A POPULATION-BASED STUDY ON VITAMIN D, BODY MASS INDEX, MORBIDITY, AND MORTALITY

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Ulla Wewer, Head of Faculty

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List of papers forming the basis of the thesis.


Preface

The work for this doctoral thesis was conducted at the Department of Clinical Biochemistry, Herlev and Gentofte Hospital, from 2011 to 2016.

I am most thankful for working under the auspices of Professor Børge G. Nordestgaard, who inspired me to become a better scientist through combining creative and systematic thinking; he has with his diligence, enthusiasm, and striving for constant betterment in conducting science shown me a path to follow in the long run. Furthermore, I thank Prof. Nordestgaard and members of the steering committees for the Copenhagen General Population Study and the Copenhagen City Heart Study for their permission to use their data for allowing me to work with data from their unique studies. I also owe thanks to Professor Stig E. Bojesen, who introduced me to the complexities of analyzing large observational studies including statistical programming and helped to improve my articles with constructive comments and by thinking outside the box. My co-authors Peter Lange, Jacob J. Freitberg, Peter Brøndum-Jacobsen, Gorm B. Jensen, and Anne Tybjærg-Hansen are thanked for their collaboration and important contributions. I thank former and present Heads of Department, Chief Physicians, Niels Fogh-Andersen and Pia R. Kamstrup, respectively, for providing excellent working conditions. Much of this work would have been impossible without the excellent technicians in the DNA research laboratory at the Department of Clinical Biochemistry, Herlev Hospital, I owe them a special thanks.

The foundation for success is often built on one’s family relations. Therefore, I am grateful for the constant love and support of my parents, Mohammed and Razia. I will be forever grateful to my wife, Maya, without her unyielding support and love this would have been impossible; in her I have truly found my better half.

For my children Noah, Iman, and Adam
Introduction

At the turn of the 19th century, investigations into the epidemiology and treatment of rickets found that exposure to sunlight or artificial light sources emitting ultraviolet B radiation (UVB) could cure rickets as could ingestion of cod liver oil, butterfat or foods irradiated with UVB. This lead researchers to postulate the existence of a new antirickets factor, which was named vitamin D. However, the vitamin D isomers were not chemically identified until 1931-1936, and the active metabolite 1,25-dihydroxyvitamin D (1,25(OH)₂D) was discovered in 1971. It was shown to be either absent (deficient production, type 1) or increased (end-organ resistance, type 2) in hereditary vitamin D dependent rickets.

Recently, the list of possible roles of vitamin D deficiency in human health has been extended beyond bone and calcium homeostasis inspired by the discovery of widespread tissue expression of the vitamin D receptor, studies showing higher incidence of e.g. cancer at latitudes with lower vitamin D production in the skin, and theories postulating that the evolution of lighter skin at higher latitudes is driven by the need for increased vitamin D production in the skin. Correspondingly, observational studies have suggested that vitamin D deficiency may be a risk factor for several diseases and increased mortality. However, the most interesting question is whether vitamin D deficiency plays a causal role in pathogenesis of these diseases or is merely a marker of poor health. This is important for public health as risk factors causally associated with diseases provide an opportunity for reduction in disease risk with intervention, whereas interventions against mere markers of disease are doomed to fail. Causality is difficult to prove in medical sciences; though, a combination of evidence from in vitro studies, animal studies, human epidemiological studies, human genetic studies, and randomized intervention studies pointing in the same direction is usually taken to indicate a causal association. In this thesis, the focus will be on...
the emerging associations of 25(OH)D with new outcomes rather than the associations with calcium homeostasis and bone health.

In contrast to the emerging role of vitamin D as a risk factor for morbidity and mortality, BMI is an extensively studied risk factor for morbidity and mortality. Several major observational studies have firmly established an association of BMI with mortality. However, several points of contention remain. Specifically, the patterns of association are known to vary with age, sex, race, geographical regions, smoking status, and history of major disease. While the handling of these potential confounders in statistical analyses has fueled the controversy over the optimal BMI ranges for health, only little attention has been given to whether these patterns are changing over time.

Furthermore, high BMI is associated with increased risk of diabetes, mortality, and low 25(OH)D, while low 25(OH)D is associated with increased risk of diabetes and mortality independently of BMI in observational analyses; thus, it seems interesting to investigate whether 25(OH)D and BMI are associated with each other, whether this association is causal, and whether they act independently or together in causing disease.

Methods

The following section focuses on general methodological issues not discussed in depth in the articles forming the basis of this review. Details on the measurement of biochemical markers, genotyping methods, specific limitations, and the statistical approaches are given in the articles.

Literature review

There has been a veritable explosion in the literature concerning vitamin D and the association of BMI with mortality. Thus, as in any review prudent choices had to be made as to which literature to reference in this exposition. Here, the focus will be on epidemiological studies, genetic studies, and randomized intervention trials at the expense of in vitro and animal studies that will only be discussed in passing. This choice was made for the simple reason that implementation in clinical practice requires evidence from the former types of studies as a minimum. Furthermore, appropriate meta-analyses will be referenced when possible rather than single studies due to the sheer number of individual studies that have been published (Figure 1).

![Figure 1. Development in publications concerning vitamin D and the association of BMI with mortality.](image)

The Mendelian randomization approach

There are numerous excellent reviews of the Mendelian randomization approach both with regard to concepts and methods, so I will refrain from reiterating their findings here. However, I will discuss the general assumptions and their testing in our studies to provide a heuristic background for understanding the results of our studies.
In essence, Mendelian randomization is a variant of instrumental variable analysis using genetic variants as instruments. Instrumental variable analysis is different from other observational designs in that it tries to eliminate confounding without even measuring confounders. This can only be achieved if the three core assumptions of instrumental variable analysis hold:

1. The instruments (genetic variants) are associated with the exposure
2. The instruments (genetic variants) are associated with the outcome only through the exposure (exclusion restriction assumption)
3. The instruments (genetic variants) are independent of confounders of the exposure-outcome relation

Of these assumptions only the first is directly testable. Assumptions 2 and 3 cannot be directly tested but can be rendered probable through use of "falsification". In addition, Mendelian randomization studies face several potential limitations that may violate these assumptions (Table 1). Examples of how some of the processes listed as limitations may violate the assumptions of Mendelian randomization studies are illustrated in Figure 2.

First, a reliable and sufficiently strong instrument variable is required to obtain unbiased estimates. Strength of an instrument can be evaluated based on estimates of the F and R² statistics. Case in point, our chosen genetic variants were associated with 25(OH)D both when used independently and combined in allele scores (Figure 3). While the F statistic is high (119-327), the variation explained by our instruments is relatively small (R² = 0.4-1%) indicating that a large sample size may be required to detect statistically significant associations. Weak instruments can lead to bias in the direction of the observational estimate. Crudely, this can be understood by differences in confounders between genotypes explaining more of the variation in the phenotype than the genetic variant for weak instruments, which will bias the instrumental variable estimates toward the biased observational estimates. Additionally, inappropriate genetic variants could be selected from the literature, which may have shown low reproducibility in the genetic variant-exposure association, and may not be associated with the exposure leading to violation of, amongst others, the first assumption. Second, violations of the other assumptions can occur in the face of pleiotropy. Pleiotropy is defined as effects of a single genetic locus on two or more (unrelated) phenotypes; the genetic locus in question can be a genetic region, e.g. a gene, or a genetic mutation. In the context of Mendelian randomization, it is usually understood as that the chosen genetic variants have other phenotypic associations beside the association with the exposure (Figure 2). However, violation of the exclusion restriction assumption only occurs if the other separate phenotypes are

<table>
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<th>Observational</th>
<th>Mendelian randomization</th>
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<td>Potentially excellent follow-up trough registries</td>
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Table 1. Strengths and limitations in principle and practice for different designs used to evaluate causal relations in human studies.
associated with the outcome. Pleiotropy has historically been classified into several subtypes and has different meaning in different fields; however, here the focus will be on classification of pleiotropy into genuine and spurious pleiotropy in a context most directly applicable to Mendelian randomization. There are many synonyms used for the different types of pleiotropy, which especially introduce ambiguity in the use of the term spurious pleiotropy (Table 2). E.g., spurious associations between genetic loci and multiple phenotypes may occur due to biases known from classical observational studies, e.g. when using registers or case-control studies, one could speculate that registration of a phenotype or an outcome may be linked with increased registration of other phenotypes or outcomes not directly affected by the exposure (or the outcome). This could lead to the false conclusion that the genetic locus in question is affecting multiple phenotypes, and is classified by some as spurious pleiotropy. Another type of pleiotropy can also be defined, so-called mediated pleiotropy, where a genetic locus affects a phenotype, which causally affects...
downstream phenotypes\textsuperscript{62}. This is used in the framework of Mendelian randomization. However, mediated pleiotropy may be called spurious pleiotropy due to historical nomenclature; however, many authors deplore the use of the term spurious for this type of pleiotropy\textsuperscript{61-64}. Genuine pleiotropy is when a single causal variant affects two independent phenotypes or when different causal variants within the same gene, or genetic region, affect independent phenotypes. This definition is extended to tagging single nucleotide polymorphisms (SNPs), since they may tag a single causal variant with independent phenotypic effects or tag independent causal variants with independent phenotypic effects within the same gene\textsuperscript{62}. Often tagging SNPs are used as instruments in Mendelian randomization studies, since instruments are frequently chosen from large genome-wide association studies where the underlying causal variants are usually unknown. Spurious pleiotropy occurs when using tagging SNPs in regions with multiple genes and strong linkage disequilibrium. In this case, a tagging SNP may concomitantly tag causal variants in different genetic loci, and that is not genuine pleiotropy given the formal definition of pleiotropy\textsuperscript{62}.

Theoretically, the bias due to pleiotropy can be both towards and away from the null hypothesis as the association of the genotype with separate phenotypes can be in the same or opposite directions and these phenotypes may be associated with synergistic or antagonistic effects on the outcome. In practice, it is often difficult to exclude pleiotropy as an explanation for findings in Mendelian randomization studies due to limited knowledge about the used genetic variant or gene function; possible avenues to investigate pleiotropic effects include review of biological knowledge of specific variants, genes, or genetic regions, testing for pleiotropy in relevant experimental study designs, and indirectly with statistical tests when using multiple genetic variants as instruments\textsuperscript{65-67}.

In our studies, some of the results, i.e. risk of diabetes and cardiovascular mortality, were different for genetic variants in \textit{DHCR7} and \textit{CYP2R1} as shown in article 6 and 7\textsuperscript{61-62}. This could be due to pleiotropic effects, but known biological functions of the genes or variants do not provide direct evidence to support or disprove this notion. Furthermore, other studies have not found convincing evidence of pleiotropy when evaluating genetic variants in \textit{DHCR7} and \textit{CYP2R1}\textsuperscript{61-62}, and we did not find any associations with cholesterol, testosterone, estradiol, or cortisol\textsuperscript{65-7}, all metabolites related to 25(OH)D in structure and metabolism; thus, it is unclear whether the different associations of \textit{DHCR7} and \textit{CYP2R1} genetic variants with diabetes and cardiovascular mortality were due to pleiotropy, play of chance, or different biological roles of endogenous and exogenous vitamin D sources.

Third, the assumptions may be violated in the presence of population stratification or correlation (linkage disequilibrium) between genetic variants acting as instruments and genetic variants independently associated with the outcome, i.e. not through the exposure. However, correlation of genetic variants acting as instruments with causal variants for the exposure of interest is useful and often assumed in Mendelian randomization studies\textsuperscript{71}. Confounding due to population stratification occurs if allele frequencies and outcome vary according to particular subgroups, e.g. due to ancestry or ethnicity, in the population, and subgroup status is independently associated with the outcome\textsuperscript{70-72}. Thus, failure to account for population subgroups could confound associations between a genetic variant and an outcome leading to spurious associations. In our study, we only included white individuals of Danish descent, i.e. an ethnically homogenous population; to account for possible population stratification, and we specifically tested whether the used genetic variants were in linkage disequilibrium with known genome-wide association hits for our specific outcomes\textsuperscript{61-7}.

Fourth, canalization, originally defined as the ability of developing a consistent phenotype in spite of varying genetic and environmental influences during development\textsuperscript{73-75}, can lead to violations of the exclusion restriction assumption. This buffering of perturbations during development could lead to an altered association of a genetic variant used as an instrument with an outcome, whereas the
association of the genetic variant with the exposure remains unchanged. E.g. under conditions of
deficiency of an important factor during development of specific tissues or organs due to specific
genetic variants, these may develop to compensate for this deficiency, and make them less sensitive
to deficiency of this factor in the future. The underlying mechanisms of canalization are actively
investigated and may involve genetic redundancy, genetic interactions and so forth74, but exact
definitions of canalization and separating it from related mechanisms can be difficult75.
Furthermore, it has been argued that the instruments used in Mendelian randomization are usually
modestly associated with phenotypes; therefore, it is uncertain that these modest effects can induce
processes associated with canalization48,49,51. While several examples of canalization exist in
laboratory experiments of simpler organisms74,75, canalization is more difficult to study in human
populations. Therefore, we cannot exclude that our results could have been affected by canalization,
although our positive findings cannot obviously be explained by canalization.
Violations of core assumptions may also occur if the genetic variant is associated with measured or
unmeasured confounders. One can adjust for confounders associated with the genotype, but the
presence of such associations could indicate that the genetic variants are associated with other
unmeasured confounders. In our studies we have tested the genotypes against a range of covariates
or confounders with little evidence of unexpected associations8,6. Additionally, other statistical
assumptions may be needed to derive “causal” estimates rather than just testing for the presence of a
causal effect only, e.g. regarding the shape of the associations of the exposure with outcome, e.g.
linearity and no interactions, and the distribution of the exposure50,52. However, these assumptions
may likewise be untestable as they may partially depend on knowledge of unmeasured confounders.
All in all, we found little evidence for violations of the core assumptions and believe our
instrumental variable analyses are valid.

