Vitamin D status and cardiovascular disease

Observational studies in patients who underwent coronary angiography

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Dissertation for the degree of philosophiae doctor (PhD)

at the University of Bergen

2016

Dissertation date: January 19th

Year: 2016

Title: Vitamin D status and cardiovascular disease

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Print: AIT OSLO AS / University of Bergen

Scientific environment

This project was conducted in the period from September 2011 to September 2015 at the Department of Clinical Medicine, Faculty of Medicine and Dentistry, University of Bergen. My main supervisor was Professor Jutta Dierkes and my co-supervisors were Professor Ottar Nygård and Dr. Stefan de Vogel.

The project was funded by the Department of Clinical Medicine, the Meltzer Research Fund and by Astri og Edvard Riisøens legat.

The candidate participated in the Postgraduate School of Clinical Medical Research at the Department of Clinical Medicine.

Acknowledgements

I would like to thank the university, faculty and institute for supporting the project and thank those who participated or contributed to generate data for this project.

I am very grateful for the help from my supervisors. I thank Professor Ottar Nygård for clarity and coordination while working with the data and PhD Stefan de Vogel for keen interest, excellent feedback and encouragement. Most of all, I would like to thank my hard-working principal supervisor Professor Jutta Dierkes for trust, guidance, honesty, humour, spirit and encouragement.

I would like to thank my co-authors, those who helped with administrative issues and anyone who showed interest in this project. A special thanks to Rune Hoff, a statistician in need and friend indeed.

To my colleagues, thank you for smiles, small talks and laughs. Friends among colleagues, these years would not have been the same without you. Kenneth Finne, I am especially glad you were there.

To my loving and joyful friends and family, especially my parents, sisters and Marion, thank you for showing interest in what I do and for making me forget all about it.

Eirik Magnus Meek Degerud

Bergen, September 2015

Abbreviations

25OHD	25-hydroxyvitamin D (calcidiol)
$1,25OH_2D$	1,25-dihydroxyvitamin D (calcitriol)
ACS	Acute coronary syndrome
AMI	Acute myocardial infarction
BECAC	Bergen Coronary Angiography Cohort
BMI	Body mass index (kg/m^2)
CAD	Coronary artery disease
CABG	Coronary artery bypass graft
CBS	Cystathionine beta synthase
CRP	C-reactive protein
CVD	Cardiovascular disease
DBP	Vitamin D binding protein
DS	Diameter stenosis
EC	Endothelial cell
eGFR	Estimated glomerular filtration rate
HUS	Haukeland University Hospital
HR	Hazard ratio
IHD	Ischaemic heart disease
IU	International units
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LDL	Low density lipoprotein
LVEF	Left ventricular ejection fraction
LQMM	Linear quantile mixed model
MLD	Minimum lumen diameter
PCI	Percutaneous coronary intervention
PPAR	Peroxisome proliferator-activated receptor
PTH	Parathyroid hormone
QCA	Quantitative coronary angiography

RCT	Randomised controlled trial
RE	Responsive elements
RAR	Retinoid acid receptor
RAS	Renin-angiotensin system
RXR	Retinoid X receptor
SAP	Stable angina pectoris
SMC	Smooth muscle cell
SNP	Single nucleotide polymorphism
SUH	Stavanger University Hospital
UVB	Ultraviolet B
UA	Unstable angina
VDR	Vitamin D receptor
VDRE	Vitamin D receptor elements
WENBIT	Western Norway B-Vitamin Intervention Trial

Abstract

Background: Vitamin D is required to maintain a healthy cardiovascular system, but it is unknown whether variation in vitamin D status in the general population is physiologically relevant to development of cardiovascular diseases (CVDs).

Aim: To study vitamin D status and investigate the associations of vitamin D status with atherosclerosis progression, all-cause and CVD mortality.

Methods: Observational data from patients in Western-Norway with suspected coronary artery disease were used (n=4116). Vitamin D status was assessed by the measurement of plasma 25-hydroxyvitamin D (25OHD) concentrations, atherosclerosis progression by repeat coronary angiography and survival data obtained from national registries.

Results: Mean 25OHD most strongly associated with seasonality, adiposity and cod liver oil consumption. Seasonal variation in 25OHD differed by age. During winter and summer ~50% and ~80% of the participants were vitamin D sufficient, respectively. When modelling baseline values, cosinor models most accurately predicted follow-up values for patients with repeated measurements of 25OHD.

Baseline concentrations of 25OHD were not associated with atherosclerosis progression after ~1 year of follow-up, but were inversely associated with a higher risk of all-cause and cardiovascular mortality after ~12 years of follow-up. Despite a linear tendency, non-linearity was observed in the relationship with all-cause mortality, with higher risk among individuals with 25OHD concentrations below 42.5 nmol/l and above 100 nmol/l in comparison to those between 42.5 – 100 nmol/l.

Conclusions: Seasonal variation has a strong influence on vitamin D status and researchers should consider cosinor models when adjusting for seasonality. A high frequency of insufficiency during winter indicates inadequate dietary intakes despite a high frequency of cod liver oil use in this population. Vitamin D status was inversely associated with a higher risk of all-cause and CVD mortality, but not

associated with subclinical progression of atherosclerosis. The relationship with all-cause mortality was J-shaped, with increased risk also among a smaller segment of participants with high 25OHD concentrations.

List of publications

I. Degerud E, Løland KH, Nygård O, Midttun Ø, Ueland PM, Seifert R, Strand E, Bleie Ø, Dierkes J. Vitamin D status was not associated with 'one-year' progression of coronary artery disease, assessed by coronary angiography in statin-treated patients. European Journal of Preventive Cardiology. Published online before print. January 30, 2014, doi: 10.1177/2047487314522137.

II. Degerud E, Hoff R, Nygård O, Strand E, Nilsen DW, Nordrehaug JE, Midttun Ø, Ueland PM, de Vogel S, Dierkes J. Cosinor modelling of seasonal variation in 25-hydroxyvitamin D concentrations in cardiovascular patients in Norway. Submitted to European Journal of Clinical Nutrition on March 20th 2015.

III. Degerud E, Nygård O, de Vogel S, Hoff R, Svingen G, Pedersen ER, Nilsen DW, Nordrehaug JE, Midttun Ø, Ueland PM, Dierkes J. Plasma 25-hydroxyvitamin D concentrations and all-cause and cardiovascular disease mortality among Caucasian patients with suspected stable angina pectoris. Manuscript.

Contents

1. Introduction

Nutrients are required by all organisms to perform the functions that constitute life. Except for a few notable exceptions, humans acquire nutrients from foods. Macronutrients, which include carbohydrates, fats and proteins, constitute the bulk in foods and provide energy and building material for almost every function in the body. Micronutrients, which are the vitamins, minerals and trace elements, are obtained in much smaller quantities and are essential to more specific functions in the body.

In the past, researchers discovered consequences of severe micronutrient deficiencies that caused clinical diseases like xerophthalmia, rickets, scurvy and beriberi. Today, they are attempting to discover consequences of milder deficiencies. Mildly deficient persons usually do not develop characteristic symptoms that are easily recognisable, but rather tend to develop certain diseases more often or at an earlier stage in life than sufficient persons. To identify subtle differences in disease risk, researchers must be able to distinguish mild deficiency from sufficiency by the help of biomarkers that accurately reflect micronutrient status. They also need to follow a large group of people over many years, as people vary in their requirement of micronutrients to sustain physiological functions.

In this thesis, we provide sequential overviews of vitamin D and cardiovascular disease. This is followed by a historical flashback on their relationship and an overview of our current understanding of how vitamin D may influence cardiovascular disease. We also describe and discuss the methodology and results of three observational studies with overlapping study populations that used the biomarker 25-hydroxyvitamin D to reflect vitamin D status. The studies focus on potential determinants of the biomarker, the longitudinal relationships of the biomarker with atherosclerosis progression and all-cause and cardiovascular mortality, as well as a methodological issue with the use of this biomarker in observational research related to seasonal variation in sun exposure.

1.1 Vitamin D

1.1.1 History

Some diseases manifest with very characteristic symptoms, such as the bowing of extremities in the children on the figure below (**Figure 1**). Due to its characteristic manifestation in humans, this symptom and the underlying disease may be traced more easily back in time through historical records.

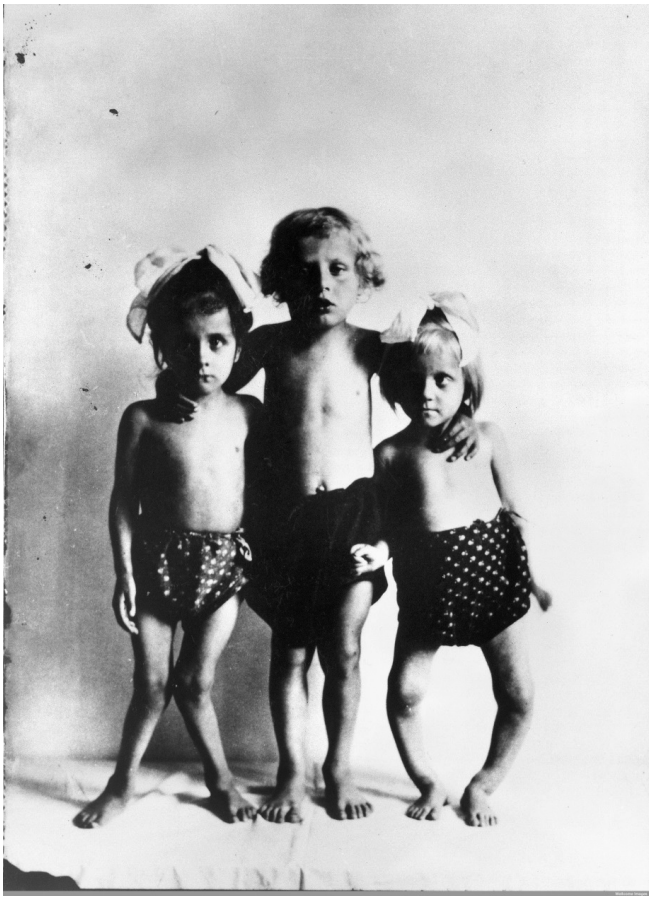

Figure 1. Three children with rickets.

Bowing of the arms or legs depended on whether the infant was crawling or walking.

Image acquired: Wellcome Library, London. http://wellcomeimages.org. Image entitled: "L0014375 Three children with rickets; anon.. Friends' Relief Mission, Vienna XII, n.d.Photograph circa 1920 – 1930". Copyright: Creative Commons Attribution only licence CC BY 4.0.

According to a historical review, this symptom is mentioned in records which date back to antiquity [1]. It was later described as “rickets” in several records from Europe between 1645 and 1668 and as a cause of death in the “Annual Bill of Mortality of the City of London” in 1634 [1]. In addition to the descriptions of rickets in historical records, it has also been confirmed in excavated remains of children of the prominent Italian renaissance family Medici from 1547-1602 [2].

Although unable to explain the cause of rickets in the 17th to 19th century, records from different parts of the world describe different remedies [1]. The records include the proposed or demonstrated efficiency of eating animal or fish liver, as well as physical activity, fresh air and sunlight. In 1918, Mellanby demonstrated that rickets was, similar to scurvy and beri beri, a disease caused by deficiency of a factor in food, and named it anti-rachitic accessory factor [3]. However, he concluded that this factor most likely was fat soluble factor A, know referred to as vitamin A, which had been shown to cure xerophthalmia, a destructive dryness of the eyes. Meanwhile, convincing observations had been made of geographical and seasonal patterns in the prevalence of rickets [1] and the curative effect of natural and artificial ultraviolet B (UVB) exposure (**Figure 2**) demonstrated [4]. The common denominator behind the curative effects of physical activity and fresh air had been identified. A series of human studies in hospital wards in Vienna between 1919 and 1922 clearly provided evidence for the separate effects of natural and artificial sunlight and cod liver oil in alleviating symptoms of rickets [5].

