95%	99.40%
Mutation carriers	7.6%	8.9%	9.0%

* The Monica10 study is a 10-year follow-up study of the Monica1 study conducted in 1982-1984.

** Baseline data. The 5-year follow-up study used in paper 1 and 3 included 4,513 persons between 35-65 years.

*** Is calculated as: 2656/4130 (Please observe that the number differs from the participation rate given in paper 2, 4, and 5)

Abbreviations: 25-OH-D, 25-hydroxyvitamin D

POWER CALCULATION

Some of the hypotheses tested in this project are inherently explorative. However, an example of a calculation of power using Power3.0 is as follows: Of the approximately 2600 individuals participating in Monica10, we assumed that 500 individuals had died before 31 December 2007. The prevalence of low (< 50 nmol/l) vitamin D status was approximately 50% (12). Choosing a power of 0.8 and a two-sided alpha-level of 0.05 we would be able to detect an odds ratio of at least 1.3 for the association between low vitamin D and cumulated mortality during the follow-up.

COX REGRESSION

Multivariate Cox regression analyses were used in paper 4 and 5 where data from the Monica10 and Inter99 studies were merged. We used age as an underlying time axis and delayed entry. The few participants lost to follow-up (emigrated, disappeared, or unknown cause of death) contributed to the risk time until the date of their last registered activity.

In paper 4, the associations of baseline vitamin D status and the incidence of IHD, stroke, and total mortality were examined. Individuals with a diagnosis of IHD or stroke at baseline were excluded from the analyses of incident IHD and stroke.

In paper 5, the association of baseline vitamin D status and cause-specific mortality was examined. Vitamin D levels were divided into quartiles before merging the two studies to account for the different methods used to measure vitamin D and different storage times. The first (lowest) quartile was used as a reference. Participants were followed and contributed with risk time in the analyses until date of death, date of emigration or 31 December 2009, whichever came first.

LINEAR REGRESSION

We used linear regression analysis to model the cross-sectional associations between vitamin D status and lipids, blood pressure, anthropometrics (paper 2), UACR and PTH (paper 3) and to examine the changes during follow-up in blood pressure, lipids (paper 1), UACR and PTH (paper 3) as a function of baseline vitamin D status. The outcomes were transformed by the natural logarithm to obtain a normal distribution. All of the prospective models were adjusted for the baseline value of the outcome to adjust for regression to the mean and allow changes in explanatory variables to be interpreted as effects on outcome changes from baseline to follow-up (88). The ß-coefficients were back-transformed and reported as percent with 95% confidence intervals (CI).

LOGISTIC REGRESSION

The cross-sectional association of vitamin D status and metabolic syndrome (paper 2) and the prospective associations between baseline vitamin D status and the dichotomous outcomes development of metabolic syndrome, hypertension and hypercholesterolaemia (paper 1) were analysed by logistic regression models. Participants with baseline hypertension were excluded from the analyses of incident hypertension, and likewise when analysing for the outcomes incident metabolic syndrome and hypercholesterolaemia.

Table 5
Classification of cardiovascular risk factors

Hypertension	Systolic blood pressure > 140 mmHg, a diastolic blood pressure > 90 mmHg or in treatment for previously diagnosed hypertension
Hypercholesterolaemia*	Total cholesterol > 6.6 mmol/l or current treatment with cholesterol-lowering medication (paper **1**).
Metabolic syndrome	Waist circumference ≥94 cm for European men and ≥80 cm for European women and at least two of the following criteria: raised triglycerides (>1.7 mmol/l or specific treatment for this lipid abnormality); reduced HDL-cholesterol (<1.03 mmol/l for men, <1.29 mmol/l for women, or specific treatment for this lipid abnormality); raised blood pressure (systolic ≥ 130 mmHg, diastolic ≥85 mmHg, or treatment of previously diagnosed hypertension); elevated fasting plasma glucose (≥ 5.6 mmol/L or previously diagnosed type 2 diabetes).

* Notice that the most frequently used clinical threshold is 5.0 mmol/l (87)

Table 6
Exposure, outcome and other covariates used for adjustments in the included studies.

Paper	Exposure	Outcome	Other covariates in model 1	Other covariates in model 2	Other covariates in model 3
1	25-OH-D	Lipids, blood pressure, metabolic syndrome, hypertension, hypercholesterolaemia	Crude*	+Gender, age, season, randomisation status, physical activity, alcohol consumption, diet, WC**, BMI**, smoking habits and education	+PTH, UACR, and 5-year changes in BMI, smoking habits, alcohol consumption, diet and physical activity
2	25-OH-D***	Lipids blood pressure anthropometrics metabolic syndrome	+Study population, age, gender, education, season, intake of fish, physical activity, smoking habits, alcohol consumption and BMI**	NA	NA
3	25-OH-D	UACR, PTH, increased UACR	Crude*	+gender, age, season, randomization status, physical activity, alcohol consumption, diet, BMI, blood pressure****, smoking habits and education, 5-year changes in BMI, smoking habits, alcohol consumption, diet and physical activity.	NA
4	25-OH-D	IHD, stroke all-cause mortality	+Study group	+Gender, education, season	+Intake of fish, physical activity, smoking habits, BMI, and alcohol consumption
5	25-OH-D	Cause-specific mortality	+Study group	+Gender, education, season, intake of fish, physical activity, smoking habits, BMI and alcohol consumption	NA

* Adjusted for the baseline value of the outcome in the prospective linear regression analyses
** Not metabolic syndrome and anthropometrics
*** Filaggrin mutation carrier status used as instrument
**** Not PTH
Abbreviations: 25-OH-D, 25-hydrocyvitamin D; BMI, body mass index; IHD, ischaemic heart disease; NA, not applicable; PTH, parathyroid hormone; UACR, urinary albumin creatinine ratio; WC, waist circumference

INSTRUMENTAL VARIABLE ANALYSIS

Since very few participants had more than one filaggrin mutation (homozygotes or compound heterozygotes), filaggrin genotype was dichotomised into non-mutation carriers or carriers of at least one of the R501X, 2282del4, or R2447X filaggrin mutations. Filaggrin genotype was used as an instrumental variable (IV) for vitamin D status. Instrumental variable analysis, e.g. two stage least squares (2SLS), is a 2-stage analysis. Serum 25-OH-D concentrations were log2-transformed (since they were used as outcome in the first stage regression) and the continuous outcomes were log-transformed to meet requirements of normality. In the first stage of the IV analyses, the log2-transformed vitamin D status was regressed on the instrument, the dichotomised filaggrin genotype, and the observed covariates. In the second stage, the outcome of interest was regressed (e.g. log[HDL]) on the predicted values from the first-stage regression and the same observed covariates as for the first stage regression. For more information, please see paper 2. The regression coefficient of the predicted values from the second stage can be interpreted as the causal effect per doubling of vitamin D on the outcome. For continuous outcomes, the regression coefficients were back-transformed and reported as percent with 95% confidence intervals (CI). For dichotomous outcomes, the estimate is only an approximation of the causal effect (89).

ETHICS

All participants gave their informed written consent, and the studies were approved by the local Ethics Committees and the Danish Data Protection Agency. The recommendations of the Declaration of Helsinki were followed.

RESULTS

The main findings are summarised in table 7.

PAPER I

Vitamin D status and changes in cardiovascular risk factors: a prospective study of a general population

Among the 4,330 individuals who participated at baseline and the 5-year follow-up of the Inter99 study, the median baseline vitamin D concentration was 48.0 nmol/l. In multivariable linear regression analyses, a 10 nmol/l higher baseline level of vitamin D was associated with small but statistically significant decreases in triglycerides and VLDL-cholesterol by 0.52% (p = 0.03) and 0.66% (p = 0.005), respectively. Vitamin D status was not significantly associated with changes in total, HDL and LDL cholesterol, and systolic or diastolic blood pressure. In multivariable logistic regression analyses, the odds ratios per 10 nmol/l higher baseline vitamin D level were 0.95 ($p<0.05$) and 0.94 (p = 0.01) for the development of the metabolic syndrome and hypercholesterolaemia, respectively. There was no association between vitamin D and hypertension.

PAPER II

Vitamin D status, filaggrin genotype, and cardiovascular risk factors: a Mendelian randomization approach

Among the 11,983 included individuals from the Monica10, Inter99 and Health2006 studies, 8.6% were carriers of at least one filaggrin mutation. The distribution of genotype was not associated with the covariates, except for vitamin D. Carrying a filaggrin mutation was significantly associated with a higher vitamin D status in all three of the studies. Instrumental variable analyses showed a 23.8% (95% confidence interval, CI: 3.0, 48.6) higher HDL cholesterol level and a 30.5% (95% CI: 0.8, 51.3) lower serum level of triglycerides per vitamin D doubling. However, these associations were not statistically significant when applying the Bonferroni adjusted significance level. The remaining lipids showed non-significant changes in a favourable direction. A doubling of vitamin D gave a non-significantly lower odds ratio = 0.26 (95% CI: 0.06, 1.17) of the metabolic syndrome. There were no

Table 7
Summary of the main findings in paper I-V regarding the prospective associations between vitamin D status, cardiovascular risk factors, disease and mortality