**Causality: comparison of observational, Mendelian randomization, and randomized intervention
studies**

Establishing causality in medicine has always been a challenge; however, with the advent of
randomized intervention studies a way to circumvent the major problems of observational studies,
i.e. quantitative epidemiological studies, was developed76,77. The major problems with observational
studies is the presence of systematic error or bias78 including 1) selection bias, i.e. differential
inclusion or follow-up in a way that distorts the exposure outcome relationship, 2) information bias,
i.e. bias occurring due to measurement error or differences in measurements between the exposed
and unexposed group, 3) confounding, i.e. non-comparability of the exposed and unexposed groups,
and 4) reverse causation, i.e. the outcome affects the exposure and not vice versa. We use general
population cohorts recruited randomly from the white Danish general population, with
measurements of exposure variables on all or randomly selected individuals, and follow-up and
outcomes are assessed through registries irrespective of exposure group. Thus, in our context
especially confounding and reverse causation is a major concern rather than selection or
information bias. Nevertheless, empirical studies show that well-conducted observational studies
give comparable estimates to randomized intervention studies79-82, and observational studies have
provided evidence for causality that few would doubt today83. However, the problem of reverse
causation, unmeasured confounders, and residual confounding will always be a problem with
observational studies and can lead to spurious findings84,87.
Crudely, it could be argued that observational studies are the weakest design for establishing
causality, while randomized intervention studies are the gold standard (Table 1). However, a
Mendelian randomization approach allows for studying causality in a slightly different setting than
randomized intervention studies. Firstly, the use of genetics influencing an exposure potentially
captures more sources of variation compared to using an intervention. Secondly, lifelong exposure
is tested rather than short term interventions. Nevertheless, these different settings also make
randomized intervention trials better at identifying interventions that may have a therapeutic effect rather than establishing causality only.

**Data sources and limitations**

**The Copenhagen City Heart Study and the Copenhagen General Population Study**

As detailed in the papers, we used 2 cohort studies recruited from the general population, the Copenhagen City Heart Study (CCHS) and the Copenhagen General Population Study (CGPS).

Individuals in the 2 studies were selected randomly from the Danish Civil Registration System to reflect the adult Danish general population, i.e. individuals aged 20-100 years, white and of Danish descent[8,9] [Personal communication with the Steering Committees of CCHS and CGPS (Børge G. Nordestgaard and Stig E. Bojesen)]; the latter classification from the Danish Civil Registration System requires that the individual is born in Denmark, has Danish citizenship, both parents are born in Denmark, and both parents have Danish citizenship[9]. The small fraction of participants in the studies not fulfilling these criteria was excluded electronically before statistical analyses in the majority of analyses. At each examination in both studies, participants filled out a comprehensive questionnaire checked by study examiners and underwent a subsequent health examination including blood samples and spirometry.

We used data from all 4 examinations of the CCHS in different combinations across the 8 articles; however, the examinations differed with regard to response rate and recruitment strategy. At all follow-up examinations all individuals invited for previous examinations were invited with additional individuals to replenish the cohort (Figure 4)[8,8]. 19329 individuals were invited in 1976-1978, in 1981-1983 an additional 500 individuals aged 20-25 years were invited, in 1991-1994 an additional 3000 individuals aged 20-49 years were invited, and in 2001-2003 an additional 2464 individuals aged 20-29 years were invited[8,9] [Personal communication with the Steering Committee of CCHS (Børge G. Nordestgaard and Stig E. Bojesen)]. 10270 plasma samples were available for 25(OH)D measurements in 1981-1983 and DNA was only available from the 1991-1994 and 2001-2003 examinations. Sample sizes in different articles differed according to the study designs as detailed in the articles.

The CGPS was started in 2003 with ongoing recruitment; we used data from those participating in 2003-2014. The response rate was approximately 45% and the cohort was recruited and examined similarly to the CCHS [Personal communication from the Steering Committee of CGPS (Børge G. Nordestgaard)]. DNA was available on almost all participants with blood samples, the difference representing individuals not consenting to genetic testing. Sample sizes in different articles differed according to the study designs as detailed in the articles, and due to ongoing measurement of 25(OH)D and ongoing recruitment and assessment of outcomes.

The Copenhagen Ischemic Heart Disease Study consists of patients from the Copenhagen area referred for coronary angiography in 1991-2011[2]; data was handled similarly to CCHS and CGPS. These individuals were only included in genetic analyses for 2 articles[6,7].

![Flowchart representing those participating in the four examinations of the Copenhagen City Heart Study (CCHS). All previously invited individuals were invited for follow-up examinations and supplemented with new participants.](imageURL)
Registries used to ascertain diagnoses

Data was used from four nationwide registries in our studies, that is, the national Danish Civil Registration System, the national Danish Patient Registry, the national Danish Cancer Registry, and the national Danish Causes of Death Registry. Individuals in these registries are identified by the unique personal identification number. Please note that the names for these registries are used with slight variations in the literature.

The national Danish Civil Registration System was established in 1968 and is an administrative registry of all permanent residents in Denmark, containing the unique personal identification number given to all permanent residents in Denmark at birth or through acquisition of residency. Other information in the registry includes name, sex, date and place of birth, citizenship, geographical origins, immigration/emigration, and vital status. Use of the unique personal identification number allows for linkage of data across national registries with information obtained from epidemiological studies.

The national Danish Cancer Registry was established in 1943 and contains data on the incidence of cancer in the Danish population. The information recorded in the registry includes year of birth, sex, cancer diagnosis, date of diagnosis, and information on tumor characteristics (e.g., morphology, topography, stage etc.). The registry uses ICD-7 coding for diagnoses until 1977, and all diagnoses have been coded for diagnoses according to ICD-10 for 1978-1994 and coded according to ICD-10 for diagnoses from 1994 and onward. The data collection procedures have undergone several changes; however, both historically and currently the registry has high coverage and accuracy.

The national Danish Patient Registry was established in 1977 and has undergone some changes in the registered information since its commencement. Initially, information on all inpatient hospitalizations was included; however, from 1995 the registry was expanded to include all hospital inpatient, outpatient, emergency care, and other hospital contacts. Furthermore, diagnoses were coded using the World Health Organization International Classification of diseases (ICD) edition 8 (ICD-8) until 1994, and from 1994 the registry has used ICD-10 codes. The national Danish Patient Registry includes information on personal identification number, hospital, department, admission and discharge date as well as information on established diagnoses and surgical procedures performed during each hospital contact. The registry has high coverage and high validity of administrative data; however, the accuracy for specific disease diagnoses varies from poor to excellent (14%-100%), typically from 65-82%.

The national Danish Causes of Death Registry was established in 1875 and records all deaths among Danish citizens. The diagnoses for underlying and contributing causes of deaths are recorded as recommended by the World Health Organization, and the ICD-system has been used since 1950 with the ICD-10 edition being implemented from 1994. However, the coding practices have changed over time, until 2007 the coding was done by the Danish Health Authority based on death certificates completed by physicians in hospitals, general practice, or forensic medicine.

After 2007 the electronic death certificate was introduced, henceforth the physician verifying death was required to submit the death certificate electronically and code the causes of death according to ICD coding. Only 0.3-0.6% of deaths have missing or incomplete death certificates.

Validity of registry diagnoses

The validity of some of the registry diagnoses used in the articles that constitute this thesis has been investigated in other studies. The national Danish Cancer Registry is among the best in the world with a completeness of 95-98%, and with morphological verification of 89% of tumors.

We used the national Danish Patient Registry in combination with self-reported diabetes, self-reported use of antidiabetic medicine, and glucose measurements to establish prevalent diabetes cases, incident cases were based on cases registered in the national Danish Patient Registry and the national Danish Causes of Death Registry. Investigations of the national Danish Patient Registry have shown that the diabetes diagnoses have a sensitivity of 64% and a positive predictive value of 19%.
97%  107. An algorithm has been proposed to better identify diabetes patients in Denmark using the national Danish Patient Registry as in this study, but combined with registers covering the primary health care system  108, laboratory data, and prescription medicine  109 as additional sources to identify diabetes cases. This approach is used in the National Diabetes Register established in 2006 to improve registration of diabetes  110. In this registry, the sensitivity is estimated to be higher than 86% and the positive predictive value is higher than 89% for data after 1995. However, we had studies from 1981-1983, 1991-1994, 2001-2003, and 2003-2012 and tried to ascertain cases in the same manner across all three studies. While the use of the newest algorithms would have improved ascertainment of incident diabetes cases in the Copenhagen General Population study, we would not have the same high quality data for incident cases in Copenhagen City Heart Study’s 2nd and 3rd examination before 1995, making ascertainment of diabetes different in the different studies. That said, in future studies we plan to also incorporate diabetes diagnoses from the National Diabetes Register. Nevertheless, the approach used in our study should be better than using the national Danish Patient Registry diagnoses alone. We show the use of our algorithm in updated data from the Copenhagen General Population study for prevalent cases in Table 3. Likewise, the dementia registry diagnoses have shown reasonable validity for the endpoints any dementia (85.8% correctness) and Alzheimer’s disease (81% correctness), but the validity for other subtypes of dementia was poor  111. However, due to policy changes the diagnoses are now usually carried out in specialized dementia clinics meaning that accuracy of the diagnoses have probably improved in recent years. The national Danish Civil Registration System records are continuously updated with vital status and, barring those who have emigrated, it should be complete for Danish residents; however, the accuracy of the national Danish Causes of Death Registry relies on the codes used in death certificates to classify cause of death by physicians  112,  113. There is no systematic evaluation on the clinical validity of diagnoses on death certificates; however, results from a comparable health care setting indicate that around 7% of recorded diagnoses may be wrong and around 3% requires recoding of the cause of death  114, but, given the low autopsy rate in Denmark (<10%), there may be up to 30% incorrect death diagnoses on death certificates as shown by an international meta-analysis comparing autopsy reports with registry diagnoses  115. Thus, while the registration of deaths is exceptionally good, the diagnoses used to classify cause-specific deaths is probably less precise indicating a need for more careful interpretation of this endpoint compared to all-cause mortality.

Table 3. Cross tabulation of the 4 variables used to construct diabetes diagnoses in the Copenhagen General Population Study.

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<tr>
<th>Self-reported diabetes</th>
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<tr>
<td>Yes (N) No (N) All (N)</td>
<td>4035 104190 108225</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Registry diagnosis at examination</td>
<td>Yes 2,152 297 2449 No 1,883 103,898 105781</td>
<td></td>
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</tr>
<tr>
<td>Self-reported antidiabetic medication</td>
<td>Yes 3025 115 3140 No 1010 104080 105090</td>
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<tr>
<td>P-Glucose ≥ 11.1</td>
<td>Yes 443 171 614 No 3592 104024 107616</td>
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*Female participants may receive metformin for other indications; however, the proportion of males was 0.48 in this subsample similar to the whole sample (0.45).

**Potential biases and limitations**

In general, for possible misdiagnoses to bias our results away from the null, they have to be differential according to exposure or outcome. For vitamin D and genotypes, differential ascertainment of diagnoses according to exposure is difficult to imagine as the used exposures are unknown by participants, doctors that diagnose the diseases, and examiners that record the questionnaire data. However, very high BMI is an easily identified phenotype and may lead to bias, as those exposed may be questioned, investigated and followed more intensely in the health care system, which could lead to spurious associations. Furthermore, another type of bias could affect our results when investigating possible causal relationships: if an exposure causes a disease that leads to follow-up in health care systems, one could imagine that other unrelated diagnoses may be found more often in these patients. One would expect the exposure to be associated with these other conditions.
diagnoses as well, even though there may be no biological pathways from the exposure to these outcomes. This could affect observational as well as Mendelian randomization studies as discussed under the related issue of false pleiotropy in registry studies (The Mendelian randomization approach). Additionally, not all diagnoses obtained from registries have been validated and incorrect registration may reduce the validity of some endpoints (see Validity of registry diagnoses). This potential problem is twofold; first, there is the possibility of unregistered disease that we do not have information on. Second, there is the possibility of misclassification. An example of the former is diabetes that is primarily diagnosed by a general practitioner. However, we have information on self-reported diagnosis and medication that can be combined with registry data to increase the completeness and perhaps the validity of this diagnosis (Table 3). An example of potential misclassification of a diagnosis is COPD. We defined COPD based on lung function measurements rather than on hospital diagnoses, as time-wise the latter may reflect the first exacerbation more than timing of a COPD diagnosis. However, our spirometric diagnoses have limitations of their own. Our spirometry measurements were pre-bronchodilator measurements and a spirometric definition of COPD will also identify individuals without symptoms. Therefore, we cannot exclude that some individuals have reversible airflow limitation, e.g. asthma, and clinically irrelevant mild obstructive pulmonary disease. A problem with general population studies is sampling bias due to possible overrepresentation of relatively healthy individuals compared to studies recruiting in a hospital setting. Individuals who do not respond to study invitations tend to have poorer socioeconomic status, lifestyle, and medical status compared to those who respond; usually, leading to an underestimation of disease prevalence and limiting the external validity. Our studies do not include immigrants and there is likely an underrepresentation of people with severe disease or substance abuse and institutionalized individuals; all subgroups that usually have lower 25(OH)D levels compared to average levels in a population and a different distribution of BMI. Therefore, those with extremely low 25(OH)D will be underrepresented in our studies, and previous studies seem to suggest that these individuals would benefit the most from supplementation (see Vitamin D deficiency: definition, measurement, and prevalence). Likewise, those with extreme BMI values may also be underrepresented as immigrants, individuals with severe disease, individuals with substance abuse, and institutionalized individuals may have a different distribution of BMI than healthy white individuals in an urban setting. This has the consequence that the generalizability of our results is reduced to relatively healthy, white Danes living in an urban setting, and extrapolation to the general population could lead to biased estimates of our exposure with several outcomes. However, internal validity will only be affected if participation leads to effect modification of the association between exposure and outcome. While, I am not aware of any evidence to suggest that the potential effects of low 25(OH)D should be qualitatively different between the mentioned subgroups of the population, the case for extreme BMI values is more controversial as discussed in connection with article 8. Furthermore, in the CCHS DNA was available in the third (1991-1994) and fourth (2001-2003) examination only, i.e. all genetic analyses are based on individuals who survived and were willing to attend these examinations. Thus, it is difficult to exclude the occurrence of survivor selection bias; however, we believe this to be a minor issue, since genotype distributions were in Hardy-Weinberg equilibrium, since the original cohort was supplemented with younger individuals at each re-examination aiming to minimize this problem, and as genotypes affecting both disease risk and death, e.g. APOE ε alleles, showed the expected associations with relevant outcomes. Most of these potential biases would probably lead to bias towards the null and could lead to false negative findings; however, as discussed in the preceding paragraphs and in the articles, it seems
that these biases should have only minor effects on our results and, likely, cannot explain positive findings.