In 1922, McCollum was able to oxidize the property of cod liver oil that cured xerophthalmia without removing its anti-rachitic properties, thereby illustrating the existence of a separate calcium-depositing vitamin, the fourth micronutrient discovered by that time [6]. Investigating the link between the anti-rachitic properties of this vitamin, named vitamin D, and the anti-rachitic properties of sunlight, several groups demonstrated that certain foods, such as liver and linseed oil, could also cure rickets if exposed to UVB radiation [7]. The technology to fortify foods with vitamin D was thereby available and contributed to reduce the prevalence of vitamin D deficiency before the structure was known.

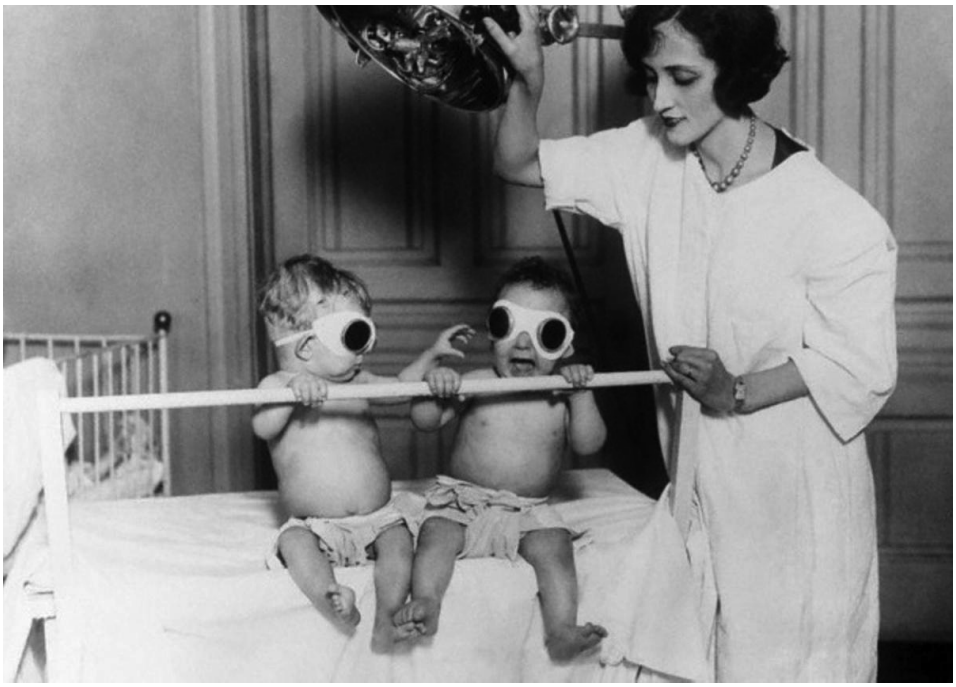

Figure 2. Treatment or prevention of rickets in children with artificial ultraviolet B radiation. To the best of the author's knowledge, this image originates from the Chicago Nursery and Half-Orphan Asylum (1925). Neither information on copyright status nor the identity of the owner or uploader was available.

1.1.2 Dietary intake and absorption

Vitamin D is acquired from dietary sources in two structural forms that only differ in side chain structure [8]. Ergocalciferol or vitamin D2 is produced by yeast and mushrooms from the fungal precursor ergosterol when exposed to UVB light while cholecalciferol or vitamin D3 is produced in the skin of animal species from the precursor 7-dehydrocholesterol when exposed to UVB light [9]. Both forms are metabolised in the same way and yield vitamin D activity.

The content of cholecalciferol in animal meat is low in comparison to the content in fatty fish meat and lean fish liver. This is the result of animal plankton synthesis of cholecalciferol and the subsequent accumulation up the marine food

chain. In Norway, fish and fish products contribute to 40% and 17% of the dietary intake of vitamin D, respectively, while the mandatory fortification of butter and margarine contribute with 30% [10]. Lesser sources are one type of skimmed milk added vitamin D (4%), eggs (4%) and cakes containing eggs and butter (6%).

Dietary sources of ergocalciferol are few, with variable amounts in mushrooms, baker's yeast and animal products, but may contribute significantly to vitamin D status in countries with a low intake of cholecalciferol [11]. The most significant contribution comes from dietary supplements. In North America, ergocalciferol has been the preferred constituent in preparations for prescriptions and both forms used in multivitamins [12]. However, a case against the potency of ergocalciferol has been ongoing since the 1930s and this form is today considered inferior to cholecalciferol [12]. Consequently, the industry is transitioning to produce products with cholecalciferol.

Absorption of dietary calciferols primarily follows dietary fats in the small intestine; including incorporation into lipid-containing micelles and diffusion into enterocytes [8]. Overall, approximately 80% of calciferols are absorbed in healthy subjects, although the rate of absorption may differ between individuals and be completely or severely diminished by intestinal fat malabsorption [13]. Although a fraction of absorbed vitamin D is transported directly to the liver through the portal vein, the majority is packed into chylomicrons and transported via the lymphatic to the circulatory system within hours after a single administration of calciferol [8, 13, 14]. Calciferols are transferred into peripheral tissue together with triglycerides when chylomicrons undergo lipolysis, which is predominantly in tissue that has a high expression of lipoprotein lipase, such as adipose tissue and muscle [8]. Vitamin D remaining in the cholesterol-rich chylomicron remnant is subsequently taken up by the liver.

1.1.3 Sunlight exposure and skin synthesis

The major site of cutaneous synthesis of vitamin D is the epidermis, where the precursor 7-dehydrocholesterol is converted to cholecalciferol upon exposure to UVB light within a wavelength of 290-315 nm [15]. The amount of UVB available for skin synthesis is determined by the solar zenith angle, which again is determined by latitude, seasonality and time of day. In a northern country like Norway, the UVB intensity is negligible in the dark season from approximately October through March [16]. During the summer in Bergen in Norway, sunlight for vitamin D production is available from approximately ten in the morning until six in the afternoon, with a narrower interval during early fall and late autumn [17]. Utilisation of available UVB in the bright season is reduced by skin melanin pigmentation and sunscreen use, which absorb or block UVB rays, respectively, and thereby prevent it from penetrating the skin. Utilisation is also reduced in older adults due to an age-dependent reduction in the availability of 7-dehydrocholesterol in the skin [15].

When UVB photons are absorbed by 7-dehydrocholesterol in the epidermal plasma membrane, it transforms to an unstable pre-vitamin that may further isomerise to cholecalciferol [18]. Synthesised cholecalciferol moves to the extracellular space and is transported into the dermal capillary bed due to affinity to vitamin D binding protein (DBP) [18, 19]. This protein is the primary and dominant carrier of the lipophilic calciferols and their subsequent metabolites in plasma, with albumin as the second minor carrier [20]. Upon skin synthesis, plasma concentrations of cholecalciferol reach a maximum after 12-24 hours [19]. While dietary cholecalciferol may be radioactively labelled, it is not possible to distinguish synthesised from endogenously present cholecalciferol. A review of different studies estimated that a full body exposure to one minimal erythema dose (the amount of UV radiation that results in slight redness of the skin) is equivalent to a dietary intake of 250 µg cholecalciferol [21]. Exposure of ~5% of the body to half the minimal erythema dose or a full body exposure for a few minutes has been estimated to be equivalent to ~10 µg vitamin D from dietary sources [22, 23].

1.1.4 Metabolism

Storage

Approximately 65% of body vitamin D is in the form of cholecalciferol, of which 75% is stored in adipose tissue [24, 25]. Extrapolations from subcutaneous adipose tissue biopsies estimate the total body fat mass of cholecalciferol to 1583 international units (IU) per kilo in morbidly obese subjects [26] and 2812 IU/kg in normal weight subjects, where 40 IU is equivalent to 1 μg [27]. The latter corresponded to a total body calciferol content of 81 000 IU. This was increased to 197 000 IU after a dietary intake of 660 000 IU over 12 weeks, resulting in a 17% storage [27]. Although the functional bioefficacy of cholecalciferol stored in adipose tissue is not well known, a study in rats indicate that only small amounts are released during isocaloric conditions [28]. The release may increase in concert with the mobilisation of triacylglycerols during fasting.

Conversion to 25-hydroxyvitamin D

With limited storage capacity and a half-life in plasma of only a few days [28], a potential loss of acquired calciferols is prevented by the rapid up-take of calciferols by the liver and subsequent hydroxylation at the carbon-25 position (C-25). The major hydroxylating enzyme in the liver is the microsomal cytochrome P450 (CYP2R1) [29]. The resulting metabolite is 25-hydroxyvitamin D (25OHD), also named calcidiol or calcifediol [30, 31]. Other enzymes may also contribute to calciferol hydroxylation [32] and explain why there are few reported cases of 25OHD deficiency due to inborn errors in metabolism [33-35].

Calcidiol is an inactive metabolite with a very strong affinity to DBP, resulting in a plasma half-life of approximately 30 days [36]. Approximately 35% of total body vitamin D is in the form of calcidiol which is distributed evenly in adipose tissue (35%), serum (30%), muscle (20%) and other tissues (15%) [24]. Serum concentrations of 25OHD are sensitive to changes in dietary intake [37] and UVB

exposure [38], and is currently the preferred biomarker of vitamin D status in clinical care and research.

Conversion to 1,25-dihydroxyvitamin D

In the kidneys, calcitriol bound to DBP is filtrated through the glomerulus and reabsorbed together with DBP in the proximal tubule by receptor mediated endocytosis [39]. The receptor responsible is megalin, a member of the low-density lipoprotein receptor superfamily [40]. Megalin is supported by the coreceptor cubilin [41]. In the proximal tubular cell, the active form of vitamin D is produced by an additional hydroxylation of 25OHD at the C-1 position, resulting in the formation of 1,25-dihydroxyvitamin D ($1,25OH_2D$, also called calcitriol) [42-47]. The hydroxylation is catalysed by the enzyme 1α-hydroxylase which is coded by the CYP271B gene [48-50]. This enzyme is distributed in many tissues, but the primary site in the body for CYP27B1 expression and responsible for the circulating concentrations of calcitriol is the proximal convoluted tubule of the kidneys [51]. Extrarenal synthesis of calcitriol normally does not influence plasma calcitriol concentrations, with the exception of macrophage synthesis during sarcoidosis that may result in severely elevated plasma concentrations [52].

Catabolism

In a five-step oxidation pathway, calcitriol is catabolised to calcitroic acid, a truncated water soluble molecule excreted through the bile [53]. The first step, and perhaps several other steps also [54], are catalysed by the enzyme 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) [55]. This enzyme also catabolises the much more abundant 25OHD, but has a stronger affinity to calcitriol [56]. The enzyme has a high concentration in the kidney mitochondria [57], but is also expressed in most cells where vitamin D has activity, which may indicate a regulatory role on cellular level [58]. In addition, CYP24A1 also performs a 23-hydroxylation as the first step in a second catabolising pathway of calcitriol which results in the formation of

$1,25OH_2D3$-26-23-lactone [59, 60]. The physiological role of CYP24A1 is illustrated in humans with mutations causing reduced enzyme activity. These individuals display chronic or transient hypercalcaemia which may lead to soft-tissue calcifications and chronic kidney disease [61, 62]. CYP24A1 deficient mice display hypercalcaemia, nephrocalcinosis and a lethality of 50% [63].