Outcome	Total number of participants	Adjusted estimate
Paper 1		
HDL-cholesterol	3426	0.12 (–0.07, 0.31)€
LDL-cholesterol	3362	–0.16 (–0.42, 0.11) €
Triglycerides	3426	–0.52 (–0.99, –0.05) €
VLDL-cholesterol	3374	–0.66 (–1.1, –0.20) €
Total cholesterol	3426	–0.08 (–0.25, 0.10) €
Systolic blood pressure	3274	–0.05 (–0.18, 0.08) €
Diastolic blood pressure	3274	0.08 (–0.06, 0.22) €
Metabolic syndrome	2623	0.95 (0.90, 1.00) &
Hypertension	2571	1.01 (0.97, 1.05) &
Hypercholesterolaemia	2363	0.94 (0.90, 0.99) &
Paper 2		
HDL-cholesterol	10601	23.8 (3.0, 48.6)¤
LDL-cholesterol	10492	-16.9 (-31.9, 1.5) ¤
Triglycerides	10601	-30.5 (-51.3, -0.8) ¤
VLDL-cholesterol	10496	-27.0 (-47.6, 1.8) ¤
Total cholesterol	10601	-9.1 (-20.2, 3.5) ¤
Systolic blood pressure	9940	-2.8 (-9.9, 4.9) ¤
Diastolic blood pressure	9940	-3.8 (-11.1, 4.1) ¤
Body mass index	10940	-2.5 (-11.8, 7.7) ¤
Waist circumference	10931	-1.9 (-9.2, 6.1) ¤
Metabolic syndrome	10931	0.26 (0.06, 1.17)#
Paper 3		
Urine albumin creatinine ratio	3493	-0.92 (-1.71, -0.13)€
Parathyroid hormone	3440	-0.11 (-0.51, 0.30) €
Increased urine albumin creatinine ratio	2603	0.96 (0.92, 0.98)&
Paper 4		
IHD	8131	1.01 (0.98, 1.05)£
Stroke	8131	1.00 (0.96, 1.05) £
All-cause mortality	8329	0.95 (0.92, 0.99) £
Paper 5		
Death caused by		
Neoplasms	8329	0.81 (0.57, 1.2)%
Endocrine, nutritional and metabolic diseases	8329	0.21 (0.04, 1.1) %
Mental and behavioural disorders	8329	0.44 (0.14, 1.4) %
Diseases of the nervous system	8329	0.75 (0.21, 2.7) %
Diseases of the circulatory system	8329	1.1 (0.70, 1.7) %
Diseases of the respiratory system	8329	0.26 (0.09, 0.75) %
Diseases of the digestive system	8329	0.28 (0.10, 0.78) %

£Hazard ratio (95% CI) per 10 nmol/l higher 25-OH-D. &Odds ratio (95% CI) per 10 nmol/l higher 25-OH-D. %Hazard ratio (95% CI) for the 4th 25-OH-D quartile compared with the 1st quartile. ¤Relative difference in % (95% CI) per 25-OH-D doubling. # Odds ratio (95% CI) per 25-OH-D doubling. € % change per 10 nmol/l higher 25-OH-D.

statistically significant causal effects of vitamin D status on body mass index, waist circumference, or blood pressure.

The ordinary regression analyses showed statistically significant associations between vitamin D status and HDL, triglycerides, BMI, waist circumference and metabolic syndrome at the Bonferroni adjusted significance level.

PAPER III

Vitamin D status and 5-year changes in urine albumin creatinine ratio and parathyroid hormone in a general population

Among the 4,330 individuals who participated at baseline and the 5-year follow-up in the Inter99 study, cross-sectional analyses showed statistically significant associations between vitamin D status and UACR and PTH. In multivariable linear regression analyses, a 10 nmol/l higher baseline level of vitamin D was associated with a small but statistically significant decrease in UACR by 0.92% (p = 0.02), but a non-significantly lower PTH. In multivarible logistic regression analyses, the odds ratio for an increased UACR was 0.96 (p=0.0006) per 10 nmol/l higher baseline vitamin D level.

PAPER IV

Vitamin D status and incident cardiovascular disease and all-cause mortality: a general population study

Among the 9,146 individuals from the Monica10 and Inter99 studies followed until 31 December 2008, there were 478 cases of ischaemic heart disease, 316 cases of stroke, and 633 deaths during follow-up. The mean follow-up time was 10 years. Cox regression analysis with age as an underlying time axis showed a significant association between vitamin D status and all-cause mortality with a hazard ratio (HR) of 0.95 (p=0.005) per 10 nmol/l

higher vitamin D level after adjustment for confounders. We found no association between vitamin D status and incident ischaemic heart disease or stroke (HR=1.01, p=0.442 and HR=1.00, p=0.920, respectively).

PAPER V

Vitamin D status and cause-specific mortality: a general population study

Among the 9,146 individuals from the Monica10 and Inter99 studies who were followed until 31 December 2009, there were 832 deaths in total. The median follow-up time was 10.3 years. Most people died from neoplasms (n=338) or circulatory disease (n=221). Vitamin D was highest among those individuals who died of diseases of the nervous system or the circulatory system and lowest among those who died of endocrine, nutritional and metabolic diseases, mental and behavioural disorders and diseases of the digestive system.

We found statistically significant inverse associations between vitamin D status and death caused by diseases of the respiratory system (ptrend=0.0042), the digestive system (ptrend=0.0040), and endocrine, nutritional and metabolic diseases (ptrend=0.035). The hazard ratios were approximately 0.25 for all three groups for the fourth vitamin D quartile compared to the first. We found non-significantly lower hazard ratios for higher vitamin D status for death caused by mental and behavioural diseases and diseases of the nervous system, but no association between vitamin D status and death caused by neoplasms or diseases of the circulatory system.

DISCUSSION

MAIN FINDINGS

In this thesis, we found a significant inverse association between vitamin D status and all-cause mortality, but no associations between vitamin D status and the incidence of IHD or stroke. Rather, we found statistically significant inverse associations between vitamin D status and death caused by diseases of the respiratory and the digestive system and by endocrine, nutritional and metabolic diseases, but no association with death caused by neoplasms or diseases of the circulatory system.

In the traditional observational study, we found vitamin D status to be significantly associated with the incidence of hypercholesterolaemia and the metabolic syndrome, but there was no association between vitamin D status and incident hypertension. Triglycerides and VLDL cholesterol levels decreased significantly with higher vitamin D levels. In the Mendelian randomisation study, we found a causal association between vitamin D status and HDL cholesterol and triglycerides; however, this was not statistically significant when applying the Bonferroni adjusted significance level. In both studies, vitamin D status seemed to be associated with a more favourable lipid profile overall, strengthening causal inference between vitamin D status and lipid profile. The inverse association between vitamin D status and metabolic syndrome was statistically non-significant in the Mendelian randomisation study. In neither the traditional nor the Mendelian randomisation study was an association found between vitamin D status and blood pressure, BMI, or waist circumference.

We found statistically significant inverse cross-sectional and prospective associations between vitamin D status and UACR and incident increased UACR. As for PTH, we found an inverse cross-sectional but no prospective association with vitamin D status. Regarding the observed effects of vitamin D on both lipids and UACR, the effect sizes were small.

The observed association between filaggrin genotype and vitamin D status in the Inter99 study is confirmation of the results reported by Thyssen et al. in the Monica10 and Health20006 studies (17).

COMPARISON WITH OTHER STUDIES

Mortality

We confirmed the findings from similar studies reporting an inverse association between vitamin D status and all-cause mortality, an association which has shown to be both strong and consistent in observational studies (26;27;90-94). Interestingly, in a Mendelian randomisation study by Trummer et al. on vitamin D and mortality, using 3 genetic markers of vitamin D status and including 3,316 middle aged with a median follow-up of 9.9 years, a joint analysis yielded a mortality hazard ratio of 1.015 (95% CI: 0.962, 1.07) per 2.5 nmol/l genetically determined 25-OH-D (95). Likewise, a study by Jorde et al. using a genetic score of 4 vitamin D associated genetic variants found that the hazard ratio of dying was 1.01 (95% CI: 0.92, 1.10) for the genotype score quartile with the lowest vitamin D status compared to the highest. Although unable to exclude it, the findings suggest that the association between vitamin D and mortality may not be causal. However, a Cochrane review and meta-analysis of RCTs found vitamin D supplementation to give a moderate reduction of total mortality compared with placebo predominantly in older populations (96). They found that 161 people needed to be treated in order to prevent one death. The results, however, must be interpreted with caution because competing risks in older people might affect the results: the results are based on the assumption that the distribution of deaths among complete cases and participants lost to follow-up is equal. In a recent update of the Cochrane review, Bjelakovic et al. found that vitamin D3 seemed to decrease mortality in elderly people, whereas vitamin D2 had no statistically significant effect; the study concluded that more RCTs are needed (30). This is in line with an umbrella review of systematic reviews and meta-analyses of observational studies and randomised trials by Theodoratou et al. who concluded that highly convincing evidence of the role of vitamin D does not exist for mortality or any other outcome (6).

Cardiovascular disease

In general, cross-sectional and prospective studies often report an inverse association between vitamin D status and CVD, although there are some inconsistencies (91;92;97). Thus, the observed lack of association between vitamin D status and cardiovascular disease (paper 4) and circulatory disease mortality (paper 5) is inconsistent with the results from at least three studies finding an inverse association between vitamin D levels and risk of all-cause and CVD mortality among older people (26;93;94) and two studies who found an inverse association between vitamin D status and incident cardiovascular disease (98;99). Possible explanations for the different results include the fact that the above-mentioned studies examined older people, or subgroups, where vitamin D could be more important regarding CVD risk.

RCTs on this matter are few and inconclusive. Most were designed to evaluate the effect of vitamin D supplementation on bone health and often, vitamin D supplementation was combined with calcium supplementation. As a meta-analysis suggested calcium supplementation to be associated with a higher risk of cardiovascular disease, it is difficult to interpret the results of supplementation with both vitamin D and calcium (100). Howev-

er, two meta-analyses showed non-significant reductions of CVD events with vitamin D supplementation (98;101). In the previously mentioned umbrella review by Theodoratou et al., the relative risk of cardiovascular disease was 0.95 (95% CI: 0.86, 1.05) in the supplementation group (6).

Cardiovascular risk factors

Our results regarding serum lipids are in line with both the results from cross-sectional studies reporting a higher vitamin D level to be associated with a favourable lipid profile and a prospective study which showed an inverse association between vitamin D status and triglycerides (53). However, a Mendelian randomisation study found no significant associations between vitamin D associated SNPs and total cholesterol or triglycerides (102), and Jorde et al. summarised evidence from the few randomised controlled trials (RCTs), examining a possible effect of vitamin D supplementation on lipid profile as inconclusive (103). None of the intervention studies included were, however, designed for evaluating the relation between vitamin D and lipids, and they may have lacked power (103).