**Differences in exposures and covariates between articles on vitamin D and outcomes**

There are some design differences between the articles with regard to classification of exposure categories. These differences reflect compromises between comparability to related previous studies, power issues, interpretability by the readership, and reviewer comments in each study. In general, 25(OH)D < 50 nmol/L was used to indicate insufficiency or deficiency (see Vitamin D deficiency: definition, measurement, and prevalence). The initial approach was to use categories of 25(OH)D that allowed us to investigate the effect of very low levels, e.g. <12.5 nmol/L or ≤5th percentile. However, while this was feasible for tobacco-related cancers and diabetes\(^3\,^5\), for other endpoints there were simply too few events in the lowest group to act as the baseline group, e.g. article 2\(^5\). Furthermore, while the category 25(OH)D <12.5 nmol/L is used by the Danish Health Authorities to define extreme vitamin D deficiency\(^1\,^10\), it is not used internationally, which was pointed out by external reviewers. Thus, we decided to collapse the categories <12.5 and 12.5-25 nmol/L in subsequent papers. Additionally, seasonally adjusted quantiles were used in several articles in conjunction with directly measured 25(OH)D, as it may be the optimal method to adjust for seasonal variation in 25(OH)D\(^3\,^11\). The use of different quantile categories, i.e. tertiles, quartiles, and quintiles, where partly dictated by a desire to make our results comparable to other major studies related in subject to our studies\(^11\,^2\,^13\), and in some cases the results of reviewer comments. In the dementia paper we investigated two endpoints\(^5\), but one of the endpoints was relatively rare, so the exposure was divided by using the median. To keep some consistency across the endpoints, we decided to use the same baseline groups for the two endpoints, but further subdivide the lower category into two equal sized groups to investigate a possible dose-response relationship for Alzheimer’s disease. The use of deciles in the article on lung function was used to show finer details in the association of 25(OH)D with lung function measures as both were continuous measures\(^4\). Overall, we found that use of 25(OH)D in nmol/L with adjustment for season or as seasonally adjusted percentiles provided for similar results; in retrospect, we could perhaps have achieved more consistent classification of exposure by using e.g. spline models supplemented by 25(OH)D in clinical categories. However, spline models also require choices that have to be justified in other ways, and can, in our experience, be more difficult to interpret for a medical readership. In Mendelian randomization studies, we consistently used 25(OH)D as a continuous exposure as discussed in Vitamin D deficiency: definition, measurement, and prevalence.

The choice of covariates for adjustment was based on *a priori* selection of variables that may confound the association of 25(OH)D with the endpoint in question (based on previous literature), partly inspired by the major risk factors for early death according to the World Health Organization, since many of these have been shown to be associated with 25(OH)D and are thought to be major causes of early deaths through disease\(^1\,^14\). However, some of the risk factors were only relevant for some diseases, e.g. biologically it is reasonable to include blood pressure in models for e.g. mortality and dementia\(^5\,^7\), whereas it may seem far-fetched to include it in studies with skin cancer as an outcome\(^2\). Additional variables were included to represent socio-economic status such as income status and education. Income status is an indirect measure for the problems of low socioeconomic status, that is, the clustering of potentially adverse life style and environmental factors; while education can serve as a similar measure, it may be a better measure of so-called cognitive reserve rather than socioeconomic status. Thus, for the dementia paper we also included education as several studies show that higher education may be independently associated with lower risk of dementia or Alzheimer’s disease\(^1\,^15\). In the tobacco-related cancer article\(^1\), education was used as a measure of socioeconomic status, in retrospect income status may have been the more
appropriate indicator of socioeconomic status; however, the results were similar whether one or the other was used. We only used time-varying covariates incorporating information from follow-up examinations for the tobacco-related cancer and diabetes article, but not subsequent articles; this decision was based on the fact that models with time varying covariates and models using only baseline measurements gave similar results, and that use of time varying covariates carry assumptions of their own, which can be untenable and bias the results if not fulfilled. One important confounder, we would have liked to have included, was use of vitamin D supplements; however, this information was not available in any of the cohorts. Though, a related sensitivity analysis in the Copenhagen General Population adjusting for vitamin supplement use did not seem to affect estimates for the mortality analyses (article 7).

Metabolism and mechanism of actions of vitamin D
Sources, synthesis, and catabolism of vitamin D
Vitamin D is a collective term used to describe the two secosteroid prohormones vitamin D$_2$ and vitamin D$_3$. The most important source of vitamin D in most parts of the world is conversion of 7-dehydrocholesterol to vitamin D$_3$ by solar UVB radiation in the basal layers of the skin (Figure 5, Table 4). However, dietary contributions become important at extreme latitudes as solar UVB radiation is inadequate to maintain sufficient vitamin D levels, circulating vitamin D$_2$ originates from (fortified) food and/or supplements as it is not produced in vivo in man.

Vitamin D does not exert physiological effects itself and is activated by hydroxylations in vivo. The initial hydroxylation at carbon 25 is mainly carried out in hepatocytes by vitamin D 25-hydroxylase (CYP2R1), while the final 1α-hydroxylation step to generate the active hormonal form 1,25(OH)$_2$D is carried out in the proximal tubuli of the kidney by 1α-hydroxylase (CYP27B1). The catabolic pathway for 25(OH)D and 1,25(OH)$_2$D is mainly initiated by vitamin D 24-hydroxylase (CYP24A1) at carbon 24, and to a lesser degree at carbon 23, and catalyzes most of the following reactions leading to the final excreted metabolites.

Other enzymes have been discovered that can substitute for vitamin D 25-hydroxylase or lead to alternative metabolic pathways, but the in vivo significance of these pathways is unresolved.

Table 4. Sources of vitamin D in vivo and dose-response relationships.

<table>
<thead>
<tr>
<th>Source</th>
<th>Explained variation (%)</th>
<th>Dose</th>
<th>Vitamin D, µg</th>
<th>Δ25(OH)D, nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sun exposure (UVB)</td>
<td>20-36%</td>
<td>1 MED</td>
<td>250</td>
<td>~40</td>
</tr>
<tr>
<td>Diet</td>
<td>2-60 %</td>
<td>100-1000 IU</td>
<td>2.5-25</td>
<td>~15-40</td>
</tr>
<tr>
<td>Supplements (D$_2$)</td>
<td>0-15%</td>
<td>800 IU</td>
<td>20</td>
<td>~12-40</td>
</tr>
</tbody>
</table>

These data are derived from multiple sources and represent simplified estimates as vitamin D production and levels depends on factors such as skin type or color, age etc. Normally diet accounts for less than 10% in most populations; however, in populations with low UVB exposure or low levels of 25-hydroxyvitamin D the relative significance of diet increases.

Regulation of vitamin D metabolism
Keratinocytes in the layers of the skin with highest de novo 7-dehydrocholesterol synthesis, the precursor of vitamin D$_3$ and cholesterol, are devoid of low density lipoprotein (LDL) receptors. Thus, uncoupling cholesterol synthesis in skin from circulating cholesterol, that is the major regulator of synthesis in the liver, but retaining negative feedback from intracellular cholesterol. However, only up to 15% of 7-dehydrocholesterol is converted to vitamin D$_3$, and prolonged exposure to UVB degrades pre-vitamin D$_3$ and vitamin D$_3$ to inactive metabolites, which makes it impossible to get vitamin D$_3$ intoxication through sun exposure. The enzymatic hydroxylations producing the active and inactive metabolites are tightly regulated (Figure 5); the 1α-hydroxylation step in the kidney is up-regulated by parathyroid hormone (PTH) and down-regulated by calcium, phosphorous, fibroblast growth factor-23 (FGF-23), and 1,25(OH)$_2$D itself, and the 24-hydroxylation step is up-regulated by 1,25(OH)$_2$D and FGF-23 leading to a classical negative feedback loop. The 25-hydroxylation step is not tightly regulated; however, evidence suggests that individuals with vitamin D depletion have greater increases in 25(OH)D after vitamin D supplementation than vitamin D replete individuals indicating that 25(OH)D is a regulator of its own synthesis.
Distribution, storage, and excretion of vitamin D
Vitamin D produced in skin and hydroxylated vitamin D metabolites are mainly bound to vitamin D binding protein (VDBP or group specific component (GC)) in plasma with a small proportion of bound by albumin and perhaps lipoproteins. Vitamin D derived from the diet is transported mostly by chylomicrons through chyle to the liver and other organs.

The different metabolites differ in their affinity to VDBP, and, consequently, in their concentrations and free fractions in plasma, which translates to shorter half-lives with higher free fractions (Table 5). Also, the degree of in vivo storage differs for the metabolites; only vitamin D is stored in significant amounts in adipose tissue, while 25(OH)D is stored in smaller amounts in different tissues.

**Table 5. Characteristics of major circulating forms of vitamin D.**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Plasma, nmol/L</th>
<th>Free fraction, %</th>
<th>Plasma t½ days</th>
<th>Storage tissues</th>
<th>VDBP, relative affinity</th>
<th>VDR, relative affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>D3</td>
<td>~5-10</td>
<td>1-3</td>
<td>1-3</td>
<td>Adipose</td>
<td>0.1-0.001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>25(OH)D</td>
<td>25-162</td>
<td>0.03</td>
<td>15-25</td>
<td>Multiple</td>
<td>1</td>
<td>0.01-0.0025</td>
</tr>
<tr>
<td>1,25(OH)2D</td>
<td>0.036-0.144</td>
<td>0.4</td>
<td>~0.2</td>
<td>-</td>
<td>0.1-0.01</td>
<td>1</td>
</tr>
<tr>
<td>24,25(OH)2D</td>
<td>~1-10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

*Data unavailable, VDBP = vitamin D binding protein, VDR = vitamin D receptor.*

**Overview of mechanism of actions of vitamin D**
1,25(OH)2D is the in vivo metabolite that is the most potent stimulator of the vitamin D receptor (VDR). The VDR gene consists of 9 exons, exon 1 contain regulatory and promoter regions, while the remaining exons encode nuclear localization, DNA binding, ligand binding, dimerization, and transactivation domains, and it belongs to the superfamily of nuclear receptors characterized by a conserved DNA binding domain and a variable ligand binding domain. Upon binding of 1,25(OH)2D, the VDR forms a heterodimer with the retinoid X receptor that binds to DNA at specific vitamin D response elements (VDREs) in the regulatory regions of different genes either initiating or repressing transcription. Chromatin immunoprecipitation studies have indicated...
that up to 1-3% of all genes may be regulated by VDR-1,25(OH)\(_2\)D interaction, and that the regulatory sites may be located up to thousands of bases from the regulated genes\(^{184,195}\).

Additionally, rapid effects of 1,25(OH)\(_2\)D stimulation have also been reported that are too fast to be the result of genomic regulation, e.g. induction of currents through ion-channels, and may be the result of activation of membrane bound VDR found in caveoli\(^{189,196,197}\). Intriguingly, there is some evidence that VDR may have effects independent of vitamin D metabolites, both through intrinsic activity and other ligands\(^{195,198}\). However, the in vivo significance of fast acting effects and vitamin D independent activities of VDR in humans have not been investigated.

There is definite evidence of concomitant CYP2B1 and VDR expression in several tissues\(^{23,24,199,200}\), indicating that 25(OH)D may be activated locally and act as an autocrine or paracrine mediator in addition to being a hormone. Hence, studies have suggested physiological effects on cells of the immune system; insulin secretion; cell proliferation, differentiation, and death; and possibly the renin-angiotensin system as reviewed elsewhere (Figure 6 and 7)\(^{154}\). The physiological and clinical significance of these "non-classical" functions are an active field of research, and large randomized intervention studies are ongoing to address whether vitamin D supplementation have clinical benefits beyond effects on calcium and bone homeostasis\(^{201}\).

**Figure 6.** Overview of established functions of vitamin D in humans. Circulating 25(OH)D is converted in the kidneys to the biologically active form, i.e. 1,25(OH)\(_2\)D. The classical functions of 1,25(OH)\(_2\)D through stimulation of the vitamin D receptor are 1) decrease in its own synthesis through negative feedback and decrease in the synthesis and secretion of parathyroid hormone by the parathyroid glands, 2) enhanced intestinal calcium absorption in the small intestine by the epithelial calcium channel TRPV6, 3) stimulation of osteoblasts causing an increase in the expression of RANKL, which binds RANK on preosteoclasts inducing differentiation to mature osteoclasts. Thus, 1,25(OH)\(_2\)D regulates uptake and release of calcium (Ca\(^{2+}\)) and phosphorous (HPO\(_4^{2-}\)) from the intestine and bone maintaining calcium and phosphorus levels in the blood. 25(OH)D = 25-hydroxyvitamin D, 1,25(OH)\(_2\)D = 1,25-dihydroxyvitamin D, VDR = vitamin D receptor, RXR = retinoic acid x receptor, RANK(L) = receptor activator of nuclear factor-\(\kappa\)B (ligand), TRPV6 = transient receptor potential cation channel, subfamily V, member 6. CaBP = Calcium binding protein. Adapted from Holick MF. N Engl J Med. 2007;357:266154.
Clinical effects beyond calcium and bone homeostasis

Some physiological and clinical effects of vitamin D supplementation have strong supporting evidence, e.g. there is little doubt that supplementation with vitamin D, 1,25(OH)_2D or 1,25(OH)_2D mimetics increases calcium and phosphate absorption and can be used to treat vitamin D deficiency, rickets (acquired and specific hereditary forms), osteomalacia, and specific forms of hyperparathyroidism. Also, calcium and vitamin D supplementation is widely prescribed for osteoporosis and fracture prevention; however, the use of vitamin D for this purpose is very much in dispute due to conflicting findings from randomized intervention trials. However, the focus of this thesis has been on other diseases and endpoints traditionally not linked to vitamin D deficiency, e.g. early death, diabetes, respiratory disease, and certain forms of cancer. While observational studies have shown strong associations of low 25(OH)D with some of these outcomes, overall few studies support that intervention with vitamin D supplementation will affect these outcomes when looking at randomized intervention trials, or secondarily genetic studies; though, some data do seem to support a potential beneficial effect on mortality, some pregnancy/neonatal outcomes, and dental health. Nevertheless, current data have several limitations including that for most non-classical outcomes the results are from post-hoc analyses of randomized interventional trials designed for other outcomes, dosing and treatment duration vary greatly between studies, mean 25(OH)D levels vary between the study populations, and many trials have been underpowered to detect a potential effect of vitamin D supplementation on these outcomes. Fortunately, several randomized intervention trials are underway that are specifically designed to investigate these non-classical outcomes, thus, higher quality data should be available within a few years for inferring effects of vitamin D supplementation on these outcomes. However, several practical issues are unresolved with regard to the current and likely future place for vitamin D supplements. A simple, but as of yet unanswered, question is what type...
of supplement should be used for treatment of vitamin D deficiency, i.e. vitamin D$_{2}$ vs D$_{3}$. Several studies have found conflicting results, some indicating equivalence of the supplements while recent meta-analyses of randomized intervention trials and new studies have indicated superiority of vitamin D$_{3}$ for treating vitamin D deficiency and, if confirmed, for prevention of mortality. Also, optimal doses and dosing intervals are still a matter of debate as different randomized studies have used different types and doses of supplementation and there is some heterogeneity in effect on the individual levels of supplementation even using similar supplementation strategies. A further complication is that optimal levels for different outcomes may differ as discussed in Vitamin D deficiency: definition, measurement, and prevalence. Additionally, findings also suggest an increased risk of hypercalcemia, hyperphosphatemia, and nephrolithiasis indicating that high levels of supplementation carry health risks of their own. The above-mentioned inconclusive results with regard to disease endpoints stand in stark contrast to the increasing world-wide clinical use of tests for 25(OH)D level in both patients and healthy individuals, even though there is insufficient evidence to support widespread screening for vitamin D deficiency. Thus, while current supplementation recommendations seem effective in raising 25(OH)D levels, the optimal supplementation strategy remains undefined and may vary according to clinical indication and risk groups, and the current wide-spread screening for vitamin D deficiency is not supported by evidence.