1.1.5 The vitamin D receptor

The vitamin D receptor

Calcitriol mediates vitamin D activity by acting as a high-affinity ligand for the vitamin D receptor (VDR) [64] and is therefore a prerequisite for vitamin D activity in a given cell. VDR is a member of a class of evolutionary conserved proteins known as the superfamily of nuclear receptors [65]. This family was originally referred to as "orphans" receptors, because their respective activators were unknown at the time of their discovery. About half of the 48 human receptors have so far been "adopted" by specific ligands, including vitamins, hormones and fatty acids, while the remaining half do not have traditional ligands [66]. The physiological effects induced by the different receptors are diverse, but they all mediate their activity by regulating the transcription of genes.

Dimerisation with retinoid-X-receptor

Nuclear receptors may function as monomers or as part of a homodimer or heterodimer with another identical or different receptor, respectively. VDR functions as part of a heterodimer with the retinoid-X-receptor (RXR) [67]. RXR is a common partner for other nuclear receptors, including the retinoic acid receptor (RAR) which ligand is vitamin A, the thyroid receptor which ligand is thyroid hormone, and peroxisome proliferator-activated receptors (PPARs) which ligands are fatty acids [68]. The marine omega-3 fatty acid docoshexanoic acids (DHA) is a direct ligand for RXR [66]. RXR has three isoforms with a wide distribution in different tissues, in

which RXRα is highly expressed in visceral tissues, RXRβ relatively abundant, while RXRγ is expressed highly in heart and muscle [69]. They are similar and assumed to have overlapping ligands and gene targets, but differ in amino-terminal domains, which could indicate individual differences [69]. The VDR-RXR heterodimer can only be activated by calcitriol ligand binding to VDR and not by ligand binding to RXR, referred to as a partnership where VDR is nonpermissive and RXR is silent [66].

Structure of vitamin D responsive elements (VDREs)

In order to regulate transcription, nuclear receptors interact with responsive elements (REs) on DNA. REs are specific nucleotide sequences and also referred to as binding sites. The receptors share binding affinity to variations of a specific sequence of six nucleotides, known as a core binding motif. The hexameric sequence is RGKTSA and the variation encapsulated by the fact that R can be either adenine or guanine, G=guanine, K=either guanine or thymine, T=thymine, S=either cytosine or guanine and A=adenine [70]. While monomeric receptors bind to a single hexamer of the motif, dimers require a repetition of the hexamer in order for both receptors to interact with the DNA. Homodimers bind palindromic sequences (i.e. AGGTCACTGGA) while RXR-heterodimers bind tandem repeats separated by nucleotides [66]. RXR-heterodimers share a strong affinity to the direct repeat of AGGTCA, however, affinity is further dictated by the number of intermittent nucleotides. The VDR-RXR heterodimer has strongest affinity to this variation of the core binding motif when the tandem repeats are separated by three intermittent nucleotides [71]. PPARs [72], the thyroid receptor, and RAR have the strongest affinity to the direct repeat of AGGTCA when separated by one, four and five intermittent nucleotides, respectively [71]. This simplistic system of affinity due to single nucleotide spacing has been conserved throughout evolution.

The cistrome of VDR

The number of potential binding sites for nuclear receptors is dictated by the length of the human genome and the occurrence of the core binding motif. An *in silico* screen of the ~3 billion bases which constitute the human genome [73, 74] would identify approximately 50 000 -100 000 potential binding sites for the average nuclear receptor [70]. However, as DNA is packed in nucleosome and chromatin, the availability of binding sites is drastically reduced *in vivo.* As the density of chromatin is dynamic and constantly changing, so is also the number of binding sites [66].

The distribution of binding sites available *in vivo* at a given moment in time is referred to as the cistrome and may be assessed by combining chromatin immunoprecipitation with DNA sequencing (ChIP-seq) [75]. In one *in vitro* study of osteoblasts, the number of sites bound by VDR increased from 1325 to 8241 upon stimulation with calcitriol [76] and in another study in lymphoblastoids increased from 623 to 2776 sites [77]. The cistrome may further increase as a result of disease activity, as demonstrated in human hepatic cells stimulated with both calcitriol and a pro-fibrotic cytokine, in which the cistrome increased from 6281 to 24984 [78]. Hence, the cistrome is ever changing and results from studies may vary depending on whether it was performed *in vivo* or *in vitro*, the type of tissue or cell, the exposure to vitamin D, as well as the definition of differentially expressed genes.

Corepressors and coactivators of VDR

Both VDR and RXR reside in the nucleus and are believed to interact with binding sites upon ligand activation or remain bound to single binding sites in the absence or presence of ligands [79]. For instance, of the 7250 *de novo* sites bound by VDR upon calcitriol stimulation of osteoblasts, 6000 sites were already pre-bound by RXR prior to calcitriol stimulation and VDR binding [76]. In the absence of a ligand, the heterodimer or RXR is believed to recruit a complex of factors that prevent transcription, known as corepressors. In the presence of ligand binding, the heterodimer recruits a complex of factors that promote transcription, known as

coactivators. Hence, the heterodimer function as a reversible switch that both decrease and increase transcriptional activity of genes [66]. The cofactors regulate different functions, including transcriptional activity, post-translational modifications and chromatin remodelling [66, 80].

Location of VDREs and gene trancription

Gene transcriptional machinery are assembled at promoter elements often situated immediately upstream to the transcription start site [81]. If there is a VDRE/binding site for VDR-RXR near the promoter of a specific gene, this indicates a regulatory role of vitamin D on that gene. In a database of many cistrome data sets, the median distance from the binding sites to the closest gene was 10 kb away [75]. More specifically, 13% of the VDR cistrome in osteoblasts was located in promoter regions (defined as within 5 kilobases (kb) upstream of the start site), 38% was located in the intron, 6% in the exon, 5% within 5 kb downstream of the gene and 39% in the intergenic regions which do not code for proteins, often referred to as “dark matter”. [76].

The total number of genes which transcription is regulated by vitamin D may be identified by gene expression studies using microarray technology. As the cistrome vary, also the number of genes reported varies from 200 to 900 [75-77, 82, 83]. Overall, VDRE binding seems to mostly enhance transcription of genes. This was illustrated by the up-regulation of 276 and ~175 genes and down-regulation of 54 and ~45 genes in osteoblasts treated with calcitriol [76] and lymphoblastoids [75], respectively.

Intergenic regions and non-coding RNAs

Out of the ~3 billion bases which constitutes the length of the human genome [73, 74], approximately three-quarters are transcribed into RNA [84], but only a few percent code for the molecular structure of proteins [85]. The activity of the much

more abundant non-protein coding RNAs, which functions are just beginning to be unraveled [86], suggests a more complex understanding of molecular biology than what has been historically referred to as the "central dogma" [85, 87]. Due to the widespread location of VDREs in intergenic regions, it is likely that vitamin D activity is relevant to the regulation of non-coding RNA transcription and thereby their function. Although an intergenic VDRE may be distal from a gene according to genomic sequence, it may in fact be much closer physically. Proteins interacting with DNA may bind two different sites simultaneously, bringing them in proximity of each other, known as DNA looping [88]. Cofactors that remodel chromatin may be particularly relevant in intergenic regions, contributing to gene regulation by chromatin interaction [66]. Nevertheless, genomic proximity of VDREs to genes appears to be a determinant of transcription regulation, as demonstrated by calcitriol stimulation *in vitro*, where sites bound by VDR where closer to differentially expressed genes than expected by random chance [75].

1.1.6 Effects of vitamin D

Bone and mineral homeostatis

Vitamin D participates in the regulation of bone and mineral homeostasis. It is crucial that the circulating concentrations of calcium remain within a narrow interval in order to sustain critical bodily functions. This requires interplay between circulating concentration of minerals, proteins, hormones and enzymes involved in vitamin D metabolism.

Receptors in the parathyroid glands sense low calcium concentrations in blood and thereupon release parathyroid hormone (PTH). PTH circulates to the kidneys and stimulates conversion of 25OHD to calcitriol by the activity of CYP27B1 [89, 90]. Transcriptional activity of calcitriol results in increased absorption of dietary calcium and phosphate in intestines, reabsorption in proximal tubules of kidneys and resorption from bone [91]. Altogether, these mechanisms prevent hypocalcaemia and hypophosphatemia. As PTH and calcitriol both have strong calcium-resorbing effects,

their joint activity could result in a rapid loss of bone minerals that could, when coupled with dietary intake and kidney reabsorption, result in increased ectopic calcification due to hypercalcaemia and hyperphosphatemia [91]. Several mechanisms are in place to prevent this scenario. Expression of VDR in parathyroid glands [92] allow calcitriol activity to inhibit PTH synthesis, a negative feedback loop that prevents hypercalcaemia [93]. Hyperphosphatemia is prevented by transcriptional activity of calcitriol on bone osteocytes and their subsequent increased expression and release of fibroblast growth factor 23 (FGF23) [94]. FGF23 reduces absorption and reabsorption of phosphate in the intestine and kidneys, respectively [95]. It also reduces circulating calcitriol by inhibiting expression of CYP271B and increasing expression of CYP24A1 [96, 97]. In addition, calcitriol also performs autoregulation [98], by enhancing the expression of its primary catabolic enzyme, CYP24A1 [99-101]. The importance of CYP24A1 in preventing hypercalcaemia is illustrated by patients with inborn errors in the CYP24A1 gene [102, 103], as mentioned earlier. In 1997, the first animal VDR knockout models were successfully created [104, 105]. Mice with global knockout were first reported to develop normally until mother's milk was withdrawn and then displayed a rachitic phenotype similar to that of human genetic diseases, causing death within 15 weeks. Normalisation of mineral ion homeostasis by dietary intervention prevented the rachitic phenotype and prolonged life [106].

Hair regeneration

The skin abundantly express VDR, which is crucial for the regeneration of hair [91]. Mutations in the VDR cause hair loss, alopecia, in both humans and mice. However, treatment with calcium does not ameliorate the disorder [106] and mutations in CYP27B1 that diminish calcitriol concentrations do not cause alopecia. This indicates that the activity of VDR on the hair follicle is independent of calcitriol [91, 107].

Other effects

With the combination of ChIP-seq and microarray expression analysis, researchers are able to identify genes that are differentially expressed upon calcitriol stimulation. The list of genes is likely to differ between cells, as they vary in expression of VDR, co-regulatory molecules and enzymes involved in either the transport of 25OHD or activation and catabolism of calcitriol [108]. The list of genes may also vary within cell types as a result of the experimental conditions, as this may change the activity of pathways relevant to the availability of VDR, RXR or binding sites. Hence, a list of differentially expressed genes is specific to the cell type studied and the experimental conditions.

By the use of bioinformatics, the list of differentially expressed genes can be linked to databases containing information about associated protein products and the functions and biological pathways the proteins are involved in. From these studies, the functions most strongly linked to vitamin D activity have been elucidated. The most strongly linked functions are involved in bone formation and mineralisation, but also fundamental cellular processes, cell signalling and the regulation of gene expression [82]. These latter non-skeletal physiological effects are often referred to as pleiotropic effects. Among the differentially expressed genes, the most extensively studied so far are those with intragenic VDREs and a strong response to vitamin D activity.