Most observational studies of vitamin D status and blood pressure are in favour of an inverse association, although the results are somewhat divergent. Thus, Kunutsor et al. performed a large meta-analysis on prospective studies and reported a significant inverse association of baseline vitamin D status with incident hypertension (104). In a Mendelian randomisation study of more than 100,000 individuals, Vimaleswaran et al. found that an increase in genetically instrumented vitamin D was associated with a statistically significant decreased risk of hypertension (105). A meta-analysis on RCTs of vitamin D supplements and blood pressure reported weak evidence to support a small effect of vitamin D supplementation on lowering the blood pressure in hypertensive patients, whereas another meta-analysis found a non-significant reduction of systolic blood pressure (80;106). Interestingly, in two recently performed RCTs by Forman et al. and Larsen et al., there was a blood lowering effect of vitamin D supplementation in hypertensive individuals with below normal vitamin D levels, in black Americans and in Danes during winter, respectively (107;108). The above-mentioned results indicate that vitamin D supplementation may have a beneficial effect on blood pressure in hypertensives with a low vitamin D status rather than in normotensives with a normal vitamin D level. This may partly explain our observed lack of association and could also explain why another recent Mendelian randomisation case-control study reported no effect (102). Further RCTs are needed to determine whether the above-mentioned findings are brought about by a causal effect of vitamin D status on blood pressure, or if they are due to e.g. chance or publication bias.

Although small cohorts have found associations between low vitamin D status and obesity, the underlying mechanisms and direction of causality are not clear (55). The inverse association might be explained by the fact that the fat soluble vitamin D is sequestered in the adipose tissue, resulting in lower levels in obese individuals, i.e. that obesity causes low vitamin D status. Interestingly, Vimaleswaran et al. performed a large bi-directional Mendelian randomisation study which showed that obesity leads to vitamin D deficiency and not vice versa (109). Likewise, Jorde et al. found no association between vitamin D associated SNPs and BMI (102). An important question – and a possible direction for further research – is the level of bioavailability of vitamin D in adipose tissue.

Our results support the inverse association between vitamin D status and the incidence of the metabolic syndrome reported by Forouhi et al. (56). However, as mentioned above, vitamin D status is associated with obesity, and since excess weight is a major component of the metabolic syndrome, the association between vitamin D status and the metabolic syndrome could be confounded by the association between vitamin D status and excess weight.

The observed association between vitamin D status and UACR is in line with a cross-sectional study by de Boer et al. who found a stepwise increase in the albuminuria prevalence with decreasing vitamin D quartiles (43). A recent prospective study by O'Seaghdha et al., however, found no association between vitamin D status and incident albuminuria (110). As for PTH, we confirmed the well-established cross-sectional association with vitamin D, but failed to show a longitudinal association.

Cause-specific mortality and disease

The observed inverse association between vitamin D status and death caused by endocrine, nutritional and metabolic diseases – diabetes mellitus accounting for three quarters – is consistent with results from previous observational studies (111). On the other hand, RCTs have largely failed to show a beneficial effect of vitamin D supplementation on glucose homeostasis (112;113). Thus, Davidson et al. found no effect of one year treatment with high dose vitamin D therapy on glucose outcomes in pre-diabetics with low vitamin D status (114).

For respiratory disease and gastrointestinal disease, the literature is sparse. Regarding respiratory disease, our results are in line with those of Romme et al. who found that vitamin D deficiency was present in the majority of patients with COPD entering pulmonary rehabilitation, Black et al. who found a dose-response relationship between the serum concentration of 25-hydroxyvitamin D and FEV1, and Ginde et al. who found an inverse association between 25-OH-D levels and recent upper respiratory tract infections (34-36). We recently found a cross-sectional but no prospective association between vitamin D status and COPD (115), and no consistent association of vitamin D with lung function (116).

Regarding digestive disease – liver disease accounting for over half – our findings are consistent with previous reports of a high prevalence of vitamin D deficiency in patients with liver disease, and low 25-OH-D levels predicting hepatic decompensation and mortality in patients with chronic liver failure (38;117). Also, we recently found vitamin D status to be inversely associated with incident liver disease (118). The lack of association between vitamin D status and cancer mortality is in line with the findings that vitamin D status was not associated with incident cancer (119).

STRENGTHS AND LIMITATIONS

A summary of the major strengths and limitations are described below. In addition, a selection of potential problems is summarised in table 8. For a more detailed discussion, please see the relevant article.

Power

A key limitation of the thesis is the shortage of power, which is clearly illustrated by the often wide confidence intervals, which makes it difficult to distinguish whether or not there is an effect. Larger studies or meta-analyses are needed to enable the detection of small to moderate effects.

Registries

We used registry-based data from the Danish National Patient Register, the Danish Registry of Causes of Death, and the Danish

Civil Registration System, which are registries with a high degree of completeness overall. The major strength of using these registries is the fact that there is virtually no loss to follow-up. Regarding the Danish Registry of Causes of Death, classification of causes of death is performed in accordance with the rules of WHO and by the ICD-10 codes that have been in use since 1994 (120). However, changes in coding practice and fewer autopsies affect the continuity and validity of cause-specific mortality.

The Danish National Patient Register, internationally considered the best of its kind, is a sound data source overall, but both definitions and content have changed over time (121). Also, the reported diagnoses underlie the payments made to the hospitals. Since the payment rates for specific diagnoses change over time, this may cause a drift in the coding of diagnoses, which will inappropriately be reflected as a change in the disease pattern (121).

Non-differential misclassification of diagnoses may have attenuated the associations and could be an explanation for the observed lack of association between e.g. vitamin D status and IHD and stroke. Madsen et al., however, found that the combination of national registries was a valid tool for monitoring the population incidence of myocardial infarction (122).

Design

Prospective cohort studies permit multiple outcomes to be assessed in the same study and allow the demonstration of temporal relationships, although these are less able to infer causality than RCTs. We used different approaches to investigate the association between vitamin D status and cardiovascular risk factors, which is a major strength. Other strengths include the large random sample of the Danish population with a high prevalence of low vitamin D levels, a relatively long-term follow-up and the use of standardised registry-based diagnoses with hardly any individuals lost to follow-up (paper 4, 5), the objective measurements of instrument (paper 2), exposure (paper 4, 1, 2, 5), and outcome (1, 2).

Mendelian randomization

In paper 2, in particular, the strengths also include the ethnic homogeneity, enabling genetic association studies, and the Mendelian randomisation approach with the potential to avoid some of the limitations of observational epidemiology (confounding, reverse causality, and regression dilution bias) for making causal inferences. Genetic variants are generally not associated with the behavioural, social, and physiological factors that confound the association between e.g. vitamin D and disease risk. The genetic variants associated with vitamin D status will not be affected by the onset of disease, and the estimates are less biased by reverse causation. Also, a genetic variant will often indicate long-term levels of exposure and will not suffer from measurement error in phenotypes that are highly variable, such as vitamin D status (15).

Several issues could violate the core assumptions of the Mendelian randomisation methodology (paper 2): developmental changes compensating for genetic variation (canalisation); linkage disequilibrium between filaggrin genotype and other causal variants; pleiotropy, which refers to a single gene having multiple biological functions (123); and epigenetic effects i.e. non-Mendelian, heritable changes in gene expression that are not accompanied by DNA sequence changes (25;124): we need to assume a random distribution of epigenetic changes at conception. It is an important limitation that we only used a single instrument. Had we found more genetic variants associated with vitamin D status that were not in linkage disequilibrium with each other, and both had been associated with the outcome, it would be unlikely that confounding would explain the association. It would have to act in a similar way for the unlinked variants, which is not plausible (125). However, vitamin D and cholesterol share the 7-dehydrocholesterol metabolic pathway (126). It may be speculated that lipid status i.e. HDL-cholesterol could affect vitamin D status and not vice versa. Likewise, it has been proposed that some statins may increase the absorption of vitamin D by stimulating the expression of cholesterol transporters. However, only few and small studies have evaluated this, and the results are inconsistent (127). Larger RCTs are needed to clarify the effect of statins on vitamin D levels.

Non-participation and loss to follow-up

Non-participation could limit the generalisability of the results, if there are differences between participants and non-participants. Baseline participation rates were relatively low, ranging from 64.3% in Monica10, to 52.5% in Inter99, and 43.8% in the Health2006 study. Non-participation is generally associated with less favourable socioeconomic characteristics (128). Thus, Bender et al. found strong socioeconomic inequality in participation at baseline and follow-up in the Inter99 study (129). Also, in population studies comparable to ours, researchers have found a higher age and a higher number of women among the participants (130). Overall, the selection bias may limit the generalisability of the results to the general population, although studies with much lower participation than ours were not biased by non-participation (131).

Loss to follow-up bias results when persons lost from a cohort study have different health response distributions from subjects who remain in the follow-up. Differential loss to follow-up may have introduced bias in the results from papers 1 and 3, where 2271 persons (33.5%) were lost to follow-up in the 5-year follow-up study of the Inter99. Compared to participants, non-participants at the 5-year follow-up had a less healthy lifestyle and a less favourable risk factor profile, including lower vitamin D levels at baseline. Also, non-participants were more likely to be female and of younger age. We have, however, no reason to believe that the persons lost to follow-up have different health responses, i.e. that vitamin D has a different role on the outcome among non-participants compared to participants, but that the loss to follow-up may have attenuated the observed associations.

Missing data

We used complete case analysis for missing covariates due to the simplicity and comparability across analyses. Limitations of this approach include loss of precision and power (132). An alternative approach to analysing incomplete data would be multiple imputations, which is the practice of "filling in" missing data with plausible values; however, the method needs to be used carefully to avoid misleading conclusions (132). It allows for the uncertainty about missing data by creating a number of different plausible imputed data sets, and appropriately combines the results from each of them (132). Covariates are referred to as "missing completely at random" (MCAR), where there are no systematic differences between the missing and the observed values; "missing at random" (MAR) which means that any systematic difference between the missing and observed values can be explained by differences in observed data; or "missing not at random" (MNAR) where even after the observed data are taken into account, systematic differences between the missing and observed data remain (132).