Genetics of 25-hydroxyvitamin D metabolism and choice of variants for Mendelian randomization studies

Inactivating mutations causing vitamin D dependent rickets have been identified in CYP2R1, CYP27B1, and VDR, while mutations in CYP24A1 can cause idiopathic infantile hypercalcemia, indicating that both activating and catabolic enzymes are important for proper function of 1,25(OH)$_{2}$D. These genetic diseases can be associated with extreme vitamin D metabolite levels indicating that there may be common genetic variants with less extreme effects on vitamin D metabolite levels and functions. Also, family and twin studies have suggested that genetics contribute significantly to 25(OH)D variability with heritability estimates of ~20-60%.

Genome-wide association studies have indeed identified genetic variants affecting plasma concentrations in relation to genes involved in vitamin D metabolism (Figure 8), i.e. VDBP (GC), DCHR7, CYP2R1, and possibly CYP24A1. However, these variants explain only ~3% of 25(OH)D variation, and a closer look at the identified genetic variants reveal that many of them are in linkage disequilibrium.

In the VDR receptor, two genetic variants have been shown to have functional effects: FokI (rs2228570) and Cdx2 (rs11568820). The former leads to a 3 amino acid shorter protein that may interact more efficiently with TFIB and be 70% more active at transcription initiation. The Cdx2 variant affects the binding affinity of the intestine specific transcription factor Cdx2, the A-allele may be more active and influence intestinal calcium absorption. Other variants such as BsmI (rs1544410), Apal (rs7975232), TaqI (rs731236), or the 3'-UTR polyA length variant have not been shown to have convincing functional effects at the molecular level, although they have been investigated extensively in genetic association studies.

Our strategy for choice of genetic variants for the Mendelian randomization studies was based on two steps. First, selection of genetic variants from the literature with a frequency > 1% that may affect 25(OH)D levels. We included both candidate gene studies and genome-wide association studies. Second, we carried out a pilot project were we tested the association of these variants with 25(OH)D in approximately 5500 individuals from the CCHS (Figure 8). Other than nominal significance we only used variants that were deemed suitable for use in Mendelian randomization studies as discussed in the section The Mendelian randomization approach; e.g. 34
Gene | SNPs | Effect on p-25(OH)D levels
<table>
<thead>
<tr>
<th>%</th>
<th>nmol/L</th>
<th>R², %</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHCR7</td>
<td>rs11234027</td>
<td>-4</td>
</tr>
<tr>
<td></td>
<td>rs7944926</td>
<td>-4</td>
</tr>
<tr>
<td>CYP2R1</td>
<td>rs10741657</td>
<td>-4</td>
</tr>
<tr>
<td></td>
<td>rs12794714</td>
<td>-4</td>
</tr>
<tr>
<td>1,25(OH)₂D</td>
<td>(rs10877012)</td>
<td>(-2)</td>
</tr>
<tr>
<td>CYP24A1</td>
<td>rs6013897</td>
<td>-2</td>
</tr>
<tr>
<td>24,25(OH)₂D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VDBP</td>
<td>rs2282679</td>
<td>-8</td>
</tr>
</tbody>
</table>

Figure 8. Genetic polymorphisms known to be associated with 25-hydroxyvitamin D. The estimates are derived from our own data6;7 (partly unpublished). We genotyped these or tagging single nucleotide polymorphisms in the 1991-1994 examination of the Copenhagen City Heart Study, of which ~5500 also had 25(OH)D measurements from the 1981-1983 examination. rs10877012 has not been replicated consistently in different studies, thus, the parentheses. Results for CYP24A1 are derived from the literature. VDBP = vitamin D binding protein. 25(OH)D = 25-hydroxyvitamin D. 1,25(OH)₂D = 1,25-dihydroxyvitamin D. 24,25(OH)₂D = 24,25-dihydroxyvitamin D. 1,24,25(OH)₃D = 1,24,25-trihydroxyvitamin D. SNPs = single nucleotide polymorphisms.

we found that some of the variants may be associated with pleiotropic effects (VDBP) or the association with concentration may be difficult to interpret (VDBP, CYP27B1, and CYP24A1). As an example we did not use variants in VDBP due to their effect on both circulating 25(OH)D and VDBP, since the latter also acts as a carrier for actin and a chemotaxin in inflammatory responses; thereby the variants affect both 25(OH)D levels and other functions of VDBP simultaneously; the latter may be independently affect risk of cardiovascular disease through vascular effects of actin metabolism and inflammation. Furthermore, while the effects on 25(OH)D levels may be large, the effects on bioavailable 25(OH)D may be nonexistent. Variants in CYP27B1 rs10877012 have not consistently been associated with 25(OH)D level, and were not statistically significant in our sample. Also, while these variants in CYP27B1 and CYP24A1 may be associated with 25(OH)D levels, the interpretation of these associations is not straightforward. If one assumes that these variants are coupled to causal variants within CYP27B1 and CYP24A1 affecting 25(OH)D level, then these differences could reflect a poorer metabolism to active or inactive metabolites, respectively; however, due to feedback mechanisms similar differences may be seen with more efficient activation of 25(OH)D or inefficient deactivation of 1,25(OH)₂D, i.e. a higher 25(OH)D could both reflect a higher vitamin D activity or a lower vitamin D activity (Figure 5). FokI (rs2228570) and Cdx2 (rs11568820) in FDR were not associated with 25(OH)D in our data. Thus, to us variants in relation to genes involved in synthesis of 25(OH)D seemed most appropriate with regard to interpretation and analysis and were selected for further study. Nevertheless, the ideal framework in a Mendelian randomization study would be a causal genetic variant in relation to a gene with a quantitative effect on its gene-product and this gene-product used as an intermediate phenotype for a specific outcome, since this provides for a direct biological plausible explanation for any results. However, our study has added uncertainty compared to this ideal scenario, since we use genetic variants identified through genome-wide association studies with unknown function as instruments, and we used a metabolite as an intermediate phenotype. The use of genetic variants identified through genome-wide association studies as instruments do have the inherent weakness that their function is usually unknown, which leads to uncertainty as to the mechanism by which the association with a potential outcome is mediated. It could be that the association is due to, e.g. pleiotropic effects or other sources of errors as already discussed (see The Mendelian...
randomization approach). We justified our use of this framework on several observations: the variants were in or near genes involved in vitamin D metabolism, mutations in some of these genes may lead to inherited diseases with altered vitamin D metabolism, and the instruments used did not seem to have any pleiotropic effects at the time we initiated our study (see The Mendelian randomization approach).

Vitamin D deficiency: definition, measurement, and prevalence

Originally vitamin D deficiency was equated with rickets or osteomalacia. However, discovery of the vitamin D metabolites enabled a redefinition of vitamin D deficiency based on measurement of 25(OH)D. Vitamin D status is evaluated by measuring 25(OH)D rather than 1,25(OH)₂D, since 25(OH)D is proportional to body stores of vitamin D, has a longer half-life, concentrations are up to 1000 times higher than 1,25(OH)₂D, it is resistant to suboptimal pre-analytical conditions, and since 1,25(OH)₂D concentration is regulated by several factors rather than vitamin D stores only (Figure 5). Several cut-points have been suggested to define deficient levels. One definition is based on the correlation of 25(OH)D and PTH concentrations, i.e. the concentration where PTH is maximally suppressed, corresponding to 25(OH)D levels of ~75 nmol/L⁵¹,⁵². However, in other studies, measuring 1,25(OH)₂D and PTH as well as other bone density or metabolism markers, the more appropriate threshold seems to be 50 nmol/L¹⁰⁶,⁵³. Also, others have defined deficiency as levels below 50 nmol/L as there is little evidence for the presence of bone disease in individuals with 25(OH)D levels of 50-75 nmol/L¹⁴⁶ and maximal calcium absorption seems to be achieved below 50 nmol/L²⁵⁴. Additional problems in defining threshold values stem from analytical difficulties, there are multiple methods with relatively large inter-laboratory variation between laboratories and methods, different determination rates of 25(OH)D₂ and 25(OH)D₃ with different assays, and interference from other vitamin D metabolites²⁵⁵-²⁵⁷. Recently, the vitamin D analyses has been standardized using reference materials and methods; thus, offering the opportunity for traceability of available assays. In essence, this means that new thresholds with greater accuracy may be defined as these traceable assays become widely available²⁵⁸-²⁵⁹. However, estimation of deficiency in different populations is complicated by additional challenges even if the measurements are accepted as accurate. First, the role of seasonal variation is substantial as shown, but definitions of 25(OH)D deficiency using absolute cut points do not account for this. Second, there can be substantial differences in 25(OH)D between populations according their distribution of (functional) genetic variants in VDBP; however, lower levels of 25(OH)D may have quantitatively similar physiological effects as higher levels depending on specific genetic variants in VDBP²⁵⁰. Therefore, there may be genotype specific cut points defining vitamin D deficiency. Lastly, the optimal threshold value for other endpoints than bone disease affected by vitamin D supplementation has not been defined³⁰. Despite all these reservations, we adhere to the general recommendations for defining insufficiency (25-49 nmol/L), and deficiency (<25 nmol/L) as is common practice in Denmark both clinically and in clinical biochemistry laboratories¹⁰⁸. In this thesis, the term deficiency will usually be used to cover both insufficiency and deficiency. As discussed in the Methods section, depending on the subject matter of specific studies some minor changes were made to this definition due to power and to enhance comparability to other studies. In the articles the term low or lower 25(OH)D was used to refer to the categories covering insufficiency or deficiency when 25(OH)D was used as an categorical variable whereas it referred to a difference on the continuous scale when 25(OH)D was used as a continuous variable. In both Mendelian randomization articles⁶-⁷ instrument variable estimates are based on assuming a linear relationship of 25(OH)D with outcome. This approach has several limitations; the model may be inadequate to describe the observed relationship between 25(OH)D and an outcome; thresholds are often important for clinical practice as decision limits and possibly treatment goals; and a difference of on a continuous scale, e.g. 20 nmol/L decrease, is
difficult to relate to clinical practice. However, our approach was necessary since non-linear Mendelian randomization models require complete information on both exposure, genotypes, and outcomes for all participants (due to prohibitory expense)\textsuperscript{260,261}, and our study size, although among the largest for vitamin D studies in published literature, was too small for analyses using thresholds rather than the continuous measure\textsuperscript{56}. However, under certain circumstances the estimates from instrumental variable analyses using linear modelling may still be interpreted as population-averaged causal effects, even if the underlying association is non-linear\textsuperscript{260}.

Using cohorts from around the World, it has been estimated that approximately 20-60\% of the World’s populations may have 25(OH)D < 50 nmol/L\textsuperscript{262,263}. We investigated the levels and seasonal variation of 25(OH)D in two large general population studies separated by 20 years, the Copenhagen City Heart Study (1981-1983 examination) and the Copenhagen General Population Study (2003-2014). There was a clear seasonal variation with highest levels in late summer and around 40-60\% had 25(OH)D < 50 nmol/L (Figure 9)\textsuperscript{2,7}. Furthermore, in a subset of the Copenhagen City Heart Study 25(OH)D with samples taken 10 and 20 years after the baseline examination, analyses showed that a baseline measurement may be representative for exposure over long time periods (Figure 10)\textsuperscript{2}. Similar results were found in another prospective cohort study studying measurements up to 14 years apart\textsuperscript{264}. 

![Seasonal variation in plasma 25-hydroxyvitamin D.](image_url)
25-hydroxyvitamin D, morbidity, and mortality: observational studies

Cancer
Several lines of evidence have suggested that 1,25(OH)2D may be involved in regulation of genetic pathways gone awry in cancer. Accordingly, molecular biology, cell, and animal studies have shown that 1,25(OH)2D regulates angiogenesis, proliferation, apoptosis, and differentiation pathways. Specifically, 1,25(OH)2D seems to decrease tumor invasion, metastasis, and angiogenesis in cell and animal models of tobacco-related cancer such as lung, bladder, and oral cancers, and vitamin D deficiency increases susceptibility to chemical carcinogenicity including chemicals related to tobacco smoke carcinogens in animal models. We tested the hypothesis that a lower 25(OH)D level is associated with higher risk of tobacco-related cancer in the general population. For this purpose, 9791 white individuals from the Copenhagen City Heart Study were followed for up to 28 years including a total of 1081 incident tobacco-related cancers and 1506 other cancers. Non-melanoma skin cancer was excluded from analysis in this article. Covariates were updated at follow-up examinations; if a participant only participated in one examination baseline values for covariates were used. However, results were similar when using baseline covariates for all individuals instead of time-varying covariates. For a 50% lower 25(OH)D the multivariable adjusted hazard ratio for any type of cancer was 1.06 (95% CI, 1.02-1.11). Dividing the end points into tobacco-related (1.20 (1.13-1.28)) and other cancers (0.95 (0.89-1.01)) showed that the association was mainly driven by tobacco-related cancers (Figure 11). Furthermore, estimates for all individual tobacco-related cancers were all in the same direction as the overall association. Additionally, analyses using categories of 25(OH)D levels rather than as a continuous exposure also showed increasing risk of tobacco-related cancer with lower 25(OH)D levels (Figure 12). Lastly, the associations were robust in all sensitivity analyses.

Other observational studies also indicate an association of lower 25(OH)D with increased risk of lung and bladder cancer, but not with other tobacco related cancers. Also, subsequent meta-analyses of observational studies investigating all cancer except non-melanoma skin cancer have shown higher risk of cancer and cancer mortality with lower 25(OH)D levels, although a meta-analysis of randomized intervention studies suggests that vitamin D supplementation may reduce cancer mortality, but not risk of cancer. However, an association with tobacco-related cancers as a group has not been investigated in other studies. In conclusion, we showed that lower 25(OH)D was associated with higher risk of tobacco-related cancers, but not with risk of other cancers.