Effects on fundamental cellular processes

Proliferation is defined as the growth and division of cells. Mitogenic signals ensure that proliferation is sufficient when needed to build, maintain and repair the organism, while a number of growth inhibitory factors restrict the clonal autonomy in order to avoid overpopulation and formation of autonomous colonies [109]. Thus, a cell will commit to proliferation only if the weight of these counter-balancing factors are shifted in one direction by sufficient stimuli [109]. A further restriction of

proliferative capacity occurs when primitive cells differentiate into more specialised cells, resulting in cell-cycle exit [110].

Anti-proliferative actions of vitamin D are mediated directly by transcriptional regulation of genes involved in cell cycle progression and indirectly by regulation of genes that promote cell differentiation [111]. It is possible that vitamin D activity may either inhibit or enhance proliferation, dependent on whether the cell is under the influence of physiological or pharmacological concentrations of calcitriol [112]. Several disease pathways are characterised by cellular hyperproliferation, such as cancer, psoriasis and diseases with an inflammatory component, implicating that vitamin D may play a role in their aetiology or for treatment [108].

Apoptosis is the process of programmed cell death [113], an integrated part of normal human physiology acting as a counterpart to mitosis and proliferation [114]. It is a genetically regulated mode of cell death that does not result in necrosis and local inflammation. Consequently, it is important in buffering the approximately 10 billion cells that arise from stem cells every day [115]. Apoptosis prevents hyperplasia and help maintain healthy tissue populations [114]. The role of apoptosis during development is studied extensively in the roundworm *Caenorhabditis elegans* where 131 of 1090 generated cells are genetically programmed to undergo apoptosis at a specific time point [116]. How and whether a cell commits to apoptosis depends upon the initiating signal, the stage of development of a specific tissue and the physiological milieu [114].

Vitamin D activity may also regulate cell growth by inhibition of apoptosis [108]. One potential mechanism is by transcriptional regulation of pro-apoptotic and anti-apoptotic members of the Bcl-2 family of proteins, which determine if cells commit to or abort apoptosis [108]. Another potential mechanism is by regulating expression of genes that initiate and carry out the proteolytic cascade which characterise apoptosis [108, 114]. Changes in normal rate of cell death may result in disorders of cell loss and cell accumulation. Disorders of cell loss include neurodegenerative disorders, AIDS and osteoporosis. while disorders of cell

accumulation include cancer, autoimmune diseases and viral infections [117]. The potential roles of vitamin D in these diseases are under investigation.

Nongenomic activity of vitamin D

Ligands of several members of the nuclear receptor superfamily exert biological activity which is too rapid to be caused by interaction with their cognate receptors in the nucleus [107]. Effects of calcitriol on ion-channel activity and enzymes involved in signal transduction has been demonstrated in various cell types [107] and may involve interaction with membrane receptors and VDRs located in the cytosol [118].

1.1.7 Vitamin D status and dietary recommendations

Vitamin D status

Scientific data on requirements and health effects of vitamin D was recently reviewed by American-Canadian [8], Nordic [119] and German-Austrian-Switzerland (D-A-CH) [120] committees with the purposes of reviewing and updating dietary recommendations within these countries. Vitamin D status was defined by the use of 25OHD concentrations and dietary recommendations set to reflect the amount of vitamin D necessary to obtain and maintain a certain level of 25OHD. The effect of vitamin D on several diseases was reviewed, including cancers, autoimmune diseases and cardiovascular diseases, but the scientific evidence only convinced the reviewers about a relevant effect on bone health. The committees concluded that there was little causal evidence that a 25OHD concentration above 50 nmol/l would provide additional benefit in terms of indicators of bone health and that this concentration would be sufficient to cover the requirement for 97.5% of the population [8]. The average requirement or median requirement that would ensure sufficiency for 50% of the population was estimated to 40 nmol/l. It should be noted that a review by the Endocrine Society Task Force weighted the literature somewhat differently. They concluded that 25OHD concentrations above 50 nmol/l led to a further reduction in

PTH concentrations, as well as a positive effect on muscle function, and suggested 75 nmol/l as the threshold for sufficiency [121].

Dietary recommendations

In order to estimate the amount of dietary vitamin D required to obtain a target 25OHD concentration, the dose-response relationship must be established, preferably in studies performed under conditions of no sunlight. The American-Canadian review performed a mixed model regression of eligible studies and estimated that the increase in 25OHD from dietary intake of vitamin D was steep and linear until 25 µg (1000 IU) per day and then started to flatten for intakes above for all individuals [8]. Hence, the dose-response is not linear, but seems to follow a logarithmic curve. In practice, this means that the amount of vitamin D required to achieve an increase in 25OHD concentration from 30 to 40 nmol/l is much less than from 80 to 90 nmol/l.

The conclusions regarding the dose-response relationships differed between committee reviews and they also differed in dietary recommendations to achieve the target concentrations of 25OHD. In order to achieve 40 nmol/l and 50 nmol/l, respectively, the American-Canadian and Nordic committees estimated that an intake of 10 µg and 15 µg would be required from dietary sources under conditions of limited sun exposure. As most Nordic individuals gain significant amounts of vitamin D during the summer period, the recommended dietary intake was set to 10 µg per day [119]. This is equivalent to the median requirement and is therefore not sufficient for 50% of those who, for reasons such as pigmentation, do not synthesise vitamin D from sunlight. The American-Canadian decided to recommend a daily allowance of 15 µg in order to include also those who do not synthesise vitamin D from sunlight [8]. The committee of the German speaking countries emphasised the results of a particular study from Ireland, performed during the winter at similar latitude as these countries [122]. In this study, only 50% of the study population achieved a concentration of 50 nmol/l from 10 µg supplemented vitamin D, while 90-95% achieved this concentration from 20 µg. Consequently, they recommended a daily

intake of 20 µg [120]. The Endocrine Society Task Force recommended a minimum dietary intake of 15 µg of vitamin D per day for individuals between 15 and 50 years of age in order to reach 75 nmol/l under conditions of sun exposure [121]. Due to the non-linear dose-response relationship between vitamin D and 25OHD, they emphasised that an intake of at least 37.5 to 50 µg would be required during conditions of no sun exposure.

Average dietary intakes of vitamin D varies considerably between countries in Europe, with a higher intake in the north in comparison to south and central regions, and a mean daily intake of 4.9 µg for men and 3.4 µg for women [123]. Consequently, dietary intakes of vitamin D from food sources are well below 10 µg for most European countries, and in order to achieve the recommended amounts, extensive food fortification or intake of dietary supplements are required. In Norway, there is a tradition for consuming cod liver oil, while the extent of food fortification is more conservative.

Safety

The foregoing review by the American-Canadian committee from 1997 considered the safety of vitamin D almost solely on the risk of acute toxic effects, where calcification in the vasculature and soft-tissue due to hypercalcaemia results in renal and cardiovascular damage [8]. In contrast, the current committee had access to observational and some trial data reporting that 25OHD concentrations from approximately 75 to 120 nmol/l associate with negative health effects, including all-cause mortality, cardiovascular risk, cancer, fractures and falls. This questions the safety of chronically excessive intakes of vitamin D. When deciding on the tolerable upper intake level, which is defined as the level of intake per day throughout a lifetime that is not likely to cause any harm to most individuals of the population, the committee therefore adopted a cautious approach. They concluded that daily intakes below 250 µg are unlikely to cause symptoms of acute toxicity, equivalent to a no observed adverse effect level, and specified the tolerable upper intake level to 100 µg

per day [8]. This was later also the conclusion by the European Food Safety Agency (EFSA) [124].

1.1.8 Research needs and information gaps

The review by the American-Canadian committee devotes a chapter on unanswered research questions and information gaps in need of closure in order to advance our understanding of vitamin D in human physiology [8]. One of the most important questions to answer is whether vitamin D is relevant to various health outcomes, and if so, to estimate the dose-response relationships. Cardiovascular diseases (CVDs) cause the most death and disability worldwide [125] and even small clinical beneficial effects from vitamin D would be meaningful. Provided that there is an effect, the dose-response relationship between 25OHD and CVDs would dictate whether it is encapsulated by current dietary recommendations for bone health or if a further increase is required.

In the other end of the scale, the committee review also emphasised the importance of elucidating any adverse health effects of chronically excessive intakes of vitamin D. Toxicity results in hypercalcemia and evident calcifications of blood vessels in renal and heart tissue, while the mere combination of vitamin D and calcium supplements increases the risk of kidney stones [126]. Chronically elevated 25OHD concentrations could result in an increased risk of CVDs, chronic kidney disease and thereby reduce life expectancy. The relationship between vitamin D status, cardiovascular disease and mortality should therefore be investigated with a two-way hypothesis in mind.

There are tendencies in the general population that warrants investigation of these questions sooner than later. In the lay press and blogosphere, uncritical and overenthusiastic interpretations of research and miraculous anecdotes are frequent. The Hormone Laboratory at Haukeland University Hospital in Bergen, Norway, has measured 25OHD in samples from hospitals and general practitioners in the region

since 2007. Despite the fact that two other hospitals in this region started their own analysis of 25OHD during this period, there has been an annual increase in measurements of 60-70% (personal communication, Kristin Viste, Hormone Laboratory, Haukeland University Hospital, Bergen, Norway). Consequently, there seems to be an interest in the curative effects of vitamin D in the general population and among health personnel. In an example from neighbouring Sweden, a son with good intentions recently administrated daily 1250 µg of vitamin D to his father sick from dementia, resulting in severe hypercalcaemia. The son had read in a book about the miracles of vitamin D and then ordered supplements online, thereby bypassing legislation that controls the potency of dietary supplements [127]. Although this was a case of vitamin D toxicity, it could be the clinical manifestation of a tendency among consumers to acquire more potent supplements to gain undocumented health effects of vitamin D.

1.2 Cardiovascular disease

1.2.1 Atherosclerosis

The human cardiovascular system is constituted by the heart and blood vessels. The heart pumps blood into the pulmonary circulation for oxygenation and then into the systemic circulation for distribution to peripheral tissue through an extensive network of blood vessels. The most important pathological condition causing CVDs is a chronic inflammatory disease of the arterial blood vessels known as atherosclerosis [128]. Atherosclerosis involves complex interplay between cells of the arterial wall as they respond to damaging stimuli, intensified by the activity of cardiovascular risk factors [129, 130]. The resulting injury occurs in defined areas on the arterial wall and is referred to as atherosclerotic lesions.

Endothelial activation

Coating the arterial wall is a monolayer of endothelial cells (ECs), which is in constant contact with the constituents of circulating blood. The endothelium responds to physical and chemical signals in an adaptive manner to regulate vascular tone, cellular adhesion, resistance to thrombosis, proliferation of cells and inflammatory processes in the arterial wall [131]. As a result of the effect of risk factors [132], changes in the physical and chemical signals cause the endothelium to shift from a quiescent phenotype to an active state, characterised by expression of chemokines, cytokines and adhesion molecules [131]. Endothelial activation comes at the expense of normal functions, i.e. the enzyme nitric oxide synthase shifts from producing the vasodilator nitric oxide to produce reactive oxygen species that contribute to inflammation [131]. The effect of tobacco smoke, which is a risk factor of atherosclerosis, is in part mediated by a reduction in nitric oxide bioavailability in ECs [133]. This particular effect of smoking has been measured as a clinically evident reduction in blood vessel dilation and blood flow [134], which is reversible by smoking cessation [135]. The shear stress produced by the laminar (streamline) blood flow is crucial for normal vascular function [136]. Disruptions of laminar flow and turbulence are important causes of endothelial activation and more common at specific arterial sites [128, 137].