Table 8
Selected examples of potential weaknesses and counter actions

Potential problem	Example	Effect on estimate	Counteraction
Reverse causation	Patients with digestive disease are prone to vitamin D deficiency; the low vitamin D levels associated with liver disease could be a consequence of the disease rather than a cause	Risk of false-positive result	Exclusion of persons with a diagnosis of digestive disease before the study
Misclassification	Baseline 25-OH-D may lose predictive power over time	Attenuation	Describe the issue in the discussion
Confounding/residual confounding	Low BMI is an independent risk factor for mortality in COPD so vitamin D status might just reflect the level of malnutrition	Risk of false-positive result	Adjustment for BMI/describe in discussion
Multiple testing	When investigating several outcomes/hypotheses, e.g. in genetic association studies.	Risk of false-positive result (type 1 error)	We provided the Bonferroni adjusted significance level and described all the performed analyses for making a balanced conclusion
Using the interventional Inter99 data as observational	If the participants at highest cardiovascular risk with presumably lowest vitamin D levels participated in the interventions and changed their cardiovascular risk factors	Attenuation	We adjusted for changes in risk factors, e.g. smoking (paper **1** and 3)
Sampling bias (a type of selection bias)	If filaggrin genotype was unequally distributed among the persons who died before the study	Risk of false-positive result	Filaggrin genotype was not associated with mortality, cardiovascular disease or cancer in previous studies (150;151)
Missing data	A total of 527 persons were excluded from the regression analyses in paper 2 due to missing data on alcohol intake	Risk of biased estimate	Additional analyses showed that filaggrin genotype was not associated with alcohol intake. Also, the genotype distribution among those with missing information on alcohol intake was similar to the overall distribution of the filaggrin genotype. Therefore, the risk of bias is small.
Regression toward the mean	If a variable is extreme the first time, it will tend to be closer to the average the second time	Risk of biased estimate	We adjusted for the baseline value of the outcome in the prospective linear regressions in paper **1** and 3 (88)

Complete case analysis may give biased estimates when covariates are MAR but not MCAR, at which point it is preferable to use multiple imputation (132). Neither complete case analysis nor multiple imputation is universally applicable, although the multiple imputation method seems appropriate across a wider range of settings (133). In cases where covariates are MNAR, bias can only be addressed by sensitivity analyses (132). Not using an alternative to complete case analysis is a limitation of our studies, which may have biased our results if our covariates were not MCAR. In the regression analyses, we excluded a number of persons due to missing data on vitamin D, filaggrin genotype or lifestyle factors such as smoking habits, e.g. 85 persons in the Monica10 study had missing data on vitamin D, filaggrin genotype or both. Enabling complete case analyses, we excluded persons with missing data on one or more of the included variables, e.g. 985 (out of 11,983) persons were excluded due to missing data in the adjusted ordinary linear regression and instrumental variable analyses of total cholesterol in paper 2, mainly due to missing data on alcohol intake (please see table 8). This may have affected our results if persons with missing data differed from those without. For some of the outcomes, we performed analyses including all individuals with exposure, outcome, age and gender and compared the results to the same analysis with the exclusion of persons with missing data on the variables used in the fully adjusted models. The estimates were comparable, thus indicating that missing data did not seriously bias our results.

Confounding

Other limitations include the risk of residual confounding, unmeasured confounders or reverse causation, all of which are inherent in an observational study. To reduce confounding, we stepwise adjusted for several covariates (potential confounders). As previously mentioned, some of the diseases are likely to cause rather than be caused by the low vitamin D status. Possible examples include liver disease and mental disease caused by alcohol abuse, which may also have reduced liver function (a substantial part of the mental and liver diseases included in paper 5 were alcohol-related). Likewise, vitamin D could simply be a marker of a healthy lifestyle. Thus, the associations may at least be explained partly by residual confounding. In addition, the inverse association between vitamin D status and all-cause mortality could still be due to residual confounding. Regarding confounding by co-morbidity, having a chronic disease may lower vitamin D levels, e.g. by decreasing dietary intake and reducing exposure to sunlight, making vitamin D status simply an indicator of chronic disease.

In paper 1, we increased the likelihood of the causality of associations (i.e. by minimising reverse causation) with vitamin D deficiency by excluding participants with hypertension, hypercholesterolaemia and the metabolic syndrome at baseline for each of the analyses.

Validity, misclassification and regression dilution bias

Regression dilution bias is caused by measurement error and poses a particular problem, as there was only a single vitamin D measurement for each participant. The predictive value of a single vitamin D measurement at baseline on future health outcomes is an important issue because it may lose predictive power over time (134). Repeated measurements could reduce this bias.

The validity of the method used for the measurement of vitamin D status is another issue, as it is recommended to store

samples for measurement of 25-OH-D at –80°C, but several studies have shown 25-OH-D to be stable under different conditions (135;136). Measuring serum 25-OH-D in general is associated with methodological concerns, and variations between methods are considerable (137). Any misclassification is, however, likely to be random and would have attenuated the associations towards the null value. The findings are likely an underestimate of the actual effect and could be part of the explanation for the lack of association between vitamin D status and incident stroke and ischaemic heart disease.

The 25-OH-D concentrations reported for the various studies are different. This could be due to several factors such as the different methods used for measuring vitamin D, evaporation during storage, or a real decrease in vitamin D levels in the population over the years. We adjusted for the different levels of vitamin D by adjusting for study population. Results of analyses for each cohort separately were consistent with the combined analyses of the three cohorts, indicating that methodological differences between the cohorts did not influence our results substantially.

Finally, 25-OH-D may not be as good a marker of active vitamin D as previously believed (138). Powe et al. found that lower levels of VDBP in black individuals seem to result in levels of bioavailable 25-hydroxyvitamin D that are equivalent to the levels in whites. Likewise, mice lacking VDBP have low levels of total 25-OH-D, but no signs of vitamin D deficiency (139). Thus, low total 25-OH-D levels do not necessarily indicate vitamin D deficiency, and further research should investigate whether VDBP should in fact be incorporated into the assessment of vitamin D status (138).

Another limitation is the single spot urine sample at baseline and follow-up for UACR determination, which is not as accurate as 24-h urine collections or first morning voids (140). However, it has been shown to be quite consistently associated with cardiovascular disease and mortality (141).

Multiple testing

The issue of multiple testing refers to the problem of finding false positive results due to multiple tests/comparisons and not due to a true association. Put simply, when we perform numerous tests, we are bound to find a significant result by chance at some point (142). This is clearly illustrated by the fact that results from highly cited clinical research are often contradicted or found to be exaggerated in subsequent studies (143) where the results of smaller studies are more often contradicted than results from larger studies (143). For cohort studies in particular, multiplicity is an issue due to multiple publications focusing on the significant findings (144).

The Bonferroni method is often used to adjust for multiple testing due to its simplicity. An important weakness of the Bonferroni method is that the interpretation of a result is dependent on the number of tests performed, and the method is criticised for being too conservative (145;146). Adjusting for multiple testing may be used in subgroup analyses (142) and in confirmative studies, but it is not recommended in explorative studies (147). However, the Bonferroni method is not appropriate when the tests are correlated, since it will be too conservative and the risk of not detecting a true effect will be too great (142).

According to "The strengthening the reporting of observational studies in epidemiology (STROBE) statement", it is important to give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies and other relevant evidence rather than necessarily adjusting for multiple testing (148). In accordance, it is important to define a priori hypotheses to decrease the risk of implausible associations (149), to describe what tests were performed and why (146), to show both crude and adjusted estimates to enable the reader to evaluate the effect of the chosen confounders (144), and to make a critical assessment of the results (145). Other alternatives to adjusting for multiple testing are considering trends and consistency in subgroup analyses rather than statistical significance and to replicate unexpected findings in separate studies (142;149). That goes both ways, however; even if an association is statistically significant, the null hypothesis may still be the most probable in the absence of other explanations (145).

CONCLUSION AND PERSPECTIVES

FUTURE RESEARCH

It is now recognised that vitamin D deficiency and insufficiency are common worldwide. It is well known that vitamin D levels are highest at the end of summer and lowest at the end of winter (4;12). It seems to make little physiological sense to have such large variations (4). The questions, therefore, are what the optimal vitamin D range is and how it can be achieved. As previously suggested, vitamin D supplementation might be beneficial in certain groups only, e.g. among old people, individuals with liver disease, etc. It may only be beneficial to improve the vitamin D status among vitamin D deficient or insufficient patients instead of increasing vitamin D status among all individuals. It is of utmost importance to rule out a possible harmful effect of supplementation.

Data of the effect of vitamin D on cardiovascular disease from RCTs are few; thus a possible causal effect remains to be investigated (6). Several large interventional trials investigating an effect on cardiovascular end-points are on-going, the results of which are expected in 2017-2020 (152;153).

An alternative to traditional observational studies and RCTs is Mendelian randomisation. Mendelian randomisation studies can in theory provide an estimate of the causal effect, and they can be performed in a representative sample with no random treatment allocation required compared with RCTs. The limited generalisability, high costs, feasibility and ethics, all of which can be limitations of RCTs, also make the Mendelian randomisation approach attractive as a supplement to RCTs (124).

An important issue in instrumental variable analyses is having a sufficiently strong instrument (154). Filaggrin genotype and other vitamin D-related genetic variants only explain a small proportion of the variance in the observed vitamin D levels compared with the variance explained by strong determinants such as sun exposure. There are several ways to move forward. Future research should focus on developing more efficient IV tools and the use of more than one IV for strengthening causal inference. Another option would be to use more accurate classifications of diseases and refined phenotypes, e.g. use 24 h ambulatory blood pressure instead of the traditional blood pressure. The inclusion of persons with missing data by e.g. the multiple imputations method which is evolving and now available in many statistical packages would also provide more powerful statistical analyses (132;155). The trend is already moving towards international consortia and individual participant data meta-analyses with a greater number of participants, enabling researchers to expose important gene-environment interactions and find and use genet-

ic variants with smaller effect sizes (156). This progress will in turn demand more sophisticated statistical methods.

CONCLUSION

A higher vitamin D status is associated with a more favourable lipid profile in both a traditional observational study and in a Mendelian randomisation approach. After adjustment for multiple testing, none of the IV associations remained statistically significant, emphasising the fact that our results need confirmation in other and larger populations. We found statistically significant inverse cross-sectional and prospective associations between vitamin D status and UACR and incident increased UACR.

The inverse association between vitamin D status and mortality was not explained by a similar association with ischaemic heart disease or stroke. Rather, we found significant inverse associations between vitamin D status and death caused by respiratory, digestive disease or death caused by endocrine, nutritional and metabolic disorders (no association with cancer or circulatory death).

Due to the explorative nature of the studies, further studies, e.g. RCTs or Mendelian randomisation studies, are needed to clarify whether vitamin D deficiency is a causal and reversible factor in the prevention of disease and mortality.