Skin cancer seems to be distinct from other types of cancer as UV radiation from sun exposure is a main environmental risk factor for non-melanoma and melanoma skin cancer. However, the wavelength of UV radiation that causes DNA damage in skin cells also induces vitamin D production in keratinocytes, which promotes differentiation and reduces proliferation in normal skin and skin cancer cell lines. Thus, an increase in vitamin D levels, or 25(OH)D, might reduce the risk of non-melanoma and melanoma skin cancer due to the aforementioned antineoplastic effects; however, if 25(OH)D primarily reflects sun exposure, then higher levels may be associated with increased rather than decreased risk of non-melanoma and melanoma skin cancer. In a general population study of 10,060 white individuals from the Copenhagen City Heart Study, including 590 incident non-melanoma skin cancer and 78 melanoma cases, we found an increased risk of both types of skin cancer. The multivariable adjusted hazard ratio for 25(OH)D ≥50 vs. <25 nmol/L were 5.04 (2.78-9.16) for non-melanoma skin cancer and 4.70 (0.96-23.30) for malignant melanoma. Similar findings have been shown in other cohort studies; however, contradictory findings have also been reported especially from case-control studies. A post hoc analysis of randomized clinical trial did not find a protective effect of vitamin D supplementation.
on skin cancer except for uncertain findings in subgroup analyses. Thus, the current body of evidence seems to support the hypothesis that 25(OH)D is a marker of sun exposure rather than a skin cancer inhibitor.

Several other types of cancers have been investigated in observational studies especially focused on breast and colorectal cancers, which suggest an increased risk with lower 25(OH)D levels. However, we could not replicate these findings in our data.

Diabetes

The hallmarks of type 2 diabetes are of a relative deficiency in insulin secretion and insulin resistance. Experimental studies have linked vitamin D deficiency to each of these pathogenic processes: 1) Evidence supporting a role for vitamin D in insulin secretion: the vitamin D receptor and the 1α-hydroxylase enzyme, the enzyme that converts 25(OH)D into 1,25(OH)2D, are present in β-cells; in vitro and in vivo studies show that vitamin D receptor knockout or vitamin D deficiency impairs glucose-induced insulin secretion; and the insulin secretory response improves after vitamin D supplementation in both animals and humans. 2) Evidence supporting a role for vitamin D in insulin resistance: 1,25(OH)2D stimulates insulin receptor expression and insulin-induced glucose transport in vitro; and vitamin D deficiency is associated with insulin resistance, whereas substitution with vitamin D in the deficient state improves insulin resistance.

We tested the hypothesis that low 25(OH)D is associated with increased risk of type 2 diabetes in the general population. For this purpose, we studied 9841 white individuals from the Copenhagen City Heart Study followed up to 29 years. Furthermore, the association of low 25(OH)D levels...
Figure 13. Hazard ratios for type 2 diabetes by 25(OH)D in clinical categories and seasonally adjusted quartiles. Multivariable models were adjusted for sex, age, smoking status (never/ever), BMI, income, and duration and intensity of leisure time physical activities. Furthermore, the model with clinical categories for 25(OH)D was adjusted for month of blood sampling. Based on 9841 individuals from the Danish general population, the Copenhagen City Heart Study, followed up to 29 years after blood sampling for measurement of 25(OH)D.

Figure 14. Meta-analysis of prospective studies on 25(OH)D and risk of type 2 diabetes. The reference category is the highest category of 25(OH)D in each study, and risk estimates are versus the lowest category of 25(OH)D in each study. On the forest plot, black box areas are proportional to the fixed-effect weight of the individual studies. The white diamonds represent the summary estimate, and CIs correspond to the width of the diamonds. Complete adjustment included adjustment for age, sex, season of blood draw, BMI or other obesity measures, smoking, and physical activity. Numbers by study names are the references. ND = no data. Adapted from Afzal S et al. Clin Chem 2013;59:381.

The strong observational association seen in our study and meta-analysis has been confirmed in subsequent studies and meta-analyses.

Analyses restricted to studies of the general population or studies with complete adjustment did not change the estimates appreciably. Analyses stratified according to study design likewise did not alter the associations substantially. There was no evidence of between-study heterogeneity (I²=1.4%, P= 0.44) or publication bias (Begg rank correlation test, P = 1.00, and Egger regression test, P = 0.58).
supplementation on risk of type 2 diabetes or glycemic indices. Several factors have been proposed to explain these seemingly contradictory results, e.g., residual confounding from obesity and/or physical activity in observational studies. In conclusion, we observed an association between low 25(OH)D and increased risk of type 2 diabetes in the general population. This finding was substantiated in a meta-analysis.

There is some evidence for a role of vitamin D deficiency in development of type 1 diabetes from birth cohort and genetic association studies. However, confirmation in randomized intervention studies is required before supplementation can be recommended, and the mechanisms involved may be through regulation of autoimmunity rather than insulin metabolism since type 1 and type 2 diabetes have distinct etiologies.

**Lung function and chronic obstructive pulmonary disease**

The pathogenic processes at the heart of chronic obstructive pulmonary disease (COPD) development are protease-antiprotease imbalance, inflammation, lung remodelling, and oxidative stress. In vitro and animal studies have indicated that lower vitamin D levels may lead to higher expression of proteases, inappropriate inflammatory responses, change towards a more fibrotic extracellular matrix, and increased oxidative stress in the lung and other tissues. Nevertheless, the association of lower 25(OH)D with lower lung function and faster lung function decline has been inconsistent in observational studies.

We tested the hypothesis that lower 25(OH)D levels are associated with lower lung function, faster lung function decline, and increased risk of COPD. For this purpose, we studied 10116 white individuals from the Copenhagen City Heart Study followed for up to 20 years, and 8391 white individuals from the Copenhagen General Population Study for cross-sectional analyses. COPD was defined using spirometry, both the GOLD criteria and the lower limit of normal criteria (LLN). In both cohorts, FVC % predicted was 7% lower and FEV1 % predicted was 7-10% lower for lowest versus highest decile of 25(OH)D (Figure 15). However, the association of 25(OH)D with FEV1/FVC was inconsistent. Analysis of lung function decline in the Copenhagen City Heart Study showed a significant interaction of age with quintiles of 25(OH)D levels on course of FEV1 % predicted and FVC % predicted (Figure 16). Individuals in the lowest quintile of seasonally adjusted 25(OH)D had a faster decline in lung function compared with individuals in the highest quintile. Based on longitudinal models, average FEV1 % predicted for individuals of age 60 years and adjusted for cumulative tobacco consumption was 87% (95% CI: 86%-88%) in the lowest quintile compared with 94% (93%-95%) in the highest quintile. The corresponding values for FVC % predicted were 92% (91%-93%) and 98% (97%-98%). The decline in FEV1/FVC did not differ according to 25(OH)D quintiles. In the cross-sectional analysis, multivariable adjusted odds ratios for COPD using GOLD criteria or LLN criteria increased with decreasing levels of 25(OH)D. The odds ratios for lowest versus highest quintile were 2.30 (1.55-3.41) and 3.06 (1.97-4.76) in the Copenhagen City Heart Study for GOLD and LLN criteria, respectively. The corresponding odds ratios were 1.82 (1.13-2.92) and 2.23 (1.35-3.69) in the Copenhagen General Population Study. In the Copenhagen City Heart Study many individuals had follow-up spirometry carried out, which was used for analysis of prospective development of COPD as a function of baseline 25(OH)D levels comprising 5341 (GOLD) and 5527 (LLN) individuals with at least two measurements. Multivariable adjusted hazard ratios for COPD for lowest versus highest quintile were 1.58 (1.05-2.40) and 2.00 (1.19-3.37) for GOLD and LLN criteria, respectively. Based on two independent samples of the general population, two novel findings were shown, that is, lower levels of 25(OH)D were associated with a higher decline in lung function and with a higher risk of spirometrically defined COPD in prospective and cross-sectional analyses. However, a limitation could be that the trajectory of lung function or disease progression among individuals, who do not attend follow-up examinations or die during follow-up, may have been different, and probably more severe, from
those included in the longitudinal analyses (see Figure 4). Given that low 25(OH)D is usually associated with more severe disease, we would expect this to bias our results towards the null; though, precise estimation of the consequences of this bias is difficult.

Figure 15. Association of FEV1 % predicted, FVC % predicted and FEV1/FVC with seasonally adjusted 25(OH)D deciles. Based on 10166 individuals from the Copenhagen City Heart Study and 8391 individuals from the Copenhagen General Population Study. FEV1, forced expiratory volume in 1 s. FVC, forced vital capacity. Adapted from Afzal S et al. Thorax 2014;69:244.

Figure 16. Relationship between age-related changes in FEV1 % predicted, FVC % predicted, and FEV1/FVC according to quintiles of seasonally adjusted plasma 25-hydroxyvitamin D. Adjusted for cumulative tobacco consumption updated at each examination. Based on one to three spirometries spanning up to 20 years in each of 10166 individuals from the Copenhagen City Heart Study. FEV1, forced expiratory volume in 1 s. FVC, forced vital capacity. Adapted from Afzal S et al. Thorax 2014;69:244.

Dementia

The pathogenetic hallmarks of Alzheimer’s disease are thought to be accumulation of dysfunctional proteins (i.e. amyloid beta and tau protein derivates) in the brain followed by oxidative damage and inflammation, leading to deranged energy metabolism, localized synaptic failure, and neuronal loss342. In vitro and animal studies have shown that 1,25(OH)2D reduces amyloid beta accumulation343-346, reduces oxidative stress-induced cell damage present in Alzheimer’s disease347;348, improves intracellular Ca2+ homeostasis dysregulated in Alzheimer’s disease349-351, upregulates neurotrophic factors351-353, and induces neuroprotective processes354-358. Vascular dementia develops as a result of cerebral infarcts359, and clinical studies have shown that vitamin D deficiency is associated with white matter hyperintensities in patients with dementia and risk of stroke360,361. Epidemiological studies also supports an association with dementia in general, since lower 25(OH)D is associated with faster cognitive decline362-365, volumetric changes in the brain seen in dementia366, and with risk of dementia in cross-sectional studies367.

We tested the hypothesis that decreased 25(OH)D is associated with increased risk of Alzheimer’s disease and vascular dementia in the general population5. For this purpose, we studied 10,186 white
individuals from the Copenhagen City Heart Study monitored for up to 30 years without losses to follow-up.

Adjusted hazard ratios for Alzheimer’s disease increased with decreasing levels of 25(OH)D by clinical categories and by seasonally adjusted percentile categories (Figure 17). Multivariable adjusted hazard ratios were 1.25 (95% CI, 0.95-1.64) for 25(OH)D <25 nmol/L vs. ≥50 nmol/L, and 1.29 (1.01-1.66) for ≤25th vs. > 50th seasonally adjusted percentile. Multivariable adjusted hazard ratios were 1.04 (1.00-1.09) per 10-nmol/L decrease in 25(OH)D and 1.08 (1.01-1.16) per 20-percentile decrease in seasonally adjusted percentiles of 25(OH)D.

Also, multivariable adjusted hazard ratios for vascular dementia were 1.22 (0.77-1.91) for 25(OH)D <50 nmol/L vs. ≥50 nmol/L, and 1.22 (0.79-1.87) for ≤50th vs. > 50th seasonally adjusted percentile. Multivariable adjusted hazard ratios were 1.02 (0.92-1.12) per 10-nmol/L decrease in 25(OH)D and 1.05 (0.90-1.23) per 20-percentile decrease in seasonally adjusted percentiles of 25(OH)D.

Finally, multivariable adjusted hazard ratios for the combined endpoint were 1.28 (1.00-1.64) for 25(OH)D <25 nmol/L vs. ≥50 nmol/L, and 1.27 (1.01-1.60) ≤25th vs. > 50th seasonally adjusted percentile. Multivariable adjusted hazard ratios were 1.04 (1.00-1.09) per 10-nmol/L decrease in 25(OH)D and 1.08 (1.01-1.15) per 20-percentile decrease in seasonally adjusted percentiles of 25(OH)D.

Thus, we observed an association of reduced 25(OH)D with increased risk of Alzheimer’s disease and vascular dementia in this prospective cohort study of the general population. Although, a subsequent prospective study has shown similar results for both Alzheimer’s disease and higher risk of other dementias using validated diagnoses, these findings need to be replicated in yet larger studies. Also, whether this association is causal remains unknown as a post-hoc analysis of a randomized intervention trial has not shown an effect of low dose vitamin D supplementation on risk of cognitive impairment.

Figure 17. Risk of Alzheimer’s disease, vascular dementia, and the combined end point by plasma 25-hydroxyvitamin D (25(OH)D) in clinical categories and seasonally adjusted percentile.

Cox regression adjusted for age, sex, smoking status, body mass index, leisure time and work-related physical activity, alcohol consumption, income level, education, baseline diabetes mellitus, hypertension, cholesterol, high-density lipoprotein cholesterol, and creatinine. CI = confidence interval. Adapted from Afzal S et al. Alzheimers Dement 2014;10:296-5.
Other diseases
The investigations into the health effects of low 25(OH)D or vitamin D supplementation include both observational studies and randomized intervention studies: the latter mostly being post hoc analyses in trials originally designed to evaluate bone or fracture endpoints. However, most results from the two designs have been conflicting with the randomized trials usually being negative. Nevertheless, some outcomes have emerged as potentially amenable to vitamin D supplementation such as dental caries and (low) birthweight.

In CCHS and CGPS, strong associations have been demonstrated in observational studies for cardiovascular disease and risk factors hereof. This includes myocardial infarction, ischemic heart disease, ischemic stroke, and risk factors such as lipoprotein levels and BMI. However, as indicated by genetic studies and randomized intervention studies, these associations may be due to confounding rather than causality.

Mortality
Observational studies show a consistent association of low 25(OH)D with increased risk of all-cause mortality and cause-specific mortality endpoints. Although, meta-analyses suggest some heterogeneity in the estimates from different studies, there is little evidence of this being due to bias. Several hypotheses has been set forth to explain the association of vitamin D deficiency with increased mortality ranging from immunoregulatory or anti-inflammatory effects to reduced resilience in disease. These explanations are primarily based on cell studies, animal studies, and observational correlations of 25(OH)D with risk factors for disease and morbidity. The effects of 25(OH)D supplementation on some of the proposed intermediate risk factors, except for maybe bone density or fracture risk, have yet to be confirmed. Thus, the association with mortality endpoints could be due to low 25(OH)D being a marker of poor health and (residual) confounding, i.e. these associations do not implicate causality.

In the CCHS and the CGPS, the observational associations have been consistent with previous reports showing an increased risk of all-cause, cardiovascular, cancer and other mortality (Figure 22). These findings and supplementary genetic analyses will be discussed in detail under the section on mortality analyses using Mendelian randomization.