LDL-particle retention and inflammation

Atherosclerosis is characterised by accumulation of mononuclear cells, which is a hallmark of chronic inflammation [138]. Upon activation, the endothelium becomes adhesive to immune cells and permeable to low-density lipoprotein (LDL) particles [130]. Parallel changes in the extracellular matrix composition of the intima layer below the endothelium contribute to trapping of migrated LDL particles [130]. Once inside the intima layer, the lipid fraction of the trapped particles is subjected to biochemical modifications, such as oxidative and enzymatically changes [138]. Monocyte-derived macrophages residing or migrating to the intima have scavenger receptors that recognise oxidatively modified LDL particles and are capable of

internalising the particles, resulting in foam cell appearance [130]. The activity of macrophages and foam cells may propagate endothelial activation and inflammation by recruitment and differentiation of immune cells, such as T lymphocytes, and is mediated by the release of cytokines, chemokines and presentation of internalised lipids [128]. Continued accumulation of immune cells and lipids appear morphologically as a yellowish fatty dot or streak on the arterial wall, which may regress without causing further disease progression [139].

Advancing lesions and plaque stability/vulnerability

In advanced atherosclerotic disease, a more profound accumulation of cells and lipids can take the shape of an atheroma, with a necrotic core of cellular debris and lipids. Lesion growth triggers migration of smooth muscle cells (SMCs) from the media to the intima arterial layer where they proliferate and produce extracellular matrix molecules. This activity results in the formation of a fibrous cap covering the atheroma, referred to as a fibroatheroma. As there is limited area for expansion inside the arterial wall, lesion growth may result in the lesion intruding into the lumen of the afflicted artery, forming a stenosis [128].

Atherosclerotic lesions do not necessarily develop in the same predictable manner and may differ widely in morphology [139]. Characteristics relevant to classification of advanced lesions include the size and shape of the necrotic core, the thickness of the fibrous cap, the cellular composition of the lesion, calcifications and the degree of stenosis [139]. Microvessels may sprout from the outermost layer of the arterial wall, the tunica externa, and into the lesions [130].

Plaque rupture is not only the underlying cause of clinical events, but is also a major contributor to lesion growth. In fact, most ruptures are subclinical and result in growth of the necrotic core by influx of cholesterol from erythrocytes and remodelling of the arterial wall from wound healing [140]. In contrast to more stable plaques that have a thick fibrous cap of collagen and smooth muscle cells, vulnerable plaques have a thin cap and a higher proportion of immune cells [140]. Inflammatory

activity regulates proteases, such as matrix metalloproteinases [141] and cysteine protease [142], which are responsible for degrading extra cellular matrix. Protease activity may theoretically result in a favourable reduction in lesion size, but also cause thinning of the fibrous cap [137]. Production of cytokines and coagulation factors by immune cells may also contribute more directly to thrombus formation [137].

1.2.2 Ischemic heart disease

Atherosclerotic plaque may obstruct arterial blood flow and cause tissue ischemia by luminal narrowing or by formation of a blood clot. Blood clots can also dislodge from the site of origin and cause ischemia in distal arteries. In the case of myocardial ischemia, the patient may experience a characteristic strangling feeling of the chest which is referred to as angina pectoris. When symptoms occur during exercise and resolve during rest, it is more likely caused by a coronary stenosis and is referred to as stable angina pectoris (SAP). When symptoms develop suddenly, during rest, or symptoms change in intensity independent of exercise, the cause is more likely thrombosis, and is referred to as unstable angina pectoris (UA).

UA may cause ischemia to a part of the heart muscle to an extent where it is unable to perform properly, but does not cause permanent damage to the heart muscle. Once ischemia causes permanent tissue damage, a heart attack or acute myocardial infarction (AMI) has occurred by definition [143]. Patients with reduced heart function either due to UA or AMI are jointly referred to as suffering from acute coronary syndrome (ACS). Together with sudden cardiac death, SAP and ACS are the conditions which constitute ischemic heart diseases (IHD). Out of all deaths attributed to cardiovascular causes worldwide, IHD is responsible for 47 percent [144].

Assessement and treatment

Patients with suspected IHD are assessed to identify the most likely cause of symptoms and to determine the treatment associated with best prognosis. Initially, an electrocardiogram or an exercise electrocardiogram test is performed to assess heart function during rest or exercise. This test may indicate whether the patient is suffering from ACS or SAP and provide meaningful differentiating information. This information is supplemented by the assessment of biomarkers in blood that reflect cardiomyocyte injury/necrosis, especially cardiac troponin T [145]. The next step is cardiac catheterisation, which involves catheter insertion through the femoral artery or radial artery, combined with coronary arteriography, which is an imaging technique. This combination allows the assessment of vessel anatomy, the underlying cause of the symptoms and determines the choice of action.

In order to restore tissue perfusion, occluded or blocked arteries can undergo revascularisation. The most common revascularisation procedure is angioplasty, where a balloon is inflated through a catheter at the target location. The full procedure of inserting a subcutaneous catheter and perform coronary angioplasty is referred to as percutaneous coronary intervention (PCI). To prevent restenosis of the vessel, it is common to insert drug-eluting stents following PCI. Revascularisation can also be performed with coronary artery bypass graft (CABG) surgery, in which perfusion is restored by redirecting the blood around the blockade by sewing on a graft, commonly a vein harvested from the patient's lower extremities.

Medications are available for treating causes and complications of IHD [146]. Thrombolytic drugs are used with the aim of dissolving the blood clot and prevent or reduce myocardial damage. The risk of future thrombosis is reduced by antiplatelet drugs as well as anticoagulants. Angiotensin-converting-enzyme inhibitors cause relaxation of blood vessels and lower blood pressure, thereby reducing cardiac work-load and oxygen demand. Beta blockers are used to prevent cardiac arrhythmias in patients who have experienced AMI. The role of the different blood lipid fractions and the therapeutic potential for manipulating their concentrations has been extensively studied, although not elucidated fully for all fractions. Statins lower LDL-

cholesterol by inhibiting the production of cholesterol in the liver, have a strong and well-documented effect on the risk of IHD and all-cause mortality, and have been administrated to patients since 1994 [147] and increasingly after the late 1990ies [148]. In contrast, more recent clinical trials of medications that raise high density lipoprotein levels or lower triglycerides levels have not been conclusive [130, 146]. Attempts to reduce the potential impact of oxidative stress by the administration of synthetic and vitamin antioxidants, such as vitamin C and E and beta-carotene, have not been successful [130, 149].

Epidemiology

When population aging and growth is taken into account, two million fewer deaths from CVD causes occurred in 2013 than expected from numbers in 1990 [144]. The reduction has been especially profound in high-income countries, with differing estimates to whether it has been caused by changes in exposure to risk factors or improved medical and surgical treatments [150]. Decreases in mortality from IHD in the US between 1980 to 2000 have been equally attributed to treatments (47%) and risk factors (44%) [151]. Lowered cholesterol levels (24%), lowered blood pressure (20%), less tobacco exposure (12%) and less physical inactivity (5%) were deemed as favourable changes in risk factor exposure, while increases in body mass index (8%) and diabetes mellitus prevalence (10%) were unfavourable changes. Nevertheless, IHD continuous to be the leading cause of death for men and women worldwide (~15%) [125]. Survivors of AMI have higher risk of future illness compared to the general population and an estimated one-year mortality of 19% and 26% and five-year mortality of 36% and 47% for men and women ≥45 years of age, respectively [143]. Hence, both the general population and CVD patients could benefit from identification of novel risk factors.

1.3 Vitamin D and cardiovascular disease

1.3.1 History

A one-sided hypothesis

Vitamin D was first mentioned in relation to human CVD in case reports of idiopathic infant hypercalcaemia. The reports succeeded widespread vitamin D fortification and cod liver oil supplementation schemes in Europe and the US starting in the 1920s [152]. Autopsies showed calcifications of blood vessels in the myocardium and heart valves [153] and excessive intake of vitamin D proposed as a causal factor. As most infants exposed to similar intake levels did not develop hypercalcaemia, it was argued that vitamin D could not be the sole factor [153]. Nevertheless, administrative action taken in 1957 resulted in a reduction to half the previous intakes among infants and children in the UK, without aggravating rickets incidence [154]. Contemporary interpretations of the relationship between vitamin D and human CVD was that *"…there is no possibility of ensuring an universal intake of vitamin D that will protect against rickets and eliminate risk of hypercalcaemia"[154]*.

In the 1970s, several case-control studies reported on the potential risk of CVD from a high vitamin D intake. Knox was the first to observe a positive correlation [155], proposing enhanced lead absorption by vitamin D as the causative mechanism [156]. Lindén (1974) observed a positive correlation in Northern Norwegians consuming 100-200 grams of fish liver (62.5-125 µg vitamin D3) two to three times per week and proposed 30 µg as a threshold of increased risk [157]. The causal relationship between vitamin D and CVD suggested by Lindén was criticised due to the retrospective study design, and heavy metals coinciding in the fish liver suggested as the causative factor [158].

Meanwhile, methods for measuring 25OHD concentration were developed and made available to researchers [159, 160]. In 1977 and 1978, two cross-sectional studies from Germany and Denmark reported no difference in 25OHD concentration between AMI patients and controls [161, 162]. In 1979, similar findings were reported in a case-control study nested within the prospective Tromsø Heart Study

[163]. Although a one-sided hypothesis about the potential hazardous effects of vitamin D formed the basis for these studies, a small inverse correlation was in fact indicated.

A two-sided hypothesis

In 1981, Scragg referred to this inverse tendency and hypothesised that vitamin D was in fact protective [164]. His hypothesis was rooted in a phenomenon observed by Hippocrates (460-370 BC), that "*All diseases occur at all seasons of the year, but certain of them are more opt to occur and be exacerbated at certain seasons*" [165]. Sporadic reports on CVD seasonality appeared in the 20th century and intensified from the 1960s [166, 167]. Although no consensus has been reached today, data suggests that both incidence and mortality vary with season [168]. Scragg hypothesised that vitamin D deficiency from limited sun exposure during the winter was the environmental risk factor responsible for the phenomenon, not temperature and respiratory diseases [164]. However, the proposed mechanisms were mostly attributed to calcium, reflecting the contemporary knowledge of vitamin D activity.

In 1990, Scragg returned with a case-control study showing lower 25OHD concentrations in AMI patients [169]. In comparison to earlier studies, this paper is a hallmark of the radical progress that occurred meanwhile in our understanding of vitamin D activity. By 1990, VDR had been identified in rat cardiac muscle [170, 171] and animal studies of diet induced deficiency had revealed a relationship between low vitamin D intake and hypertrophy of the ventricular muscle of the heart [172, 173]. However, only a few epidemiological studies in the form of case-control studies were published in the 17 years that followed the first publication by Scragg [174-177].

Not until 2008 came the first longitudinal observational studies of hard endpoints of CVD, such as AMI, stroke and cause-specific mortality [178-181]. Accompanying these findings are studies of soft endpoints, such as measures of atherosclerosis progression. Parallel to epidemiological observations, a range of

experimental studies *in vitro* and in animal models have paved a mechanistic foundation for understanding how vitamin D activity may influence atherosclerosis and the risk of CVD events.