SUMMARY

Vitamin D is essential for bone mineralisation, but a growing body of evidence points at a broader role; vitamin D deficiency has been found to be associated with mortality and several diseases ranging from cardiovascular disease to autoimmune diseases and liver diseases. The evidence is, however, inconclusive and the possible pathways remain unresolved.

The aims of the thesis were to investigate the association of vitamin D status to 5-year changes in cardiovascular risk factors such as blood pressure, lipid profile, the metabolic syndrome and urine albumin creatinine ratio (UACR); the association of a known genetic determinant of vitamin D status to cardiovascular risk factors; the association of vitamin D status to the incidence of cardiovascular disease (CVD) and all-cause mortality; and the association of vitamin D status to cause-specific mortality.

Data from the 3 population-based studies Monica10 (n=2,656, 1993-94), Inter99 (n=6,794, 1999-2001) and Health2006 (n=3,471, 2006-2008) conducted at the Research Centre for Prevention and Health were used. The studies included questionnaires, physical examinations, and blood tests. Vitamin D status was measured at baseline. Participants were genotyped for the most frequent filaggrin mutations. Registry-based diagnoses and causes of death were obtained from The Danish National Patient Register and the Danish Registry of Causes of Death, respectively. Linear, logistic, Cox and instrumental variable regressions were used to model the associations between vitamin D status and cardiovascular risk factors, disease and mortality.

With a 10 year mean follow-up time, we found a significant association between vitamin D status and all-cause mortality with a HR=0.95 (p=0.005) per 10 nmol/l higher vitamin D level. We found no association between vitamin D status and incidence of ischaemic heart disease or stroke (HR=1.01, p=0.442 and HR=1.00, p=0.920, respectively).

We found a baseline level of vitamin D that was 10 nmol/l higher to be associated with a decrease in triglycerides and very low density lipoprotein cholesterol by 0.52% (p = 0.03) and 0.66% (p = 0.005), respectively. The odds ratios per 10 nmol/l higher baseline vitamin D level were 0.95 (p<0.05) and 0.94 (p = 0.01) for the development of the metabolic syndrome and hypercholesterolaemia, respectively. There was no association between vitamin D and blood pressure.

With filaggrin genotype as an instrumental variable, we found a 23.8% (95% confidence interval, CI: 3.0, 48.6) higher HDL cholesterol level and a 30.5% (95% CI: 0.8, 51.3) lower serum level of triglycerides per doubling of vitamin D. These associations were no longer statistically significant when applying the Bonferroni-adjusted significance level. The remaining lipids showed non-significant changes in a favourable direction. A doubling of vitamin D gave a non-significantly lower odds ratio=0.26 (95% CI: 0.06, 1.17) of the metabolic syndrome. There were no statistically significant causal effects of vitamin D status on blood pressure or anthropometrics.

With a total of 832 deaths and a 10.3 year median follow-up time, we found significant associations between vitamin D status and death caused by respiratory diseases, digestive diseases, and endocrine, nutritional and metabolic diseases with hazard ratios (HRs) 0.26 (ptrend=0.0042), 0.28 (ptrend=0.0040), and 0.21 (ptrend=0.035), respectively, for the fourth vitamin D quartile compared to the first. We found non-significantly lower HRs for death caused by mental and behavioural diseases and diseases of the nervous system, but no association between vitamin D status and death caused by diseases of the circulatory system or neoplasms.

We found that a baseline level of vitamin D that was 10 nmol/l higher was associated with a small but statistically significant decrease in UACR by 0.92% (p = 0.02), but a non-significantly lower PTH. The odds ratio for an increased UACR were 0.96 (p=0.0006) per 10 nmol/l higher baseline vitamin D level.

Our studies support the idea that vitamin D can affect lipid status in a favourable direction and the incidence of metabolic syndrome and increased UACR but neither blood pressure nor anthropometrics. Vitamin D status was inversely associated with mortality, but this was not explained by an association with cardiovascular disease. Rather, the association seemed to be caused by an inverse association with death caused by digestive disease, endocrine, metabolic and nutritional diseases, and respiratory disease. Further studies, e.g. RCTs or Mendelian randomisation studies, are needed to clarify whether low vitamin D status is a causal and reversible factor to prevent disease and mortality.

ABBREVIATIONS

BMI	body mass index
CI	confidence interval
COPD	chronic obstructive pulmonary disease
CVD	cardiovascular disease
DAG	directed acyclic graph
GFR	glomerular filtration rate
HDL	high density lipoprotein
HR	hazard ratio
ICD	International Classification of Disease
IHD	ischaemic heart disease
IOM	Institute of Medicine
IV	instrumental variable
LDL	low density lipoprotein
MAR	missing at random
MCAR	missing completely at random
MNAR	missing not at random
NA	not applicable

OR	odds ratio
PTH	parathyroid hormone
RCT	randomised controlled trial
SNP	single nucleotide polymorphism
STROBE	the strengthening the reporting of observational studies in epidemiology
25-OH-D	25-hydroxyvitamin D
2SLS	two stage least squares
VDBP	vitamin D binding protein
VLDL	very low density lipoprotein

REFERENCES

1 Holick MF. Vitamin D deficiency. N Engl J Med 2007 Jul 19;357(3):266-81.

2 Haddad JG. Plasma vitamin D-binding protein (Gc-globulin): multiple tasks. J Steroid Biochem Mol Biol 1995 Jun;53(1-6):579-82.

3 Steingrimsdottir L, Gunnarsson O, Indridason OS, Franzson L, Sigurdsson G. Relationship between serum parathyroid hormone levels, vitamin D sufficiency, and calcium intake. JAMA 2005 Nov 9;294(18):2336-41.

4 Pludowski P, Holick MF, Pilz S, Wagner CL, Hollis BW, Grant WB, et al. Vitamin D effects on musculoskeletal health, immunity, autoimmunity, cardiovascular disease, cancer, fertility, pregnancy, dementia and mortality-A review of recent evidence. Autoimmun Rev 2013 Aug;12(10):976-89.

5 Avenell A, Mak JC, O'Connell D. Vitamin D and vitamin D analogues for preventing fractures in post-menopausal women and older men. Cochrane Database Syst Rev 2014;4:CD000227.

6 Theodoratou E, Tzoulaki I, Zgaga L, Ioannidis JP. Vitamin D and multiple health outcomes: umbrella review of systematic reviews and meta-analyses of observational studies and randomised trials. BMJ 2014;348:g2035.

7 Husemoen LL, Skaaby T, Thuesen BH, Jorgensen T, Fenger RV, Linneberg A. Serum 25(OH)D and incident type 2 diabetes: a cohort study. Eur J Clin Nutr 2012 Dec;66(12):1309-14.

8 Kienreich K, Tomaschitz A, Verheyen N, Pieber T, Gaksch M, Grubler MR, et al. Vitamin d and cardiovascular disease. Nutrients 2013;5(8):3005-21.

9 Seamans KM, Cashman KD. Existing and potentially novel functional markers of vitamin D status: a systematic review. Am J Clin Nutr 2009 Jun;89(6):1997S-2008S.

10 Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Guidelines for preventing and treating vitamin D deficiency and insufficiency revisited. J Clin Endocrinol Metab 2012 Apr;97(4):1153-8.

11 Brot C, Darso P. Sundhedsstyrelsens anbefalinger vedrørende forebyggelse, diagnostik og behandling af D-vitaminmangel. Rationel Farmakoterapi 2010 Jun.

12 Thuesen B, Husemoen L, Fenger M, Jakobsen J, Schwarz P, Toft U, et al. Determinants of vitamin D status in a general population of Danish adults. Bone 2012 Mar;50(3):605-10.

13 Hilger J, Friedel A, Herr R, Rausch T, Roos F, Wahl DA, et al. A systematic review of vitamin D status in populations worldwide. Br J Nutr 2013 Aug 9;1-23.

14 Skaaby T, Husemoen LL, Pisinger C, Jorgensen T, Thuesen BH, Fenger M, et al. Vitamin D status and incident cardiovascular disease and all-cause mortality: a general population study. Endocrine 2013 Jun;43(3):618-25.

15 Smith GD, Timpson N, Ebrahim S. Strengthening causal inference in cardiovascular epidemiology through Mendelian randomization. Ann Med 2008;40(7):524-41.

16 Wang TJ, Zhang F, Richards JB, Kestenbaum B, van Meurs JB, Berry D, et al. Common genetic determinants of vitamin D insufficiency: a genome-wide association study. Lancet 2010 Jul 17;376(9736):180-8.

17 Thyssen JP, Thuesen B, Huth C, Standl M, Carson CG, Heinrich J, et al. Skin barrier abnormality caused by filaggrin (FLG) mutations is associated with increased serum 25-hydroxyvitamin D concentrations. J Allergy Clin Immunol 2012 Nov;130(5):1204-7.

18 Mildner M, Jin J, Eckhart L, Kezic S, Gruber F, Barresi C, et al. Knockdown of filaggrin impairs diffusion barrier function and increases UV sensitivity in a human skin model. J Invest Dermatol 2010 Sep;130(9):2286-94.

19 Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. Nat Genet 2006 Apr;38(4):441-6.

20 Thyssen JP, Linneberg A, Carlsen BC, Johansen JD, Engkilde K, Hansen T, et al. A possible association between a dysfunctional skin barrier (filaggrin null-mutation status) and diabetes: a cross-sectional study. BMJ Open 2011 Jan 1;1(1):e000062.

21 Berg ND, Husemoen LL, Thuesen BH, Hersoug LG, Elberling J, Thyssen JP, et al. Interaction between filaggrin null mutations and tobacco smoking in relation to asthma. J Allergy Clin Immunol 2012 Feb;129(2):374-80, 380.

22 van den Oord RA, Sheikh A. Filaggrin gene defects and risk of developing allergic sensitisation and allergic disorders: systematic review and meta-analysis. BMJ 2009;339:b2433.

23 Conen D, Vollenweider P, Rousson V, Marques-Vidal P, Paccaud F, Waeber G, et al. Use of a Mendelian randomization approach to assess the causal relation of gamma-Glutamyltransferase with blood pressure and serum insulin levels. Am J Epidemiol 2010 Dec 15;172(12):1431-41.