25-hydroxyvitamin D, morbidity, and mortality: Genetic studies using Mendelian randomization
Diabetes
Vitamin D deficiency has been associated with obesity and diabetes in observational studies, including our study, as discussed previously. However, observational associations are susceptible to confounding and reverse causation. Thus, the association between low 25(OH)D and increased risk of diabetes could be the result of obesity causing both low 25(OH)D and higher risk of diabetes, i.e. confounding, or diabetes causing lower 25(OH)D through a disease related process, i.e. reverse causation. One way of disentangling these associations could be the use of a Mendelian randomization design, which is largely free of confounding and reverse causation (see the section on The Mendelian randomization approach).

We tested the hypotheses that the lower 25(OH)D levels caused by genetic variants are associated with increased risk of diabetes, and that the effect of genetic variants associated with high BMI on diabetes is partly mediated through reduction of 25(OH)D level. The latter hypothesis requires that genetically increased BMI is associated with low 25(OH)D level and with increased risk of diabetes. The associations tested and the design is illustrated in Figure 18.

The multivariable adjusted hazard ratio for type 2 diabetes was 1.96 (95% CI, 1.26-3.03) for 25(OH)D level of \( \geq 25 \) nmol/L versus \( \geq 50 \) nmol/L. The corresponding hazard ratio was 6.44 (5.30-7.83) for BMI of \( \geq 30 \) kg/m² versus <25 kg/m² (Figure 18, arrow D).
In genetic analyses, a 10 kg/m² higher BMI was associated with a 11.1 nmol/L (2.6-19.6) lower 25(OH)D (Figure 18, arrow C), with a corresponding observational estimate of 9.1 nmol/L (8.4-9.7; Figure 19). Genetically lower 25(OH)D was not associated with BMI.

Increasing BMI allele score was associated with higher risk of diabetes (p for trend <0.0001; Figure 18, arrow E and Figure 20), as was an increasing allele score for DHCR7 (p for trend=0.04). In contrast, we report no significant associations between CYP2R1 genotypes or allele scores and risk of diabetes.

For type 2 diabetes, the odds ratio for a genetically determined 20 nmol/L lower 25(OH)D level via endogenous production (DHCR7) was 1.51 (0.98-2.33) and via liver conversion (CYP2R1) it was 1.02 (0.75-1.37; Figure 18, arrow E and Figure 21), with a corresponding observational multivariable adjusted odds ratio of 1.16 (1.08-1.25).

The odds ratio for type 2 diabetes was 19.4 (6.4-59.1) for a genetically determined 10 kg/m² higher BMI, with a corresponding observational multivariable adjusted odds ratio of 4.33 (3.70-5.07). Use of any diabetes as the outcome produced similar results.

Mediation analyses showed that 3% (1%-5%; p=0.01) of the observational association of BMI with risk of type 2 diabetes was mediated through low 25(OH)D level. Corresponding genetic mediation analysis using a BMI allele score instead of measured BMI, showed similar results with an estimate of 4% (0%-7%; p=0.02). For any diabetes, the results were much the same.

To our knowledge, this is the first study to report a relation between DHCR7 genotypes associated with low 25(OH)D level via endogenous production and increased risk of type 2 diabetes; this result was not statistically significant, although the relation was significant when assessing any diabetes.
Thus, these results should be viewed as hypothesis-generating. Furthermore, we showed that higher BMI probably causes lower 25(OH)D and confirmed its causal association with type 2 diabetes. Our approach to test mediation was simple, but could be potentially flawed compared to a two-step Mendelian randomization. Typically, mediation analyses assume no unmeasured confounding between exposures, mediators, and outcomes or no measurement error, which are often not fulfilled in observational analyses. E.g., by conditioning on observed 25(OH)D rather than genetically determined 25(OH)D, we can introduce an association between BMI allele score and confounders of the 25(OH)D and diabetes association and, thus, introduce collider stratification bias.

However, we have the building blocks for inference by two-step Mendelian randomization as shown by the Mendelian randomization analyses of BMI with 25(OH)D and 25(OH)D with diabetes, which support a possible mediation by 25(OH)D. At the time of writing, specific methods for quantification of mediation in Mendelian randomization had not been rigorously studied but have been developed subsequently, at least for linear associations without interactions. Thus, we used the BMI allele score as exposure in two parallel articles from our group instead of a model based purely on observational data only. Nevertheless, the mediation analyses should be considered preliminary and susceptible to bias; as indicated in article 6 we consider these analyses to be hypothesis generating rather than definitive.

As discussed previously, several potential mechanisms link lower 25(OH)D with higher risk of both type 1 and type 2 diabetes. However, randomized intervention studies have shown contradictory results for vitamin D supplementation on risk of diabetes, and recent meta-analyses do not support an effect of vitamin D supplementation on glycemic control. Also, a recent Mendelian randomization study using a larger sample size could not replicate our findings indicating the need for further investigation.
Figure 21. Estimates of risk of type 2 diabetes and any diabetes for a 20 nmol/L lower 25-hydroxyvitamin D concentration and a 10 kg/m² higher BMI, for observational and genetic measures. Corresponds to Figure 18 arrows D and E. Observational estimates were done by logistic regression including incident cases only; genetic estimates were by instrumental variable analyses. P value was evaluated by a Hausman test. 25(OH)D=plasma 25-hydroxyvitamin D.

Cardiovascular disease

Several mechanisms have been proposed to explain the association of vitamin D deficiency with increased risk of cardiovascular disease. Observationally, low 25(OH)D is associated with an atherogenetic lipoprotein profile, higher blood pressure, increased body mass index, and low-grade inflammation, all risk factors that have been shown to be causally associated with increased risk of cardiovascular disease either through randomized intervention studies or Mendelian randomization studies. This has generated the hypothesis that vitamin D may be a regulator of lipoprotein metabolism, blood pressure, adiposity, and/or inflammation. Here the association with hypertension, myocardial infarction, and ischemic heart disease will be discussed. Vitamin D has been implicated in processes thought to be involved in causing hypertension, e.g. regulation of renin secretion, regulation of PTH secretion, and vascular smooth muscle Ca²⁺ metabolism. However, the hypothesis is mainly based on in vitro studies or animal studies showing: 1) expression of the VDR in juxtaglomerular cells of the kidney, 2) VDR activation causes decreased renin secretion in vitro, 3) mice with VDR knockout show increased renin expression, hypertension, and myocardial hypertrophy, 4) increased PTH causes increased blood pressure, and 5) vitamin D induced changes in Ca²⁺ may regulate contractibility of vascular smooth cells. Also, results from meta-analyses of observational studies show an overall increase in blood pressure or increased risk of hypertension with lower 25(OH)D levels. Conversely, meta-analyses of randomized intervention studies have shown either no or minor effects of vitamin D supplementation on blood pressure; however, in these studies there is some indication that only individuals with high blood pressure and low 25(OH)D may benefit from supplementation. A Mendelian randomization study has investigated this question using a multi-cohort study with a combined sample size of up to ~140 000 individuals. The results from that study showed that a 10% increase in 25(OH)D in instrumental variable analysis, was associated with a change of -0.29
mmHg in diastolic blood pressure (-0.52 to -0.07), a change of -0.37 mmHg in systolic blood pressure (-0.73 to 0.003), and a decreased odds of hypertension (OR 0.92, 0.87-0.97). In a smaller Mendelian randomization study using summary data the results was inconclusive\textsuperscript{391}. In our own studies, we found a weak potentially causal association with systolic and diastolic blood pressure, but not with ischemic stroke\textsuperscript{392}. Indicating a limited effect of 25(OH)D on clinically relevant ischemic stroke events.

Low 25(OH)D levels have also been associated with increased risk of ischemic heart disease and myocardial infarction in numerous observational studies\textsuperscript{30,109}. Although, randomized intervention studies of vitamin D supplementation to reduce the risk of ischemic heart disease are ongoing\textsuperscript{301}, secondary analyses of studies of vitamin D supplementation with bone health as the primary outcome, and meta-analyses of such studies, have not been able to demonstrate a cardioprotective effect\textsuperscript{30,208,210,393}. Also, randomized intervention studies with vitamin D supplementation have often been conducted with concomitant calcium supplementation; the latter may increase the risk of cardiovascular disease and myocardial infarction in particular\textsuperscript{394}. In a Mendelian randomization study, we tested the hypothesis that genetically reduced 25(OH)D is associated with increased risk of ischemic heart disease and myocardial infarction\textsuperscript{309}. For this purpose we used the combined populations of CCHS, CGPS, and CHIDS with 92,416 individuals of Danish descent, of whom 14,455 developed ischemic heart disease and 7,061 myocardial infarction.

The multivariable adjusted hazard ratios for lowest vs highest quartile of 25(OH)D were 1.82 (95% CI: 1.42-2.32) for ischemic heart disease. The genetic variants in DHCR7 and CYP2R1 or the combined allele score were, however, not associated with increased risk of ischemic heart disease. In instrumental variable analysis, the odds ratio for ischemic heart disease for a genetically 25-nmol/l decrease in 25(OH)D was 0.98 (0.76-1.26), with a corresponding observational hazard ratio by Cox regression of 1.07 (1.01-1.13). Similarly, with myocardial infarction as the outcome, observational analyses suggested an increased risk with lower 25(OH)D, whereas genetic analyses suggested no causal effect.

Thus, the results from the two stronger designs for establishing causality seem to indicate that lower 25(OH)D does not cause ischemic heart disease or myocardial infarction. Nevertheless, lower 25(OH)D may be associated with increased risk of hypertension or higher blood pressure\textsuperscript{68,392}, but more studies are required as most randomized studies have not shown a convincing effect of vitamin D supplementation on blood pressure\textsuperscript{390}. However, the differences between genetic and randomized studies may reflect biology: perhaps low 25(OH)D may predispose to hypertension trough irreversible effects early in life, 25(OH)D may only affect blood pressure in individuals with high blood pressure and low vitamin D levels, or the genetic variants may be pleiotropic. Hopefully, future research will clarify the source of these conflicting results.

**Mortality**

Several lines of evidence, including experimental, epidemiological, and randomized studies, suggest an association of low 25-hydroxyvitamin D concentrations with mortality endpoints. Firstly, vitamin D activating enzymes and the vitamin D receptor are present in many tissues\textsuperscript{23,24,199,200}, and vitamin D is estimated to regulate 1-3\% of all gene expression\textsuperscript{184,195}. Secondly, vitamin D has been implicated in the regulation of proliferation, differentiation, and apoptosis in several cell types\textsuperscript{154,194,195,197,199,206}. Thirdly, animal studies indicate that knockout of the vitamin D receptor may reduce lifespan and accelerate ageing\textsuperscript{97,398}. Fourthly, observational studies have implicated low vitamin D concentrations as a risk factor for a wide range of diseases and increased all-cause, cardiovascular, and cancer mortality\textsuperscript{29,30,399}. Finally, a meta-analysis of randomized intervention studies indicates that vitamin D\textsubscript{3} may decrease all-cause and cancer mortality, especially among vitamin D deficient populations\textsuperscript{208}. 62
We tested the hypothesis that genetically lower 25(OH)D levels are associated with increased mortality. We tested firstly whether 25(OH)D levels were associated with all-cause and cause specific mortality, secondly and thirdly whether the selected genotypes were associated with 25(OH)D levels and with all-cause and cause specific mortality, and finally whether the selected genotypes were associated with increased all-cause and cause specific mortality consistent with their effect on 25(OH)D levels by using instrumental variable analysis.

A 20 nmol/L lower plasma 25(OH)D was associated with all-cause and cause-specific mortality in the two studies separately and in pooled analyses (Figure 22). The pooled multivariable adjusted hazard ratios were 1.19 (95% CI, 1.14-1.25) for all-cause mortality, 1.18 (1.09-1.28) for cardiovascular mortality, 1.12 (1.03-1.22) for cancer mortality, and 1.27 (1.15-1.40) for other mortality. Plasma 25(OH)D levels were 4.6 (3.3-5.9) nmol/L lower for four versus no variant alleles in DHCR7 and 6.1 (5.2-7.0) nmol/L lower for four versus no variant alleles in CYP2R1 (Figure 3).

Combining genotypes by 25(OH)D lowering variant alleles showed an 8.4 (7.4-9.5) nmol/L decrease for 6-8 versus 0-1 variant alleles in DHCR7/CYP2R1 allele score. Each increase in DHCR7/CYP2R1 allele score was associated with a 1.9 (1.7-2.1) nmol/L decrease in 25(OH)D level.

The hazard ratio per one DHCR7/CYP2R1 allele score increase was 1.02 (1.00-1.03) for all-cause mortality, 0.98 (0.96-1.01) for cardiovascular mortality, 1.03 (1.00-1.06) for cancer mortality, and 1.03 (1.00-1.06) for other mortality (Figure 23).

The odds ratio for a genetically determined 20 nmol/L lower 25(OH)D level was 1.30 (1.05-1.61) for all-cause mortality, with a corresponding observational multivariable adjusted odds ratio of 1.21 (1.11-1.31) (Figure 24).

### Table: Mortality endpoint

<table>
<thead>
<tr>
<th>Mortality endpoint</th>
<th>Individuals</th>
<th>Deaths</th>
<th>Hazard ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All-cause</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCHS</td>
<td>9902</td>
<td>7132</td>
<td>1.17 (1.12-1.24)</td>
</tr>
<tr>
<td>CGPS</td>
<td>25432</td>
<td>1386</td>
<td>1.20 (1.06-1.37)</td>
</tr>
<tr>
<td>Pooled</td>
<td>35334</td>
<td>8518</td>
<td>1.19 (1.14-1.25)</td>
</tr>
<tr>
<td><strong>Cardiovascular</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCHS</td>
<td>9902</td>
<td>2877</td>
<td>1.17 (1.08-1.28)</td>
</tr>
<tr>
<td>CGPS</td>
<td>25432</td>
<td>317</td>
<td>1.32 (1.09-1.76)</td>
</tr>
<tr>
<td>Pooled</td>
<td>35334</td>
<td>3194</td>
<td>1.18 (1.09-1.28)</td>
</tr>
<tr>
<td><strong>Cancer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCHS</td>
<td>9902</td>
<td>2161</td>
<td>1.14 (1.04-1.25)</td>
</tr>
<tr>
<td>CGPS</td>
<td>25432</td>
<td>380</td>
<td>1.00 (0.79-1.26)</td>
</tr>
<tr>
<td>Pooled</td>
<td>35334</td>
<td>2541</td>
<td>1.12 (1.03-1.22)</td>
</tr>
<tr>
<td><strong>Other</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CCHS</td>
<td>9902</td>
<td>1915</td>
<td>1.23 (1.11-1.37)</td>
</tr>
<tr>
<td>CGPS</td>
<td>25432</td>
<td>310</td>
<td>1.38 (1.02-1.86)</td>
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<tr>
<td>Pooled</td>
<td>35334</td>
<td>2125</td>
<td>1.27 (1.15-1.40)</td>
</tr>
</tbody>
</table>

**Figure 22. Association of plasma 25-hydroxyvitamin D concentrations with all cause and cause specific mortality in general population.**

Analysis using Cox regression adjusted for age, sex, smoking status, cumulative tobacco consumption, alcohol consumption, leisure time physical activity, systolic blood pressure, body mass index, income, diabetes, plasma cholesterol, season (month and year of blood sample), and study (in pooled analyses). Maximum follow-up 32 years in Copenhagen City Heart Study (CCHS) and 9.4 years in Copenhagen General Population Study (CGPS). 25(OH)D=25-hydroxyvitamin D. Adapted from Afzal S et al. BMJ 2014;349:g6330.
Figure 23. All cause and cause specific mortality according to DHCR7/CYP2R1 allele score. Analyses were carried out using Cox regression adjusted for age, year of birth, sex, and study and were based on individuals from Copenhagen City Heart Study, Copenhagen General Population Study, and Copenhagen Ischemic Heart Disease Study combined. Adapted from Afzal S et al. BMJ 2014;349:g6330.