1.3.2 Mechanistic framework

Distribution of VDR

The prerequisite for activity of vitamin D in atherosclerosis is the expression of VDR in relevant tissue. VDR is expressed in almost all tissue [182] including heart [171], and more specifically in myoblasts [170, 183], endothelial cells [184], smooth muscle cells [185] and immune cells [186, 187]. It has been discussed whether promiscuous antibodies used to detect VDR produced false positive results of VDR distribution in muscle cells [188], but improved release of VDR from DNA by the use of a hyperosmolar lysis buffer did confirm previous studies [189].

Lessions from VDR knockout mice

Knockout mice with either global or cell-specific deletion of VDR display a phenotype of moderate hypertension and left ventricular hypertrophy [190-192], increased thrombogenicity [193] and progression of atherosclerosis [194, 195]. Thus, calcitriol may theoretically have a reverse role and prevent the development of this phenotype. However, it should be emphasised that findings in VDR knockout mice do not prove a role for calcitriol under normal physiological conditions in humans.

A role for calcitriol in preventing cardiac hypertrophy could be mediated by inhibition of myocyte proliferation and differentiation, a mechanisms supported after observing that mice with cardiomyocyte-specific knockout develop cardiac hypertrophy [192]. Macrophage-specific knockout mice had more cholesterol and foam cells in the arterial walls, and the researchers observed macrophage-specific uptake of cholesterol [195]. Thus, calcitriol may prevent uptake of LDL-cholesterol and reduce inflammation by regulating macrophage activity. Aortas dissected from

VDR knockout mice show increased expression of adhesion molecules and proinflammatory cytokines, evident of endothelial activation [194]. Two mechanisms for how calcitriol may influence endothelial activation are suggested from studies of VDR knockout mice, by the regulation of the renin-angiotensin system (RAS) and by a direct effect on endothelial cells.

Endothelial dysfunction can be caused by RAS activity. Renin is the rate-limiting substrate of RAS, which chief effector is angiotensin II [196]. Angiotensin II is a vasoconstrictor that, when formed in the circulation, causes systemic constriction of blood vessels. This leads to increased peripheral resistance to blood flow and a compensatory elevation of blood pressure. Angiotensin II can also be formed by local RAS activity in many tissues [197], including aortic vascular SMCs [198] and macrophages [199]. Under normal circumstances, vasoconstriction by angiotensin II activity is counterbalanced by nitric oxide production in the endothelial monolayer [131, 197]. However, over activity of RAS may provoke endothelial activation and contribute to atherosclerosis, tissue remodelling and thrombogenicity [196, 197].

When noticing that VDR knockout mice urinated more than wild-type counterparts [200], Li discovered upregulation of renin mRNA expression in renal juxtaglomerular cells [191]. This was later also confirmed in cardiomyocytes [190] and macrophages [194]. Downregulation of renin expression by calcitriol occurs through transrepression, in which calcitriol prevents cyclic AMP response element-binding protein (CREB) from interacting with a responsive element on the enhancer region in the renin gene promoter [201]. This prevents CREB from enhancing renin expression. Observational studies report associations of both phenotypic and genotypic determined 25OHD concentrations with blood pressure [202], but a recent meta-analysis of RCTs concluded that vitamin D supplementation is ineffective as an agent for lowering blood pressure and treat hypertension [203]. Nevertheless, calcitriol may prevent endothelial activation by regulating local and systemic RAS activity.

Calcitriol may also prevent endothelial activation by a direct effect on endothelial cells. The expression of the enzyme nitric oxide synthase in endothelial cells from mice with endothelial-specific knockout of VDR was reduced, resulting in less nitric oxide synthesis. [204]. As a result, these mice displayed impaired endothelium-dependent vasorelaxation and responded to blood pressure-inducing angiotensin II administration more adversely.

2. Aims

The overall aims of this project were to study the vitamin D status of cardiovascular patients in Western-Norway and to investigate the longitudinal relationship between vitamin D status and cardiovascular disease.

Specific aims

Paper 1:

In patients with stable angina pectoris and acute coronary syndrome who underwent repeat coronary angiography

- Assess the relationship between 25OHD and progression of atherosclerosis

Paper 2:

In patients with suspected stable angina pectoris

- Assess the seasonal variation in 25OHD concentrations
- Use cosinor models to identify correlates of 25OHD concentrations and the seasonal variation in 25OHD
- Evaluate cosinor modelling as a method to adjust for seasonal variation in 25OHD by predicting repeat measurements of 25OHD

Paper 3:

In patients with suspected stable angina pectoris:

- Assess the relationship between 25OHD and future risk of all-cause and CVD mortality

3. Methods

3.1 Study population

3.1.1 Source population

The source population to this thesis was patients who underwent elective coronary angiography due to suspected coronary artery disease (CAD) either at Haukeland University Hospital (HUS) or Stavanger University Hospital (SUH) roughly between 2000 and 2004. From this source population, patients were recruited to participate in the Bergen Coronary Angiography Cohort (BECAC), the Western Norway B-Vitamin Intervention Trial (WENBIT), and the WENBIT re-angiography sub-study (WENBIT-RA). These studies have overlapping populations and an overview is presented in **figure 3**.

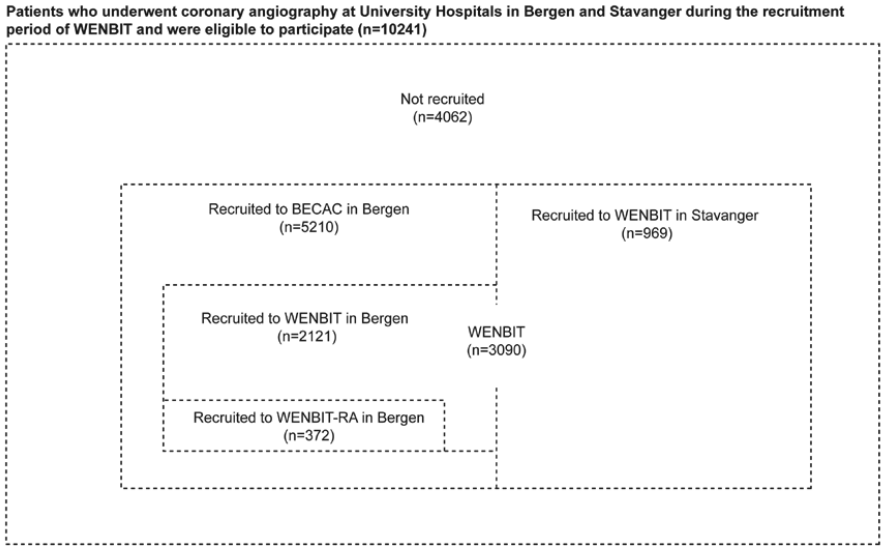

Figure 3. Schematic overview of the source population and participation in overlapping studies.

BECAC was designed as a prospective cohort study and the overall aim was to investigate prognostic markers of cardiovascular events. Patients who underwent elective coronary angiography at HUS between January 2000 and April 2004 were asked to participate.

Among patients who agreed to participate in BECAC, some were asked to participate in WENBIT, a randomised controlled trial (RCT). The primary aim of the trial was to assess the effect of homocysteine-lowering B-vitamin treatment on a composite endpoint of cardiovascular events. WENBIT was a two-centre trial and also recruited patients who underwent angiography at SUH between September 2000 and April 2004. This thesis also included patients who were enrolled in a pilot study (WENBIT-90 substudy) between April 1999 and September 1999. Patients reluctant or unable to attend long-term follow up due to alcohol abuse, mental illness or known malignant disease were not eligible for participation in WENBIT. Within the source population to WENBIT at Stavanger and Bergen, 10241 were eligible for participation. In WENBIT, scheduled follow-up exams were conducted after one month and one year.

The WENBIT-RA was a nested substudy in WENBIT conducted at and among patients recruited at HUS. The aim of the substudy was to assess the effect of the WENBIT treatment on atherosclerosis progression. To ensure recruitment of patients with significant atherosclerosis at baseline, only patient who underwent PCI at the baseline angiography were asked to participate. Substudy participants agreed to undergo a repeat angiography scheduled to coincide with the one-year follow in WENBIT, thus allowing the study of atherosclerosis progression. Most of the patients were recruited to WENBIT-RA at the baseline WENBIT exam, but some were also recruited during the scheduled WENBIT follow-up exams. Patients who underwent angiography during follow-up due to clinical indications were also included, as long as it occurred at least 90 days after the baseline. Patients with a high risk of future complications or a coronary anatomy that could cause difficulties when performing a repeat angiography were excluded.

BECAC, WENBIT and WENBIT-RA were conducted according to the principles of the Helsinki Declaration and approved by the Regional Committee for Medical and Health Research Ethics and the Norwegian Data Inspectorate. All participants gave broad written consent for the use of the collected data in future research.

3.1.2 Study populations

To achieve the aims of this thesis we used data from the source population, including data from BECAC, WENBIT and WENBIT-RA. The study populations in the three papers therefore vary somewhat in respect to cardiovascular phenotype and study site, but mostly, patients with suspected or verified SAP were included (**Figure 4**).

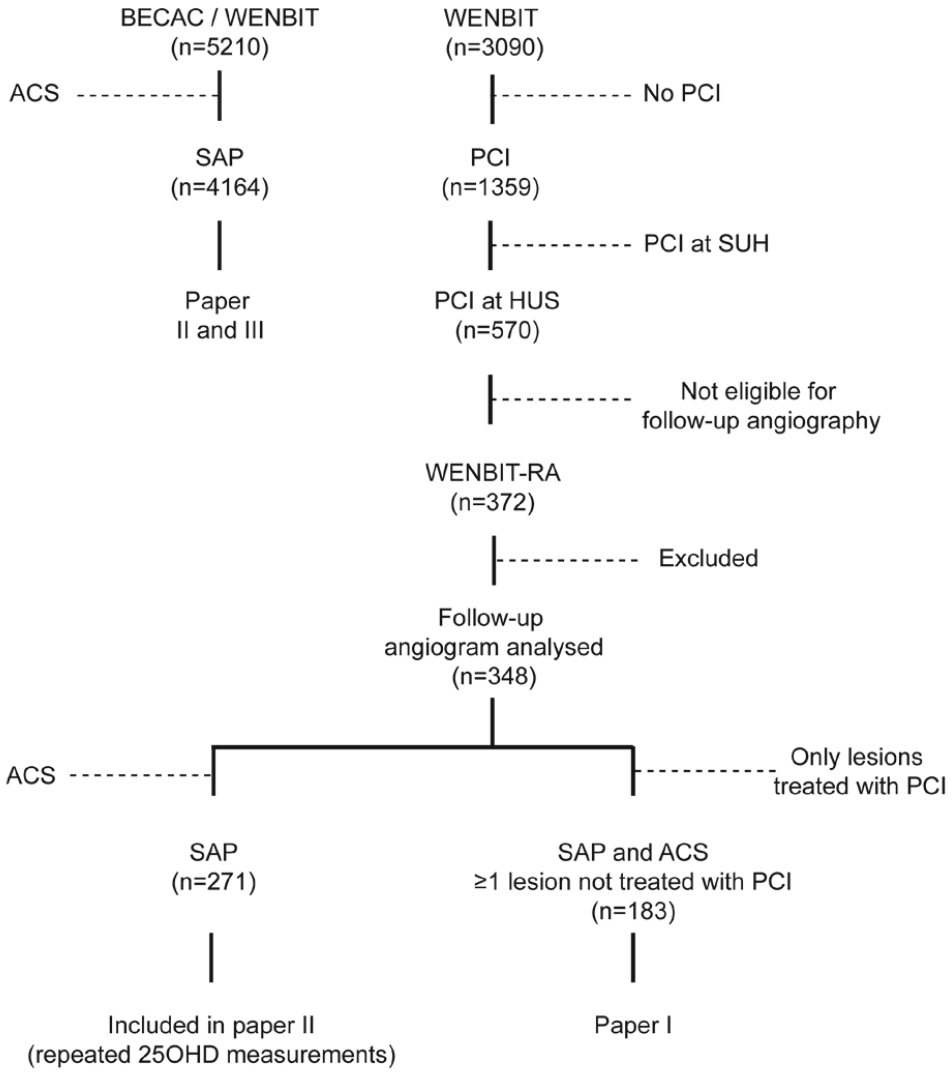

Figure 4. Flow of subjects with suspected SAP and ACS from BECAC, WENBIT and WENBIT-RA to the study populations in paper I, II and III.