24 Lawlor DA, Harbord RM, Sterne JA, Timpson N, Davey SG. Mendelian randomization: using genes as instruments for making causal inferences in epidemiology. Stat Med 2008 Apr 15;27(8):1133-63.

25 Smith GD, Ebrahim S. 'Mendelian randomization': can genetic epidemiology contribute to understanding environmental determinants of disease? Int J Epidemiol 2003 Feb;32(1):1-22.

26 Ginde AA, Scragg R, Schwartz RS, Camargo CA, Jr. Prospective study of serum 25-hydroxyvitamin D level, cardiovascular disease mortality, and all-cause mortality in older U.S. adults. J Am Geriatr Soc 2009 Sep;57(9):1595-603.

27 Hutchinson MS, Grimnes G, Joakimsen RM, Figenschau Y, Jorde R. Low serum 25-hydroxyvitamin D levels are associated with increased all-cause mortality risk in a general population: the Tromso study. Eur J Endocrinol 2010 May;162(5):935-42.

28 Autier P, Gandini S. Vitamin D supplementation and total mortality: a meta-analysis of randomized controlled trials. Arch Intern Med 2007 Sep 10;167(16):1730-7.

29 Rejnmark L, Avenell A, Masud T, Anderson F, Meyer HE, Sanders KM, et al. Vitamin D with calcium reduces mortality: patient level pooled analysis of 70,528 patients from eight major vitamin D trials. J Clin Endocrinol Metab 2012 Aug;97(8):2670-81.

30 Bjelakovic G, Gluud LL, Nikolova D, Whitfield K, Wetterslev J, Simonetti RG, et al. Vitamin D supplementation for prevention of mortality in adults. Cochrane Database Syst Rev 2014;1:CD007470.

31 Ma Y, Zhang P, Wang F, Yang J, Liu Z, Qin H. Association between vitamin D and risk of colorectal cancer: a systematic review of prospective studies. J Clin Oncol 2011 Oct 1;29(28):3775-82.

32 Kilkkinen A, Knekt P, Heliovaara M, Rissanen H, Marniemi J, Hakulinen T, et al. Vitamin D status and the risk of lung cancer: a cohort study in Finland. Cancer Epidemiol Biomarkers Prev 2008 Nov;17(11):3274-8.

33 Abbas S, Chang-Claude J, Linseisen J. Plasma 25-hydroxyvitamin D and premenopausal breast cancer risk in a German case-control study. Int J Cancer 2009 Jan 1;124(1):250-5.

34 Black PN, Scragg R. Relationship between serum 25-hydroxyvitamin d and pulmonary function in the third national health and nutrition examination survey. Chest 2005 Dec;128(6):3792-8.

35 Romme EA, Rutten EP, Smeenk FW, Spruit MA, Menheere PP, Wouters EF. Vitamin D status is associated with bone mineral density and functional exercise capacity in patients with chronic obstructive pulmonary disease. Ann Med 2012 Apr 2.

36 Ginde AA, Mansbach JM, Camargo CA, Jr. Association between serum 25-hydroxyvitamin D level and upper respiratory tract infection in the Third National Health and Nutrition Examination Survey. Arch Intern Med 2009 Feb 23;169(4):384-90.

37 Arteh J, Narra S, Nair S. Prevalence of vitamin D deficiency in chronic liver disease. Dig Dis Sci 2010 Sep;55(9):2624-8.

38 Malham M, Jorgensen SP, Ott P, Agnholt J, Vilstrup H, Borre M, et al. Vitamin D deficiency in cirrhosis relates to liver dysfunction rather than aetiology. World J Gastroenterol 2011 Feb 21;17(7):922-5.

39 Pappa HM, Bern E, Kamin D, Grand RJ. Vitamin D status in gastrointestinal and liver disease. Curr Opin Gastroenterol 2008 Mar;24(2):176-83.

40 Annweiler C, Rolland Y, Schott AM, Blain H, Vellas B, Herrmann FR, et al. Higher Vitamin D Dietary Intake Is Associated With Lower Risk of Alzheimer's Disease: A 7-Year Follow-up. J Gerontol A Biol Sci Med Sci 2012 Apr 13.

41 Llewellyn DJ, Lang IA, Langa KM, Muniz-Terrera G, Phillips CL, Cherubini A, et al. Vitamin D and risk of cognitive decline in elderly persons. Arch Intern Med 2010 Jul 12;170(13):1135-41.

42 Hogberg G, Gustafsson SA, Hallstrom T, Gustafsson T, Klawitter B, Petersson M. Depressed adolescents in a case-series were low in vitamin D and depression was ameliorated by vitamin D supplementation. Acta Paediatr 2012 Feb 28.

43 de Boer IH, Ioannou GN, Kestenbaum B, Brunzell JD, Weiss NS. 25-Hydroxyvitamin D levels and albuminuria in the Third National Health and Nutrition Examination Survey (NHANES III). Am J Kidney Dis 2007 Jul;50(1):69-77.

44 Petta S, Camma C, Scazzone C, Tripodo C, Di M, V, Bono A, et al. Low vitamin D serum level is related to severe fibrosis and low responsiveness to interferon-based therapy in genotype 1 chronic hepatitis C. Hepatology 2010 Apr;51(4):1158-67.