<table>
<thead>
<tr>
<th>Mortality endpoint</th>
<th>Individuals</th>
<th>Deaths</th>
<th>Hazard ratio (95% CI)</th>
<th>P for observational vs genetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>All-cause</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pooled</td>
<td>95766</td>
<td>10349</td>
<td>1.02 (1.00-1.03)</td>
<td></td>
</tr>
<tr>
<td>Cardiovascular</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pooled</td>
<td>95766</td>
<td>3231</td>
<td>0.98 (0.96-1.01)</td>
<td></td>
</tr>
<tr>
<td>Cancer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pooled</td>
<td>95766</td>
<td>2639</td>
<td>1.03 (1.00-1.06)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pooled</td>
<td>95766</td>
<td>2585</td>
<td>1.03 (1.00-1.06)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 24. Observational and genetic risk estimates for all cause and cause specific mortality for 20 nmol/L lower 25-hydroxyvitamin D concentrations. Observational analyses were by logistic regression and genetic estimates by instrumental variable analyses. Observational analyses were adjusted for the same variables as in Figure 22 as well as age. Genetic analyses were adjusted for age, year of birth, sex, and study. Genetic estimates were based on individuals from Copenhagen City Heart Study, Copenhagen General Population Study, and Copenhagen Ischemic Heart Disease Study combined. 25(OH)D=25-hydroxyvitamin D whereas observational estimates were based on the former 2 populations. Adapted from Afzal S et al. BMJ 2014;349:g6330.

Corresponding genetic and observational odds ratios were 0.77 (0.55-1.08) and 1.13 (1.03-1.24) for cardiovascular mortality, 1.43 (1.02-1.99) and 1.10 (1.02-1.19) for cancer mortality, and 1.44 (1.01-2.04) and 1.17 (1.06-1.29) for other mortality.

These results are in line with meta-analyses of randomized intervention studies that show a reduction in risk of all-cause mortality with vitamin D supplementation. Oddly, supplementation with vitamin D did not produce similar results. Furthermore, the benefits of supplementation were highest for institutionalized individuals and for cancer mortality.

However, there are several problems with current randomized intervention studies: few studies were designed to investigate mortality as an endpoint, the larger studies used low-dose interventions, and the individuals were not recruited based on occurrence of vitamin D deficiency. However, several ongoing randomized intervention studies tackle some of these shortcomings and will provide higher quality data to examine the effect of vitamin D supplementation on mortality endpoints. In this study as well as the Mendelian randomization study on diabetes, we observed a discrepancy between estimates derived from DHCR7 and CYP2R1 allele scores, in this case for cardiovascular mortality. As discussed in the methods section, this could represent pleiotropic effects, but consideration of evidence from other studies and given the fact that we in another study did not find discrepant associations with ischemic heart disease or myocardial infarction, final conclusions regarding possible pleiotropic effects must be deferred until further studies have been carried out. If pleiotropic effects are confirmed, the estimates from this study must be reevaluated accounting for these effects, if at all possible.

In conclusion, genetically low 25(OH)D levels were associated with increased all-cause mortality, cancer mortality, and other mortality but not with cardiovascular mortality. These findings are compatible with the notion that genetically low 25-hydroxyvitamin D concentrations may be
causally associated with mortality due to cancer and other causes, but also suggest that the observational association with cardiovascular mortality could be the result of confounding. The clinical implication of our findings remain limited, as widespread vitamin D supplementation can be recommended only after confirmation of our results in future genetic and randomized intervention studies.

**25-hydroxyvitamin D, BMI, and risk factors for morbidity and mortality**

The association of vitamin D with intermediary phenotypes relevant for morbidity, e.g. adiposity, inflammation, hypertension, and an atherogenic lipoprotein profile, has been demonstrated in multiple studies. Establishing such pathways are especially important for elucidating possible mechanisms by which vitamin D may regulate physiological and pathological processes. However, randomized intervention trials do not show an effect of vitamin D supplementation on most of these phenotypes, indicating the presence of reverse causation or confounding. Also, some of these phenotypes are difficult to intervene on, and other types of studies, such as Mendelian randomization studies, may be of use to investigate causality in such circumstances. The potential mechanisms by which vitamin D deficiency may be associated with hypertension have already been discussed in the section **Cardiovascular disease**.

The association between body mass index, or rather adiposity, and vitamin D is thought to be bidirectional. However, the supposed underlying mechanisms governing these associations are unclear. It is known that vitamin D and 25(OH)D seems to be stored in adipose tissue (see **Distribution, storage, and excretion of vitamin D**), and it is hypothesized that adipose tissue could sequester vitamin D, since obese individuals have lower response to vitamin D supplementation than expected; nevertheless, it has been shown that this association can be explained by a simple dilution effect, without a need to invoke an active sequestration mechanism, as the volume of distribution for vitamin D and 25(OH)D will be higher in obese individuals than normal weight individuals. On the other hand, vitamin D has been suggested to affect numerous mechanisms regulating deposition of fat and energy metabolism and as already mentioned bone development all of which may affect body shape and size. Nonetheless, most of these potential mechanisms should be classified as speculative as they are either based on cross-sectional studies, theoretical considerations, or data from cell or animal studies. One way of disentangling such pathways is to carry out bidirectional Mendelian randomization studies, e.g. using genetic variants affecting BMI one can determine whether BMI causally affects 25(OH)D levels, and simultaneously using genetic variants affecting 25(OH)D, one can determine whether 25(OH)D levels causally affect BMI. We tested this hypothesis in our previously mentioned paper (**Diabetes**, Figure 19). The results of our study indicated that obesity could be a contributing cause to vitamin D deficiency, whereas changes in plasma 25(OH)D may not affect BMI. Similar findings have been demonstrated in another Mendelian randomization study. Also, results from the few available randomized intervention trials do not seem to support an effect of vitamin D supplementation on BMI.

Vitamin D deficiency has been associated to low-grade inflammation and suggested mechanisms include direct effects of 1,25(OH)2D on macrophages, dendritic cells, and T lymphocytes. Specifically, VDR have been found on macrophages, dendritic cells, T lymphocytes, and some myeloid progenitor cells. Furthermore, CYP27B1 is expressed in macrophages, dendritic cells, and T lymphocytes allowing for local activation of 25(OH)D to 1,25(OH)2D to function as an autocrine and paracrine mediator. The net effect of 1,25(OH)2D stimulation seems to be downregulation of pro-inflammatory and upregulation of anti-inflammatory cytokine secretion from macrophages, differentiation of monocytes to macrophages, decreased antigen presentation from dendritic cells, differentiation of T-lymphocytes to regulatory T-lymphocytes induced by...
changes in cytokine secretion\textsuperscript{414}, and increased secretion of antimicrobial peptides such as calthelcidin from macrophages\textsuperscript{415}. Overall, these processes are expected to reduce local and systemic inflammation and secondarily markers of systemic inflammation such as C-reactive protein (CRP). While observational studies have found an inverse association between 25(OH)D and CRP levels, the results from randomized intervention trials and Mendelian randomization studies have been conflicting\textsuperscript{381,416-419}. Meta-analyses of randomized intervention trials investigating the effects of vitamin D supplementation on CRP levels and specific cytokines have found that vitamin D supplementation either have a weak or no effect on levels of CRP and pro-inflammatory cytokines\textsuperscript{381,416-418}, and the only relatively small Mendelian randomization study investigating the association of 25(OH)D levels with CRP levels was negative\textsuperscript{417}. Thus, whether the results from the cell and animal studies can be translated to corresponding effects in humans is presently unknown. Finally, a lower 25(OH)D level have been associated with an atherogenic lipoprotein profile, e.g. higher LDL cholesterol and lower HDL cholesterol\textsuperscript{400}. The proposed mechanisms by which vitamin D may affect lipoprotein concentrations are derived from cell studies and theoretical considerations, e.g. VDR activation may lead to inhibition of apo-AI synthesis in cell studies and it has been hypothesized that indirect effects on lipoprotein metabolism may be mediated through reduced PTH and increased insulin secretion/sensitivity\textsuperscript{400,420}. However, there may be mechanisms by which lipid metabolism may affect 25(OH)D levels as well. Vitamin D and cholesterol may share active transport mechanisms in the intestine, and given the abundance of cholesterol in many diets compared to vitamin D, this could lead to reduced absorption of vitamin D and lower plasma 25(OH)D\textsuperscript{421}. Furthermore, studies show that VDBP with 25(OH)D binds to very-low-density lipoprotein (VLDL) coupling the concentration of these molecules\textsuperscript{415}. Furthermore, as already mentioned dietary vitamin D is transported by chylomicrons; thus, one could speculate that higher VLDL and chylomicron concentrations may directly affect 25(OH)D levels through delivery to tissues or possibly metabolism in the liver. In humans, results from observational studies have been conflicting\textsuperscript{400}. Though, a large prospective trial investigating time trends in plasma 25(OH)D and changes in several lipoprotein types did not find an association of 25(OH)D with lipoprotein levels\textsuperscript{422}. Also, a meta-analysis of randomized intervention trials found little effect of vitamin D supplementation on the lipoprotein profile, except for a minor increase in LDL cholesterol\textsuperscript{423}. A genetic study carried out by our group showed that genetic variants associated with increased lipoprotein concentrations may cause lower 25(OH)D levels, while genetic variants associated with 25(OH)D had no effect on lipoprotein levels except for a minor effect on HDL cholesterol\textsuperscript{427}. Thus, current evidence does not support a causal role for vitamin D in regulating lipoprotein levels; rather, higher lipoprotein levels may reduce 25(OH)D levels.

In contrast to the discussed studies on 25(OH)D levels, evidence from genetic and randomized intervention trials is consistent with the hypothesis that higher BMI causes hypertension, low-grade inflammation, hypertriglyceridemia, diabetes, and cardiovascular disease. The mechanisms invoked to explain these associations range from obesity induced changes in the immune system over extracellular fluid volume expansion to changes in the nervous system\textsuperscript{424-426}; however, a review of these mechanisms is outside the scope of this review as the focus is on secular trends in the association of BMI with mortality risk. It has been demonstrated in randomized intervention trials that weight loss in overweight and obese individuals reduces risk of hypertension and diabetes\textsuperscript{427,430}. Additionally, Mendelian randomization studies have found that high BMI increases risk of several cardiovascular diseases, including ischemic heart disease, heart failure, and stroke, and higher BMI increases risk factors for cardiovascular disease, including hypertension, hyperlipidemia, and diabetes\textsuperscript{42,380,384,386,387,431-433}. Since hypertension, hyperlipidemia, and diabetes are associated with increased risk of cardiovascular disease\textsuperscript{383,385,434,435}, and cardiovascular disease is the leading cause of death worldwide, the association of obesity with increased mortality risk is
thought to be mediated primarily through diabetes and other risk factors for cardiovascular disease. Thus, several lines of evidence indicate that obesity causes diseases thought to be associated with early death.

**BMI and mortality: delineation and limitations**

An exact definition of obesity is difficult, but is usually understood as a harmful accumulation of fat in the body. A logical question would be how and where to measure this fat accumulation. Several suggestions for correct measurements have been suggested, e.g. total body fat, abdominal fat vs. subcutaneous fat, visceral fat, and fat free mass vs. fat mass. Likewise, suggested methods to measure obesity range from simple to extremely complicated techniques, e.g. BMI (height and weight), waist and hip circumference and ratio thereof, DEXA scans, full body CT/MR scans, and density measurements of the body. Despite given these more advanced options for measuring and defining obesity, we still believe that BMI is a good and simple measure of obesity as discussed in subsequent paragraphs. In any case, BMI is a measure that many individuals within the general population understand and sometimes even use themselves.

Current cut-points of BMI for defining obesity categories were reached by consensus in an expert panel under WHO (Table 6). Even though BMI is a suboptimal predictor of body fat, it seems to be equivalent to e.g. waist and hip circumference for predicting cardiovascular disease, and newer more complex obesity measurements have yet to be investigated in large studies for superiority in predicting disease compared to simpler obesity measures. However, potential downsides are that exact cut-points for categorization may depend on ethnicity as indicated by WHO itself and by large observational studies in USA, Europe, and Asia as well as that BMI is a statistical measure of obesity rather than a physiological measurement. Thus, from a clinical point of view there is little compelling evidence to replace BMI with other obesity measures as BMI is easy to measure precisely, other measures are not superior for disease prediction, and there is vast experience with using BMI in clinical practice and guidelines.

The association of BMI with mortality in the general population has primarily been derived from observational studies, which makes it difficult to substantiate claims of causality with concrete evidence beyond logical extension from the studies discussed in the previous section on BMI and risk factors for mortality. This is partly due to the complications in use of randomized intervention studies to study the effect of weight loss on mortality, e.g. it is difficult to maintain weight loss in intervention groups and weight loss often occur in both intervention and control groups reducing power. Furthermore, it would be unethical to withhold treatment for known risk factors for death, e.g. hypertension and diabetes, in intervention and control groups. This could lead to reduced power results if the effect of obesity on mortality is mediated by these intermediate risk factors, or if all the effect of obesity on mortality is mediated through treatable intermediate risk factors, an effect of weight loss would be impossible to see when using mortality as an outcome and including treatment of mediating risk factors in both control and intervention groups. Finally, interventions used to reduce weight are seldom specific, e.g. lifestyle intervention often include both increased physical activity and diet changes, making it difficult to quantify the effects of weight-loss alone.