When studying atherosclerosis progression in paper I, subjects with verified SAP and ACS from WENBIT-RA were included. The aim was to study progression in lesions not treated with PCI. To include more lesions, angiograms at baseline and follow-up were analysed to identify eligible lesions.

Subjects from BECAC and WENBIT referred to baseline angiography with suspected SAP were used in paper II and III. Hence, individuals with ACS were excluded in these investigations. In paper II, we also aimed to evaluate how accurate cosinor modelling could predict patients' future vitamin D status. For that we used data from participants in WENBIT-RA with verified SAP and repeat measurements of 25OHD available from the follow-up exams.

3.2 Exposures

3.2.1 Clincial and demographical data

Prior to baseline coronary angiography, trained study personnel collected clinical and demographical data, including anthropometrical and blood pressure measurements. Self-administrated questionnaires validated by hospital records assessed medical history and medication. Questionnaires also enquired on vitamin D supplement intake and leisure time physical activity, but not on outdoor activity specifically (**Appendix**).

Body composition was determined by body mass index (BMI, kg/m^2) and defined as normal weight (<25), overweight (25-30) and obesity (≥30). Current smoking was defined as a self-reported smoker or having stopped smoking less than 90 days ago in paper I. In addition to self-report, plasma cotinine concentration above 85 nmol/l (~15 ng/ml) were also used to define current smoking in paper II and III [205]. Estimated glomerular filtration rate (eGFR) was used as a measure of kidney function and calculated with the formula suggested by the Chronic Kidney Disease Epidemiology Collaboration [206]. Diabetes mellitus type I and II were based on pre-

existing diagnoses and combined into one variable. Hypertension was defined by systolic blood pressure (>140 mm Hg) and/or diastolic blood pressure (>90 mm Hg) and/or antihypertensive therapy. Hypercholesterolemia was defined as familial hypercholesterolemia or untreated total serum cholesterol (≥6.5 mmol/l = 251.4 mg/dl). Heart function was assessed by left ventricular ejection fraction (LVEF), as measured by ventriculography during cardiac catheterization for most participants, or by ultrasonic echocardiography. LVEF is a percentage estimate of how much of the blood in the ventricle that is pumped out on each contraction. The extent of CAD was assessed from baseline angiograms and quantified by the number of blood vessels with ≥50% luminal narrowing. This cut-off should be distinguished from the cut-off criteria for eligible lesions in paper I (≥30% luminal narrowing at baseline or follow-up).

3.2.2 Laboratory data

Blood samples were collected by study personnel at baseline and follow-up exams. Blood sampling was conducted non-fasting and prior to coronary angiography at HUS, and fasting and after coronary angiography at SUH. Fresh samples were used to measure routine blood parameters at the study site laboratories while samples for biobanking were stored at -80°C. Serum concentrations of apolipoprotein A-1, apolipoprotein B and C-reactive protein (CRP) were analysed with the Hitachi 917 system (Roche Diagnostics, GmbH, Mannheim, Germany). Plasma cotinine concentrations were measured by liquid chromatography tandem-mass spectrometry (LC-MS/MS) at Bevital AS [207].

3.2.3 Measurement of 25OHD2 and 25OHD3

Analyses of plasma 25OHD2 and 25OHD3 concentrations were performed between 2011 and 2012 at Bevital AS (www.bevital.no) by LC-MS/MS [208]. Baseline samples were measured for all BECAC/WENBIT participants and also follow-up

samples for nested participants in WENBIT-RA. The lower limit of detection and quantification were 3.3 nmol/l and 6.6 nmol/l for both forms, respectively. Measurements below the lower limit of quantification were excluded. In paper I, only 25OHD3 was used to reflect exposure to vitamin D. In paper II and III, measurements of 25OHD2 and 25OHD3 were combined to reflect total 25OHD.

3.3 Endpoints

3.3.1 Atherosclerosis progression

Atherosclerosis progression was used as endpoint in paper I and assessed by coronary angiography at baseline and follow-up. Quantitative coronary angiography (QCA) analysis was performed on 16 coronary artery segments by two trained technicians and supervised by a cardiologist. The software used was Quantcor QCA, CAAS II, version 5.0, Pie Medical Imaging, Maastricht, The Netherlands. A prerequisite for studying lesion progression is adequate visualisation of the lesion on both angiograms. In the current study, a stenotic lesion was eligible for inclusion if at baseline or follow-up had a reference diameter ≥2 mm and resulted in ≥30% diameter reduction compared to a healthy section of the same coronary artery segment. Reanalysis by both observers was performed if they disagreed upon the eligibility of a specific lesion. Lesions were projected at the end of a diastole and, in case of differing projections, the one showing a more severe stenosis chosen. Quality control of QCA analyses ensured that the report referred to the correct stenosis, segment and angiogram.

From QCA analyses, minimum lumen diameter (MLD) and diameter stenosis (DS) of eligible lesions were measured and used as endpoints in paper I (**Figure 5**). Inter-rater reliability for the measurement of DS was assessed at baseline and follow-up. Reanalysis was performed for the top 10% with the largest inter-rater difference.

3.3.2 All-cause and cardiovascular mortality

The risks of all-cause and cardiovascular disease mortality were used as endpoints in paper III. CVD mortality included code I00 to I99 and R96 from the International Statistical Classification of Diseases, 10th revision (ICD-10). Information was obtained from the Cause of Death Registry at Statistics Norway (www.ssb.no) and recorded until January 1st, 2013.

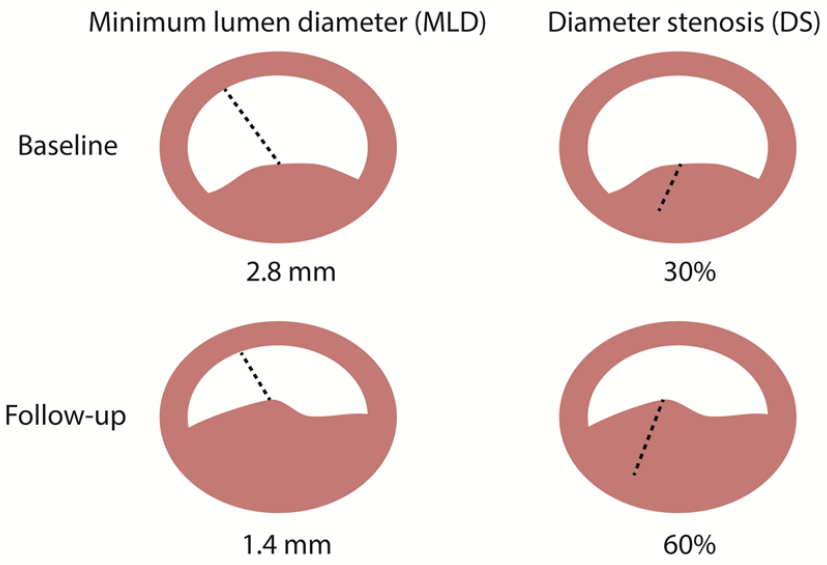

Figure 5. Endpoints derived from repeat coronary angiography for the study of atherosclerosis progression in paper I.

3.4 Statistical analysis

Descriptive statistics of the study populations at baseline were presented with mean and standard deviation (SD) for continuous variables and with median and inter quartile range (IQR) for some biochemical variables in paper I. Categorical variables were presented with counts and percentages. When testing for similarities at baseline, analysis of variance (ANOVA) and the chi-square test were used for continuous and categorical variables, respectively. Probability values were two-sided and considered

statistically significant if the probability of observing a difference due to chance was less than 5%. No adjustments were made to account for multiple comparisons.

3.4.1 Statistical modelling

Regression refers to the process of fitting models to observed data in order to capture meaningful relationships between variables, such as the exposure and outcome of interest. The choice of model depends on the nature of the relationship, whether it is linear or non-linear. Furthermore, different models require different mathematical methods in order to be fitted to the data. These methods requires assumptions that should be met in order for the regression to be accurate and for the results to be generalised to the target population [209].

Linear regression

The conditional probability distribution P(Y | X = x) is the probability distribution of the response variable (Y) when the exposure variable (X) takes on a particular value (x) [210]. It may be expressed as a function, i.e. 25OHD concentrations as a linear function of dietary intakes of vitamin D and/or other exposure variables. Linear regression, which is one of the most frequently used methods in epidemiological research, models the mean of the conditional probability distribution. It uses the least squares approach to minimise the distances from the fitted mean line to the observed values, thus minimising the residuals. Because it models the mean of the conditional probability distribution, results will be more accurate if this distribution is normally distributed. Results will also be more accurate if observed values are divided in equal proportions above and below the mean line for all values of the exposure (X), referred to as homogeneity or homoscedasticity. Normality and homoscedasticity are therefore referred to as required assumptions justifying the use of linear regression. During model validation, researchers inspect the residuals and may observe heterogeneity and non-normality [209]. This is not necessarily a reason to reject the model and the results altogether. A linear tendency in the data may still be captured despite some degree of violations of the model assumptions.

Quantile regression

If the conditional probability distribution is non-linear, i.e. U-shaped, linear regression may be misleading. It can for instance result in a null relationship between an outcome and exposure, despite strong inverse linear tendencies at both the higher and lower end of the exposure distribution. This is relevant when analysing the effect of micronutrient status, as they may cause disease if status is too low (deficiency) and cause disease if too high (acute toxicity or chronically elevated). Thus a health outcome may be adversely affected both by low and high values of the exposure. If we want to estimate another quantile of the distribution, rather than the conditional mean, quantile regression is an optional method that performs regression by minimising the sum of absolute residuals [211]. Quantile regression may be used to assess the conditional median, the lowest or highest part (i.e. the 10^{th} or 90^{th} percentile), or any other quantile. In contrast to linear regression, quantile regression does not require normalisation of skewed variables before analysis in order to improve accuracy. It is important to distinguish quantile regression of the conditional distribution from stratification directly on the distribution of an exposure variable, as they are incomparable [210]. Because of its utility, quantile regression has been applied to estimate reference curves for growth charts for children as a function of age, sex and other relevant variables that may influence growth [212].

Mixed effects modelling

In nutrition, we are interested in elucidating the effects of diet and nutrients on disease risk. The effect of a nutrient varies from person to person due to randomness. Randomness can for instance be the effect of genetic variation. If we obtain a large representative sample of the target population, this minimises the risk of the randomness being unequally distributed, and allows us to elucidate fixed effects of nutrients, which can be generalised to the target population.