45 Barchetta I, Angelico F, Del BM, Baroni MG, Pozzilli P, Morini S, et al. Strong association between non alcoholic fatty liver disease (NAFLD) and low 25(OH) vitamin D levels in an adult population with normal serum liver enzymes. BMC Med 2011;9:85.
46 Targher G, Bertolini L, Scala L, Cigolini M, Zenari L, Falezza G, et al. Associations between serum 25-hydroxyvitamin D3 concentrations and liver histology in patients with non-alcoholic fatty liver disease. Nutr Metab Cardiovasc Dis 2007 Sep;17(7):517-24.
47 de Boer IH, Katz R, Chonchol M, Ix JH, Sarnak MJ, Shlipak MG, et al. Serum 25-hydroxyvitamin D and change in estimated glomerular filtration rate. Clin J Am Soc Nephrol 2011 Sep;6(9):2141-9.
48 Annweiler C, Beauchet O. Vitamin D-mentia: randomized clinical trials should be the next step. Neuroepidemiology 2011;37(3-4):249-58.
49 Buell JS, Dawson-Hughes B, Scott TM, Weiner DE, Dallal GE, Qui WQ, et al. 25-Hydroxyvitamin D, dementia, and cerebrovascular pathology in elders receiving home services. Neurology 2010 Jan 5;74(1):18-26.
50 Dobak J, Grzybowski J, Liu FT, Landon B, Dobke M. 1,25-Dihydroxyvitamin D3 increases collagen production in dermal fibroblasts. J Dermatol Sci 1994 Aug;8(1):18-24.
51 Adams JS, Hewison M. Unexpected actions of vitamin D: new perspectives on the regulation of innate and adaptive immunity. Nat Clin Pract Endocrinol Metab 2008 Feb;4(2):80-90.
52 Alvarez JA, Ashraf A. Role of vitamin d in insulin secretion and insulin sensitivity for glucose homeostasis. Int J Endocrinol 2010;2010:351385.
53 Jorde R, Figenschau Y, Hutchinson M, Emaus N, Grimnes G. High serum 25-hydroxyvitamin D concentrations are associated with a favorable serum lipid profile. Eur J Clin Nutr 2010 Dec;64(12):1457-64.
54 Forman JP, Giovannucci E, Holmes MD, Bischoff-Ferrari HA, Tworoger SS, Willett WC, et al. Plasma 25-hydroxyvitamin D levels and risk of incident hypertension. Hypertension 2007 May;49(5):1063-9.
55 Parikh SJ, Edelman M, Uwaifo GI, Freedman RJ, Semega-Janneh M, Reynolds J, et al. The relationship between obesity and serum 1,25-dihydroxy vitamin D concentrations in healthy adults. J Clin Endocrinol Metab 2004 Mar;89(3):1196-9.
56 Forouhi NG, Luan J, Cooper A, Boucher BJ, Wareham NJ. Baseline serum 25-hydroxy vitamin d is predictive of future glycemic status and insulin resistance: the Medical Research Council Ely Prospective Study 1990-2000. Diabetes 2008 Oct;57(10):2619-25.
57 Lopez AD, Mathers CD, Ezzati M, Jamison DT, Murray CJ. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. Lancet 2006 May 27;367(9524):1747-57.
58 Kim AS, Johnston SC. Global variation in the relative burden of stroke and ischemic heart disease. Circulation 2011 Jul 19;124(3):314-23.
59 Jaimungal S, Wehmeier K, Mooradian AD, Haas MJ. The emerging evidence for vitamin D-mediated regulation of apolipoprotein A-I synthesis. Nutr Res 2011 Nov;31(11):805-12.
60 Després JP, Poirier P, Bergeron J, Tremblay A, Lemieux I, Alm+®ras N. From individual risk factors and the metabolic syndrome to global cardiometabolic risk. European Heart Journal Supplements 2008 Mar;10(suppl B):B24-B33.
61 Alberti KG, Zimmet P, Shaw J. The metabolic syndrome--a new worldwide definition. Lancet 2005 Sep 24;366(9491):1059-62.
62 Grundy SM, Brewer HB, Jr., Cleeman JI, Smith SC, Jr., Lenfant C. Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. Arterioscler Thromb Vasc Biol 2004 Feb;24(2):e13-e18.
63 Mallika V, Goswami B, Rajappa M. Atherosclerosis pathophysiology and the role of novel risk factors: a clinicobiochemical perspective. Angiology 2007 Oct;58(5):513-22.
64 Alexander RW. Theodore Cooper Memorial Lecture. Hypertension and the pathogenesis of atherosclerosis. Oxidative stress and the mediation of arterial inflammatory response: a new perspective. Hypertension 1995 Feb;25(2):155-61.
65 Greenland P, Alpert JS, Beller GA, Benjamin EJ, Budoff MJ, Fayad ZA, et al. 2010 ACCF/AHA guideline for assessment of cardiovascular risk in asymptomatic adults: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. J Am Coll Cardiol 2010 Dec 14;56(25):e50-103.
66 Lambers Heerspink HJ, Brinkman JW, Bakker SJ, Gansevoort RT, de ZD. Update on microalbuminuria as a biomarker in renal and cardiovascular disease. Curr Opin Nephrol Hypertens 2006 Nov;15(6):631-6.
67 Sheng CS, Hu BC, Fan WX, Zou J, Li Y, Wang JG. Microalbuminuria in relation to the metabolic syndrome and its components in a Chinese population. Diabetol Metab Syndr 2011;3(1):6.
68 Ibsen H, Olsen MH, Wachtell K, Borch-Johnsen K, Lindholm LH, Mogensen CE. Reduction in albuminuria translates to reduction in cardiovascular events in hypertensive patients with left ventricular hypertrophy and diabetes. J Nephrol 2008 Jul;21(4):566-9.
69 de ZD, Remuzzi G, Parving HH, Keane WF, Zhang Z, Shahinfar S, et al. Albuminuria, a therapeutic target for cardiovascular protection in type 2 diabetic patients with nephropathy. Circulation 2004 Aug 24;110(8):921-7.
70 Hagstrom E, Hellman P, Larsson TE, Ingelsson E, Berglund L, Sundstrom J, et al. Plasma parathyroid hormone and the risk of cardiovascular mortality in the community. Circulation 2009 Jun 2;119(21):2765-71.
71 Muldowney S, Kiely M. Vitamin D and cardiometabolic health: a review of the evidence. Nutr Res Rev 2010 Dec 1;1-20.
72 Cho HJ, Kang HC, Choi SA, Ju YC, Lee HS, Park HJ. The possible role of Ca2+ on the activation of microsomal triglyceride transfer protein in rat hepatocytes. Biol Pharm Bull 2005 Aug;28(8):1418-23.
73 Li YC, Qiao G, Uskokovic M, Xiang W, Zheng W, Kong J. Vitamin D: a negative endocrine regulator of the renin-angiotensin system and blood pressure. J Steroid Biochem Mol Biol 2004 May;89-90(1-5):387-92.
74 Tomaschitz A, Pilz S, Ritz E, Grammer T, Drechsler C, Boehm BO, et al. Independent association between 1,25-dihydroxyvitamin D, 25-hydroxyvitamin D and the renin-angiotensin system: The Ludwigshafen Risk and Cardiovascular Health (LURIC) study. Clin Chim Acta 2010 Sep 6;411(17-18):1354-60.
75 Zagura M, Serg M, Kampus P, Zilmer M, Eha J, Unt E, et al. Aortic stiffness and vitamin D are independent markers of aortic calcification in patients with peripheral arterial disease and in healthy subjects. Eur J Vasc Endovasc Surg 2011 Nov;42(5):689-95.
76 Shi H, Norman AW, Okamura WH, Sen A, Zemel MB. 1alpha,25-Dihydroxyvitamin D3 modulates human adipocyte metabolism via nongenomic action. FASEB J 2001 Dec;15(14):2751-3.
77 Fassina G, Maragno I, Dorigo P, Contessa AR. Effect of vitamin D2 on hormone-stimulated lipolysis in vitro. Eur J Pharmacol 1969 Feb;5(3):286-90.
78 Chiu KC, Chu A, Go VL, Saad MF. Hypovitaminosis D is associated with insulin resistance and beta cell dysfunction. Am J Clin Nutr 2004 May;79(5):820-5.
79 Pittas AG, Chung M, Trikalinos T, Mitri J, Brendel M, Patel K, et al. Systematic review: Vitamin D and cardiometabolic outcomes. Ann Intern Med 2010 Mar 2;152(5):307-14.
80 Witham MD, Nadir MA, Struthers AD. Effect of vitamin D on blood pressure: a systematic review and meta-analysis. J Hypertens 2009 Oct;27(10):1948-54.
81 Osler M, Linneberg A, Glumer C, Jorgensen T. The cohorts at the Research Centre for Prevention and Health, formerly 'The Glostrup Population Studies'. Int J Epidemiol 2011 Jun;40(3):602-10.
82 Pedersen CB. The Danish Civil Registration System. Scand J Public Health 2011 Jul;39(7 Suppl):22-5.
83 Andersen TF, Madsen M, Jorgensen J, Mellemkjoer L, Olsen JH. The Danish National Hospital Register. A valuable source of data for modern health sciences. Dan Med Bull 1999 Jun;46(3):263-8.
84 Juel K, Helweg-Larsen K. The Danish registers of causes of death. Dan Med Bull 1999 Sep;46(4):354-7.
85 Aadahl M, von Huth SL, Pisinger C, Toft UN, Glumer C, Borch-Johnsen K, et al. Five-year change in physical activity is associated with changes in cardiovascular disease risk factors: the Inter99 study. Prev Med 2009 Apr;48(4):326-31.
86 Grundy SM. A changing paradigm for prevention of cardiovascular disease: emergence of the metabolic syndrome as a multiplex risk factor. European Heart Journal Supplements 2008 Mar;10(suppl B):B16-B23.
87 Perk J, De BG, Gohlke H, Graham I, Reiner Z, Verschuren M, et al. European Guidelines on cardiovascular disease prevention in clinical practice (version 2012). The Fifth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of nine societies and by invited experts). Eur Heart J 2012 Jul;33(13):1635-701.
88 Vickers AJ, Altman DG. Statistics notes: Analysing controlled trials with baseline and follow up measurements. BMJ 2001 Nov 10;323(7321):1123-4.
89 Didelez V, Sheehan N. Mendelian randomization as an instrumental variable approach to causal inference. Stat Methods Med Res 2007 Aug;16(4):309-30.
90 Ford ES, Zhao G, Tsai J, Li C. Vitamin D and all-cause mortality among adults in USA: findings from the National Health and Nutrition Examination Survey Linked Mortality Study. Int J Epidemiol 2011 Aug;40(4):998-1005.
91 Kilkkinen A, Knekt P, Aro A, Rissanen H, Marniemi J, Heliovaara M, et al. Vitamin D status and the risk of cardiovascular disease death. Am J Epidemiol 2009 Oct 15;170(8):1032-9.
92 Melamed ML, Michos ED, Post W, Astor B. 25-hydroxyvitamin D levels and the risk of mortality in the general population. Arch Intern Med 2008 Aug 11;168(15):1629-37.
93 Pilz S, Dobnig H, Nijpels G, Heine RJ, Stehouwer CD, Snijder MB, et al. Vitamin D and mortality in older men and women. Clin Endocrinol (Oxf) 2009 Nov;71(5):666-72.
94 Semba RD, Houston DK, Bandinelli S, Sun K, Cherubini A, Cappola AR, et al. Relationship of 25-hydroxyvitamin D with all-cause and cardiovascu-