Interestingly, in diabetes patients antidiabetic medication with appreciable weight-loss seems to reduce risk of cardiovascular disease and death; however, intensive lifestyle intervention with weight loss did not reduce risk of death. Thus, while obesity causally increases risk for diabetes and several cardiovascular diseases as discussed in 25-hydroxyvitamin D, BMI, and risk factors for morbidity and mortality, concrete evidence on its effect on mortality is difficult to attain. However, methods using instrumental variable analyses, using e.g. off-spring cohorts or genetic variants, may be helpful in this regard, but few studies have been published. As discussed in...
among obese individuals. However, the few previous studies investigating secular trends in the association of BMI with mortality have used differently recruited cohorts or have incomplete follow-up leading to difficulties in interpretability. In our study, we tested the hypothesis that the BMI values associated with lowest all-cause mortality has increased over time in the general population. For this purpose, 3 cohorts were used that were recruited from the white Danish general population in 1976-1978 (CCHS, 1st examination), 1991-1994 (CCHS, 3rd examination), and 2003-2013 (CGPS).


There was a nonlinear association of BMI with all-cause mortality in all 3 cohorts as both high and low BMI were associated with high all-cause mortality. However, the BMI associated with the lowest mortality increased over time (Figure 25). The association of BMI with cardiovascular mortality and other mortality showed a similar pattern (Figure 26); however, the association with cancer mortality was not U-shaped, and the BMI associated with the lowest mortality could not be determined. The BMI associated with the lowest all-cause mortality was 23.7 (95% CI, 23.4-24.3) in 1976-1978, 24.6 (24.0-26.3) in 1991-1994, and 27.0 (26.5-27.6) in 2003-2013 (Figure 25). The corresponding BMI estimates for cardiovascular mortality were 23.2 (22.6-23.7), 24.0 (23.4-25.0), and 26.4 (24.1-27.4), respectively, and for other mortality, 24.1 (23.5-25.9), 26.8 (26.1-27.9), and 27.8 (27.1-29.6), respectively (Figure 26).

**Table 6. Body mass index categories used to define overweight and obesity according to WHO.**

<table>
<thead>
<tr>
<th>Classification</th>
<th>BMI (kg/m²)</th>
<th>Principal cut-off points</th>
<th>Additional cut-off points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Underweight</td>
<td>&lt;18.50</td>
<td>&lt;18.00</td>
<td></td>
</tr>
<tr>
<td>Severe thinness</td>
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<td></td>
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</tr>
<tr>
<td>Moderate thinness</td>
<td>18.00 - 18.49</td>
<td>18.50 - 22.99</td>
<td></td>
</tr>
<tr>
<td>Mild thinness</td>
<td>17.00 - 18.49</td>
<td>23.00 - 24.49</td>
<td></td>
</tr>
<tr>
<td>Normal range</td>
<td>18.50 - 24.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overweight</td>
<td>≥25.00</td>
<td>25.00 - 27.49</td>
<td>27.50 - 29.99</td>
</tr>
<tr>
<td>Obese</td>
<td>≥30.00</td>
<td>30.00 - 32.49</td>
<td>32.50 - 34.99</td>
</tr>
<tr>
<td>Obese class I</td>
<td></td>
<td>35.00 - 37.49</td>
<td></td>
</tr>
<tr>
<td>Obese class II</td>
<td></td>
<td>37.50 - 39.99</td>
<td></td>
</tr>
<tr>
<td>Obese class III</td>
<td></td>
<td>≥40.00</td>
<td></td>
</tr>
</tbody>
</table>

**BMI associated with lowest mortality in Denmark, 1976-2013**

Several very large epidemiological studies have established a strong association of BMI with increased mortality; however, the precise values of BMI associated with lowest mortality remain controversial. Especially, the interpretation and analysis of the U-shaped association of BMI with mortality has fueled the controversy; the increased risk of mortality observed with BMI in the low normal range has been suggested to be caused by residual confounding due to concurrent disease or decline in BMI due to disease, while others have suggested this is caused by a detrimental effect of low BMI on disease. Consequently, there is also disagreement on the most optimal analytical techniques to analyze these data: many large studies restrict their analyses to “healthy never-smokers” to exclude confounding, while others argue that this leads to biases of its own. Nevertheless, whether the association of BMI with mortality has changed over time is poorly understood. Previous studies indicate that while average BMI is increasing over time, prevalence of obesity related risk factors associated with cardiovascular disease may be decreasing...
To assess follow-up dependent changes in the BMI associated with the lowest mortality, the estimates from 2 years of follow-up to maximum follow-up were plotted for the 1976-1978 and 2003-2013 cohorts (Figure 27). Graphically, there was no evidence of convergence or a follow-up-dependent influence on the estimates, indicating that the BMI associated with the lowest mortality was consistently different between the 2 cohorts.

Using WHO categories of BMI, with BMI of 18.5 to 24.9 as the reference, showed corresponding results: the risk of all-cause mortality decreased from 1976-1978 through 1991-1994 to 2003-2013 for both BMI of 25 to 29.9 and BMI of 30 or greater (Figure 28). However, the multivariable-adjusted hazard ratios for BMI less than 18.5 vs BMI of 18.5 to 24.9 were similar across all 3 time periods. The multivariable-adjusted hazard ratios for all-cause mortality for BMI of 25 to 29.9 vs BMI of 18.5 to 24.9 were 1.04 (95% CI, 0.99-1.08) in the 1976-1978 cohort, 0.97 (0.91-1.04) in the 1991-1994 cohort, and 0.86 (0.82-0.92) in the 2003-2013 cohort. The corresponding hazard ratios for BMI of 30 or greater vs BMI of 18.5 to 24.9 were 1.31 (1.23-1.39) in the 1976-1978 cohort, 1.13 (1.04-1.22) in the 1991-1994 cohort, and 0.99 (0.92-1.07) in the 2003-2013 cohort.

Potential limitations of this study include that there was different follow-up time in the 3 cohorts; however, sensitivity analyses indicated that the differences between the 1976-1978 and 2003-2013 cohorts could not be explained by different follow-up time. Another limitation is that the 1976-1978 and 1991-1994 cohorts overlapped, which could raise concern regarding survivor selection bias in the latter cohort beyond that normally observed in studies of the general population. Yet, comparison of the 1976-1978 and 2003-2013 cohorts alone would give similar conclusions. The participation rate was lower in the 2003-2013 cohort compared with the older cohorts, which could indicate a greater risk of healthy participant bias compared with the older cohorts. Though, for this to be a viable source of bias, a selective participation of healthier individuals in the overweight and obese groups compared with the normal weight group should be observed.

Subsequently, letters to the Editor also suggested alternative methods to handle the different ages structures expected to occur over time in the cohorts due to different follow-up time; however, we found little evidence of this changing our results substantially. Furthermore, others suggested that the only proper analysis would be restriction to never-smokers without baseline disease, exclusion of the first years of follow-up and aged less than 70 years, but as already discussed, the findings could not be attributed to differences in follow-up time, as shown in Figure 27 and eFigure 4 in the article. Second, an analysis restricted to never smokers without baseline disease still placed the BMI associated with the lowest mortality in the overweight range (26.1) for the most recent cohort (2003-2013) (eFigure 2 in the article). However, these sensitivity analyses had limited statistical power and further restriction would not provide usable results (e.g., the additional suggested restrictions would reduce the sample size to 14453 individuals [15% of original] and 63 deaths [1% of original] in the 2003-2013 cohort). Also, the use of these restrictions may not be effective in reducing bias or confounding and may introduce biases of their own. Additionally, we specifically wished to investigate the reported association between BMI and mortality in the general population setting and not in selected subgroups only. Informally, our article also links to the ongoing discussion of whether BMI as an appropriate classifier for obesity. We did not specifically address this question; however, it is possible that a higher BMI, especially in the overweight range, may represent a different body phenotype now than it did in e.g. 1970’s. However, we tried to investigate the question from the point of view of current clinical practice, where BMI is the initial, and in some cases perhaps only, obesity measure used widely for identifying and following obese individuals outside of specialized clinics.

Solid lines are multivariable-adjusted hazard ratios, and dashed lines indicate 95% confidence intervals derived from restricted cubic spline regression with knots chosen by Akaike information criterion as described in Methods. A body mass index (BMI) of 25 was used as the reference (calculated as weight in kilograms divided by height in meters squared). There is no BMI data marker and error bar for cancer mortality because the 95% confidence intervals for the hazard ratios overlap almost the entire upper range of BMI in all 3 cohorts. The graphs are truncated at the 1st and 99th percentiles. The Cox regression was adjusted for age, sex, smoking status, cumulative tobacco consumption, alcohol consumption, leisure-time physical activity, income, and plasma cholesterol level. Adapted from Afzal S et al. JAMA 2016;315:1989-1996.


Solid lines are multivariable-adjusted hazard ratios, and dashed lines indicate 95% confidence intervals derived from restricted cubic spline regression with knots chosen by Akaike information criterion as described in Methods. A body mass index (BMI) of 25 was used as the reference (calculated as weight in kilograms divided by height in meters squared). There is no BMI data marker and error bar for cancer mortality because the 95% confidence intervals for the hazard ratios overlap almost the entire upper range of BMI in all 3 cohorts. The graphs are truncated at the 1st and 99th percentiles. The Cox regression was adjusted for age, sex, smoking status, cumulative tobacco consumption, alcohol consumption, leisure-time physical activity, income, and plasma cholesterol level. Adapted from Afzal S et al. JAMA 2016;315:1989-1996.
**Perspectives and conclusion**

The aim of this review was to summarize and discuss our findings on 25(OH)D and body mass index as risk factors for morbidity and mortality in the general population. For this purpose several different designs within the realm of classical observational epidemiology and newer methods (in medical research) such as Mendelian randomization studies (instrumental variable analyses) were used. First, articles 1, 2, 3, and 5 represent the investigations of 25(OH)D as a risk factor for cancer, diabetes, and dementia in prospective studies. Second, article 4 consisted of cross-sectional and longitudinal analyses for lung function, lung function decline, and risk of spirometrically defined COPD, essentially using that we had similarly recruited cohorts and overlapping individuals within these cohorts. Also, article 8 showcases another benefit of having similar studies recruited over a longer time period, which enabled us to investigate the secular trend in the association of BMI with mortality in a relatively strong design. Third, articles 6 and 7 demonstrate the use of Mendelian randomization studies in our cohorts as a way of evaluating possible causal pathways. Overall, these articles illustrate the benefits of having several large cohorts recruited over time from the general population for public health research. The data allowed us to combine comprehensive information from baseline questionnaires with genotype and biochemical measurements and physical examinations with follow-up in nation-wide registries; ultimately, the goal of our investigations, as in most epidemiological research, was to produce data that may translate to betterment of public health and clinical practice.

The usefulness of observational studies in this regard is that they are excellent for determining correlations and for establishing hypotheses regarding possible causality based on these observations. Our studies indicated strong, dose-response associations of low vitamin D levels with several common diseases such as several cancers, diabetes, lung function decline, and dementia and with risk factors for disease, e.g., BMI. However, this multitude of associations is somewhat concerning...
in itself as the lack of specificity may indicate that low 25(OH)D is a marker of disease or unhealthy lifestyle rather than a cause of disease. In our studies we tried to adjust for all possible confounders; however, as discussed previously it is difficult to account for residual confounding. Furthermore, reverse causation is difficult to eliminate as a problem, while some of the sensitivity analyses we carried out may partially account for this problem, little is known about alternations in metabolic states before diagnosis of a disease especially the timing of their start and their extent. Of course, the hypotheses generated by our observational studies are testable by others in similar designs and, perhaps more importantly, in different designs that may be better suited to delineate causal pathways, i.e. genetic and randomized intervention studies. Thus, we tested some of the hypotheses using Mendelian randomization studies. The mortality endpoints are the most intriguing as observational, Mendelian randomization, and randomized intervention trials seem to agree on the direction and magnitude of risk. Specifically, we found a specific increase in non-cardiovascular deaths, which is in agreement with randomized intervention trials that found that the effect of vitamin D supplementation was strongest on cancer deaths. This makes the case for actual causality stronger and could indicate that more wide-spread supplementation may be useful in a public health setting, as supplementation is cheap and with few harmful effects in recommended doses. However, this is only feasible if ongoing vitamin D trials confirm these potential beneficial effects. Other Mendelian randomization studies with diabetes or hypertension as an endpoint has also suggested possible causality; however, conflicting studies have been published regarding these endpoints. Lastly, several other Mendelian randomization studies have not been able to confirm the causality for some of the proposed associations of low 25(OH)D with disease outcomes, e.g. the association with colorectal cancer or ischemic heart disease could not be replicated in a Mendelian randomization studies. Accordingly, the number of negative studies gives reason for pause as this indicates that many of the observational associations may be the result of confounding or reverse causation, and that low 25(OH)D may indeed be a marker of poor health, while causally affecting only few extraskeletal outcomes. Equally interesting have been Mendelian randomization studies testing the effect of risk factors on 25(OH)D levels. Our group has published two such studies showing that higher BMI and an atherogenic lipoprotein profile reduces 25(OH)D levels. This provides a partial explanation for the persistent association with cardiovascular disease seen for low 25(OH)D levels and the lack of effect on cardiovascular disease in randomized vitamin D supplementation studies. Since both higher BMI and an atherogenic lipoprotein profile causally increases risk of cardiovascular disease and reduces levels of 25(OH)D, a consistent association is expected in observational studies between low 25(OH)D and high risk of cardiovascular disease, perhaps even after adjustment for these risk factors due to residual confounding.

Thus, the current weight of evidence suggests that low 25(OH)D is probably a marker of poor health as it is reduced by some risk factors for cardiovascular disease and lowered in many diseases; however, there seems to be consistent extraskeletal effects of vitamin D also, as low 25(OH)D may increase mortality and vitamin D supplementation may affect, e.g. mortality and neonatal outcomes. Given the ongoing large studies on vitamin D using genetic or randomized intervention designs, clarification may be underway for usefulness of vitamin D supplementation in improving health.

Other than showing an effect of higher BMI in reducing 25(OH)D, this thesis include our findings on secular trends in the association of BMI with mortality. We show, in homogenously recruited cohorts, that the BMI associated with lowest mortality may have increased from around 24 to 27 kg/m² over these decades. This finding may have considerable public health implications if confirmed in other studies and other health care settings; however, several questions remain unanswered. First, does this finding indicate that current treatment of obesity related complications
almost completely compensate for their effect on mortality in the overweight group? Second, does this secular trend represent differences in lifestyles in the investigated time periods? Third, have risk factor burdens for e.g. cardiovascular disease changed over time differently across BMI categories? We aim to investigate some of these questions in future studies.

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