lar disease mortality in older community-dwelling adults. Eur J Clin Nutr 2010 Feb;64(2):203-9.
95 Trummer O, Pilz S, Hoffmann MM, Winkelmann BR, Boehm BO, Marz W, et al. Vitamin D and mortality: a Mendelian randomization study. Clin Chem 2013 May;59(5):793-7.
96 Bjelakovic G, Gluud LL, Nikolova D, Whitfield K, Wetterslev J, Simonetti RG, et al. Vitamin D supplementation for prevention of mortality in adults. Cochrane Database Syst Rev 2011;(7):CD007470.
97 Messenger W, Nielson CM, Li H, Beer T, Barrett-Connor E, Stone K, et al. Serum and dietary vitamin D and cardiovascular disease risk in elderly men: A prospective cohort study. Nutr Metab Cardiovasc Dis 2011 Apr 3.
98 Wang TJ, Pencina MJ, Booth SL, Jacques PF, Ingelsson E, Lanier K, et al. Vitamin D deficiency and risk of cardiovascular disease. Circulation 2008 Jan 29;117(4):503-11.
99 Hosseinpanah F, Yarjanli M, Sheikholeslami F, Heibatollahi M, Eskandary PS, Azizi F. Associations between vitamin D and cardiovascular outcomes; Tehran Lipid and Glucose Study. Atherosclerosis 2011 May 23.
100 Bolland MJ, Grey A, Avenell A, Gamble GD, Reid IR. Calcium supplements with or without vitamin D and risk of cardiovascular events: reanalysis of the Women's Health Initiative limited access dataset and meta-analysis. BMJ 2011;342:d2040.
101 Avenell A, MacLennan GS, Jenkinson DJ, McPherson GC, McDonald AM, Pant PR, et al. Long-term follow-up for mortality and cancer in a randomized placebo-controlled trial of vitamin D(3) and/or calcium (RECORD trial). J Clin Endocrinol Metab 2012 Feb;97(2):614-22.
102 Jorde R, Schirmer H, Wilsgaard T, Joakimsen RM, Mathiesen EB, Njolstad I, et al. Polymorphisms related to the serum 25-hydroxyvitamin D level and risk of myocardial infarction, diabetes, cancer and mortality. The Tromso Study. PLoS One 2012;7(5):e37295.
103 Jorde R, Grimnes G. Vitamin D and metabolic health with special reference to the effect of vitamin D on serum lipids. Prog Lipid Res 2011 Oct;50(4):303-12.
104 Kunutsor SK, Apekey TA, Steur M. Vitamin D and risk of future hypertension: meta-analysis of 283,537 participants. Eur J Epidemiol 2013 Mar;28(3):205-21.
105 Vimaleswaran KS, Cavadino A, Berry DJ, Jorde R, Dieffenbach AK, Lu C, et al. Association of vitamin D status with arterial blood pressure and hypertension risk: a mendelian randomisation study. Lancet Diabetes Endocrinol 2014 Jun 25.
106 Burgaz A, Orsini N, Larsson SC, Wolk A. Blood 25-hydroxyvitamin D concentration and hypertension: a meta-analysis. J Hypertens 2011 Apr;29(4):636-45.
107 Forman JP, Scott JB, Ng K, Drake BF, Suarez EG, Hayden DL, et al. Effect of vitamin D supplementation on blood pressure in blacks. Hypertension 2013 Apr;61(4):779-85.
108 Larsen T, Mose FH, Bech JN, Hansen AB, Pedersen EB. Effect of cholecalciferol supplementation during winter months in patients with hypertension: a randomized, placebo-controlled trial. Am J Hypertens 2012 Nov;25(11):1215-22.
109 Vimaleswaran KS, Berry DJ, Lu C, Tikkanen E, Pilz S, Hiraki LT, et al. Causal relationship between obesity and vitamin D status: bi-directional Mendelian randomization analysis of multiple cohorts. PLoS Med 2013;10(2):e1001383.
110 O'Seaghdha CM, Hwang SJ, Holden R, Booth SL, Fox CS. Phylloquinone and vitamin D status: associations with incident chronic kidney disease in the Framingham Offspring cohort. Am J Nephrol 2012;36(1):68-77.
111 Forouhi NG, Ye Z, Rickard AP, Khaw KT, Luben R, Langenberg C, et al. Circulating 25-hydroxyvitamin D concentration and the risk of type 2 diabetes: results from the European Prospective Investigation into Cancer (EPIC)-Norfolk cohort and updated meta-analysis of prospective studies. Diabetologia 2012 Aug;55(8):2173-82.
112 Mitri J, Muraru MD, Pittas AG. Vitamin D and type 2 diabetes: a systematic review. Eur J Clin Nutr 2011 Sep;65(9):1005-15.
113 George PS, Pearson ER, Witham MD. Effect of vitamin D supplementation on glycaemic control and insulin resistance: a systematic review and meta-analysis. Diabet Med 2012 Aug;29(8):e142-e150.
114 Davidson MB, Duran P, Lee ML, Friedman TC. High-dose vitamin D supplementation in people with prediabetes and hypovitaminosis D. Diabetes Care 2013 Feb;36(2):260-6.
115 Skaaby T, Husemoen LL, Thuesen BH, Pisinger C, Jorgensen T, Fenger RV, et al. Vitamin d status and chronic obstructive pulmonary disease: a prospective general population study. PLoS One 2014;9(3):e90654.
116 Thuesen BH, Skaaby T, Husemoen LL, Fenger M, Jorgensen T, Linneberg A. The association of serum 25-OH vitamin D with atopy, asthma, and lung function in a prospective study of Danish adults. Clin Exp Allergy 2014 Feb 27.
117 Putz-Bankuti C, Pilz S, Stojakovic T, Scharnagl H, Pieber TR, Trauner M, et al. Association of 25-hydroxyvitamin D levels with liver dysfunction and mortality in chronic liver disease. Liver Int 2012 May;32(5):845-51.
118 Skaaby T, Husemoen LL, Borglykke A, Jorgensen T, Thuesen BH, Pisinger C, et al. Vitamin D status, liver enzymes, and incident liver disease and mortality: a general population study. Endocrine 2013 Nov 23.
119 Skaaby T, Husemoen LL, Thuesen BH, Pisinger C, Jorgensen T, Roswall N, et al. Prospective population-based study of the association between serum 25-hydroxyvitamin-D levels and the incidence of specific types of cancer. Cancer Epidemiol Biomarkers Prev 2014 Apr 30.
120 Helweg-Larsen K. The Danish Register of Causes of Death. Scand J Public Health 2011 Jul;39(7 Suppl):26-9.
121 Lynge E, Sandegaard JL, Rebolj M. The Danish National Patient Register. Scand J Public Health 2011 Jul;39(7 Suppl):30-3.
122 Madsen M, Davidsen M, Rasmussen S, Abildstrom SZ, Osler M. The validity of the diagnosis of acute myocardial infarction in routine statistics: a comparison of mortality and hospital discharge data with the Danish MONICA registry. J Clin Epidemiol 2003 Feb;56(2):124-30.
123 Palmer TM, Lawlor DA, Harbord RM, Sheehan NA, Tobias JH, Timpson NJ, et al. Using multiple genetic variants as instrumental variables for modifiable risk factors. Stat Methods Med Res 2012 Jun;21(3):223-42.
124 Ogbuanu IU, Zhang H, Karmaus W. Can we apply the Mendelian randomization methodology without considering epigenetic effects? Emerg Themes Epidemiol 2009;6:3.
125 Smith GD, Timpson N, Ebrahim S. Strengthening causal inference in cardiovascular epidemiology through Mendelian randomization. Ann Med 2008;40(7):524-41.
126 Perez-Castrillon JL, Abad ML, Vega G, Del Pino MJ, de LD, Duenas LA. Vitamin d levels and lipid response to atorvastatin. Int J Endocrinol 2010;2010:320721.
127 Yavuz B, Ertugrul DT. Statins and vitamin D: A hot topic that will be discussed for a long time. Dermatoendocrinol 2012 Jan 1;4(1):8-9.
128 Strandhagen E, Berg C, Lissner L, Nunez L, Rosengren A, Toren K, et al. Selection bias in a population survey with registry linkage: potential effect on socioeconomic gradient in cardiovascular risk. Eur J Epidemiol 2010 Mar;25(3):163-72.
129 Bender AM, Jorgensen T, Helbech B, Linneberg A, Pisinger C. Socioeconomic position and participation in baseline and follow-up visits: the Inter99 study. Eur J Prev Cardiol 2012 Dec 11.
130 Jorgensen T, Borch-Johnsen K, Thomsen TF, Ibsen H, Glumer C, Pisinger C. A randomized non-pharmacological intervention study for prevention of ischaemic heart disease: baseline results Inter99. Eur J Cardiovasc Prev Rehabil 2003 Oct;10(5):377-86.
131 Nohr EA, Frydenberg M, Henriksen TB, Olsen J. Does low participation in cohort studies induce bias? Epidemiology 2006 Jul;17(4):413-8.
132 Sterne JA, White IR, Carlin JB, Spratt M, Royston P, Kenward MG, et al. Multiple imputation for missing data in epidemiological and clinical research: potential and pitfalls. BMJ 2009;338:b2393.
133 White IR, Carlin JB. Bias and efficiency of multiple imputation compared with complete-case analysis for missing covariate values. Stat Med 2010 Dec 10;29(28):2920-31.
134 Jorde R, Sneve M, Hutchinson M, Emaus N, Figenschau Y, Grimnes G. Tracking of serum 25-hydroxyvitamin D levels during 14 years in a population-based study and during 12 months in an intervention study. Am J Epidemiol 2010 Apr 15;171(8):903-8.
135 Wielders JP, Wijnberg FA. Preanalytical stability of 25(OH)-vitamin D3 in human blood or serum at room temperature: solid as a rock. Clin Chem 2009 Aug;55(8):1584-5.
136 Ocke MC, Schrijver J, Obermann-de Boer GL, Bloemberg BP, Haenen GR, Kromhout D. Stability of blood (pro)vitamins during four years of storage at -20 degrees C: consequences for epidemiologic research. J Clin Epidemiol 1995 Aug;48(8):1077-85.
137 Snellman G, Melhus H, Gedeborg R, Byberg L, Berglund L, Wernroth L, et al. Determining vitamin D status: a comparison between commercially available assays. PLoS One 2010;5(7):e11555.
138 Powe CE, Evans MK, Wenger J, Zonderman AB, Berg AH, Nalls M, et al. Vitamin D-binding protein and vitamin D status of black Americans and white Americans. N Engl J Med 2013 Nov 21;369(21):1991-2000.
139 Safadi FF, Thornton P, Magiera H, Hollis BW, Gentile M, Haddad JG, et al. Osteopathy and resistance to vitamin D toxicity in mice null for vitamin D binding protein. J Clin Invest 1999 Jan;103(2):239-51.
140 Witte EC, Lambers Heerspink HJ, de ZD, Bakker SJ, de Jong PE, Gansevoort R. First morning voids are more reliable than spot urine samples to assess microalbuminuria. J Am Soc Nephrol 2009 Feb;20(2):436-43.
141 Skaaby T, Husemoen LL, Ahluwalia TS, Rossing P, Jorgensen T, Thuesen BH, et al. Cause-specific mortality according to urine albumin creatinine ratio in the general population. PLoS One 2014;9(3):e93212.
142 Bland JM, Altman DG. Multiple significance tests: the Bonferroni method. BMJ 1995 Jan 21;310(6973):170.
143 Ioannidis JP. Contradicted and initially stronger effects in highly cited clinical research. JAMA 2005 Jul 13;294(2):218-28.
144 Pocock SJ, Collier TJ, Dandreo KJ, de Stavola BL, Goldman MB, Kalish LA, et al. Issues in the reporting of epidemiological studies: a survey of recent practice. BMJ 2004 Oct 16;329(7471):883.

145 Rothman KJ. No adjustments are needed for multiple comparisons. Epidemiology 1990 Jan;1(1):43-6.
146 Perneger TV. What's wrong with Bonferroni adjustments. BMJ 1998 Apr 18;316(7139):1236-8.
147 Bender R, Lange S. Adjusting for multiple testing--when and how? J Clin Epidemiol 2001 Apr;54(4):343-9.
148 von EE, Altman DG, Egger M, Pocock SJ, Gotzsche PC, Vandenbroucke JP. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. PLoS Med 2007 Oct 16;4(10):e296.
149 Austin PC, Mamdani MM, Juurlink DN, Hux JE. Testing multiple statistical hypotheses resulted in spurious associations: a study of astrological signs and health. J Clin Epidemiol 2006 Sep;59(9):964-9.
150 Husemoen LL, Skaaby T, Jorgensen T, Thyssen JP, Meldgaard M, Szecsi PB, et al. No Association between Loss-of-Function Mutations in filaggrin and Diabetes, Cardiovascular Disease, and All-Cause Mortality. PLoS One 2013;8(12):e84293.
151 Skaaby T, Husemoen LL, Thyssen JP, Meldgaard M, Thuesen BH, Pisinger C, et al. Filaggrin loss-of-function mutations and incident cancer: A population-based study. Br J Dermatol 2014 Mar 14.
152 Kupferschmidt K. Uncertain verdict as vitamin D goes on trial. Science 2012 Sep 21;337(6101):1476-8.
153 Pilz S, Rutters F, Dekker JM. Disease prevention: vitamin D trials. Science 2012 Nov 16;338(6109):883.
154 Pierce BL, Ahsan H, Vanderweele TJ. Power and instrument strength requirements for Mendelian randomization studies using multiple genetic variants. Int J Epidemiol 2011 Jun;40(3):740-52.
155 Daniel RM, Kenward MG, Cousens SN, de Stavola BL. Using causal diagrams to guide analysis in missing data problems. Stat Methods Med Res 2012 Jun;21(3):243-56.
156 Riley RD, Lambert PC, Abo-Zaid G. Meta-analysis of individual participant data: rationale, conduct, and reporting. BMJ 2010;340:c221.