In the analysis of atherosclerosis progression in the first paper, we have data with clustered measurements: some subjects were represented by several atherosclerotic lesions, while some were only represented by one. This we refer to as within-subject clustering of lesions. The probability of any subject taking the place of

each atherosclerotic lesion was therefore not random. Hence, the random effects in the target populations were not distributed equally for each lesion in the analysis.

To deal with this issue in statistical analysis, it was necessary to introduce a level of randomness for this random variation to occur. This is referred to as multilevel analysis and can be achieved by the use of mixed effects modelling [209]. In a mixed model, a categorical variable representing the clustering can be modelled as a random effect term to account for the specific cluster an observation belongs to. In our case the clustering variable was "subject", meaning all subjects in the study have different intercept values.

In the first paper, we used mixed effects modelling to account for within-subject clustering of lesions in combination with conditional median regression to deal with skewed variables and outliers that could be more influential in an analysis with fewer subjects. The analysis was performed with the *lqmm* package version 1.02 for R statistical software [213].

3.4.2 Modelling and adjusting for seasonal variation

Assuming that 25OHD concentrations vary throughout the year in a sinusoidal pattern (Figure 6), we applied cosinor models to analyse seasonality [214]. Cosinor modelling transforms the time variable into a sine and cosine term and then regresses the observations of 25OHD concentrations onto these terms using linear regression. These two terms represent the sinusoidal pattern in an oscillating curve with amplitude and phase that can be calculated directly from the regression coefficients. The amplitude is an estimate of seasonal variation and defined as the distance from the mean to the highest or lowest value of the curve (y-axis), while phase is the value of the time variable (x-axis) when the curve is at its highest location (peak). The use of these parameters allowed the study of seasonal variation in 25OHD concentrations in paper II.

Interaction terms between covariates and sine and cosine terms enable assessment of whether the sinusoidal pattern differs for covariate levels [215]. This allowed for the identification of potential determinants of the mean annual 25OHD concentration and the seasonal variation in 25OHD concentration in paper II.

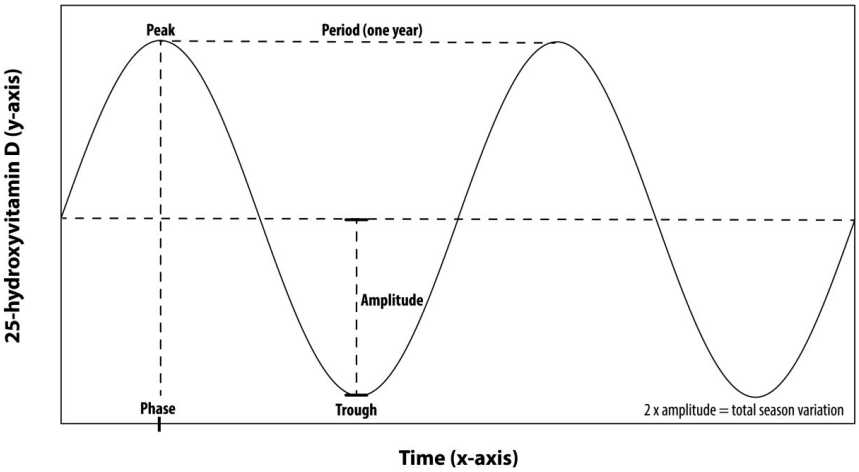

Figure 6. The relationship between 25-hydroxyvitamin D concentrations (y-axis) and time (x-axis) displayed as a sinusoidal pattern in an oscillating curve with amplitude and phase.

Assuming that the seasonal variation of the study population is representative for all participants, and knowing a patient's 25OHD concentration at a particular month, his or hers concentration at a later point can be predicted. By comparing prediction to true follow-up measurements collected in WENBIT-RA, it was possible to assess the accuracy of cosinor models in paper II.

Each participant's baseline measurement of 25OHD can be adjusted for seasonal variation in the study sample by using cosinor models. To the mean annual 25OHD concentration of the study population, deviations from the sine curve are added for each subject – yielding an estimate of each subject's yearly average. This is useful when including 25OHD as an exposure variable in statistical models, when

measurements are spread throughout the year for different subjects. This approach was used in the third paper, prior to descriptive and inferential statistical analysis. In the first paper, adjustment for season was performed by including season as a four-level categorical variable in inferential statistical analysis. In order to reflect months during which sun exposure results in eligible or negligible synthesis of vitamin D, all three papers defined seasons as from January through March, April through June, July through September, and October through December.

3.4.3 Survival analysis

In paper III, Kaplan-Meier curves were used to present the unadjusted cumulative survival function according to 25OHD concentrations for all-cause and CVD mortality, while Cox-proportional hazard models were used to assess hazard ratios (HRs). Schoenfeld residuals were checked to test the assumption of proportional hazards. The functional forms of the relationship between 25OHD concentrations and the adjusted HRs of these endpoints were assessed by modelling 25OHD concentrations with a penalised smoothing spline [216] in Cox models, using the default algorithm in the *survival* package version 2.37-4 for R statistical software [217].

3.4.4 Threshold analysis

The functional relationships between 25OHD and all-cause and CVD mortality were assessed for candidate thresholds, defined as the most optimal division of participants with a higher and lower risk of the outcome [218]. Wald statistics were extracted and ranked from adjusted Cox models in which the 25OHD concentration was modelled as a dichotomous variable for incremental increases of the 25OHD distribution [219]. The cut-off that resulted in the highest Wald statistics was proposed as a candidate threshold. This approach was used because it had a clear definition and a transparent and reproducible method.

4. Summary of publications

4.1 Study population

Table 1 shows baseline characteristics of the study population. Data from participants with suspected SAP from BECAC/WENBIT (n=4116 and n=4114, respectively) were used in the second and third paper. Data from WENBIT-RA were used in the first and second paper from participants with SAP and ACS (n=183) and participants with SAP (n=271), respectively. The lower number of participants in the first paper was due to additional lesion inclusion criteria.

As a result of study design, participants in WENBIT-RA differed from participants in BECAC/WENBIT with suspected SAP. All were enrolled in Bergen, had significant CAD and underwent PCI at baseline (not shown). In addition, the proportion of males, 25OHD concentrations, and use of medications were notably higher ($P < 0.05$). Within WENBIT-RA, participants with ACS (n=46) had more systemic inflammation ($P < 0.05$), as judged by serum CRP, than patients with SAP (n=137).

Table 1. Baseline characteristics of the study populations

Characteristics	BECAC/WENBIT SAP (n=4114)	WENBIT-RA SAP (n=271)	WENBIT-RA SAP and ACS (n=183)
Demography and lifestyle			
Age (years)	61.7 ± 10.4	60.8 ± 10.2	60.3 ± 10.0
Male sex, n (%)	2959 (71.9)	218 (80.4)	154 (84.2)
Study site, n (%)			
Bergen	3367 (81.8)	271 (100)	183
Stavanger	747 (18.2)	-	-
Regular consumption of cod liver oil, n (%)	1190 (33.6)	98 (37.5)	62 (40.5)
Smoking, n (%)	1302 (31.6)	82 (30.3)	67 (36.6)
Physical activity, ≥2 hours per week, n (%)	2135 (68.8)	185 (70.3)	-
Clinical characteristics			
Body mass index (kg/m^2)	26.8 ± 4.0	26.9 ± 3.4	27.1 ± 3.3
Systolic blood pressure, mm Hg	141 ± 20.8	142 ± 21.6	143 ± 22.6
Left ventricular ejection fraction, (%)	64.0 ± 11.4	65.4 ± 8.2	63.5 ± 9.4
Extent of coronary artery disease, n(%)			
No stenotic vessels	1029 (25.1)	-	-
One vessel	947 (23.1)	137 (50.6)	77 (42.1)
≥Two vessels	2121 (51.8)	134 (49.4)	106 (57.9)
Medical history and cardiovascular risk factors			
Diabetes, n (%)	490 (11.9)	29 (10.7)	17 (9.3)
Hypertension, n (%)	1927 (46.8)	121 (44.6)	79 (43.2)
Hypercholesterolemia, n (%)	2227 (57.9)	162 (62.8)	108 (62.4)
Previous AMI, n (%)	1657 (40.3)	99 (36.5)	50 (27.3)

Previous PCI, n (%)	790 (19.2)	59 (21.8)	34 (18.6)
Previous CABG, n (%)	473 (11.5)	10 (3.7)	6 (3.3)
Laboratory			
25OHD, nmol/l	59.4 ± 20.3	65.9 ± 21.4	64.1 ± 20.9
eGFR, ml/min per 1.73^2	87.8 ± 17.2	90.9 ± 14.6	92.4 ± 13.1
C-reactive protein, mg/l	3.70 ± 7.19	2.73 ± 4.2	8.20 ± 17.1
CRP ≥10 mg/l, n (%)	278 (6.8)	13 (4.8)	34 (18.6)
S-Low-density lipoprotein cholesterol, mmol/l	3.10 ± 1.03	3.01 ± 0.86	3.12 ± 0.86
S-High-density lipoprotein cholesterol, mmol/l	1.29 ± 0.38	1.29 ± 0.33	1.23 ± 0.34
S-Apolipoprotein B100, g/l	0.90 ± 0.25	0.86 ± 0.22	0.90 ± 0.24
S-Apolipoprotein A1, g/l	1.32 ± 0.27	1.34 ± 0.24	1.29 ± 0.26
S-Triglycerides, mmol/l	1.78 ± 1.22	1.83 ± 1.96	1.88 ± 2.22
Medication at discharge, n (%)			
Statin therapy	2982 (72.4)	255 (94.1)	171 (93.4)
β-Adrenergic receptor antagonists	2982 (72.5)	207 (76.4)	141 (77.0)
ACE inhibitors	852 (20.7)	35 (12.9)	27 (14.8)
Acetylsalicylic acid	3355 (81.6)	264 (97.4)	178 (97.3)
ADP receptor antagonists	621 (15.1)	145 (53.5)	120 (65.6)

Continuous variables are presented as mean ± standard deviation and categorical variables as numbers (n) and percentages (%). SAP; stable angina pectoris, ACS; acute coronary syndrome, AMI; acute myocardial infarction, PCI; percutaneous coronary intervention, CABG; coronary artery bypass graft surgery, 25OHD; 25-hydroxyvitamin D2 and D3 combined, eGFR; estimated glomerular filtration rate, CRP; C-reactive protein, ACE inhibitors; angiotensin-converting enzyme inhibitors ADP; adenosine 5'-diphosphate receptor antagonists.

4.2 Summary of individual papers

4.2.1 Paper I

Vitamin D status was not associated with 'one year' progression of coronary artery disease, assessed by coronary angiography in statin-treated patients.

In the first paper, our aim was to assess the relationship between vitamin D status, represented by baseline 25OHD3 concentrations, and atherosclerosis progression. Atherosclerosis progression was assessed by QCA analysis from repeat coronary angiography and MLD and DS chosen as study endpoints. From 348 eligible individuals, we used data from 183 participants with at least one non-PCI treated lesion that fulfilled lesion inclusion criteria. The remaining 165 subjects did not have eligible lesions and were excluded. From the 183 participants, we identified in total 309 eligible lesions for statistical analysis. Accordingly, 106 participants (58%) were represented by more than one lesion.

During a mean ± SD follow-up time of 302 ± 79 days, we observed significant atherosclerosis progression, as evident from a decrease in MLD from 1.92 ± 0.55 to 1.75 ± 0.51 mm and increase in DS from 37.6 ± 9.64 to 42.0 ±10.4 percentage points ($P < 0.001$). The baseline median (interquartile range) 25OHD3 concentration was 63.9 (48.1-78.5) nmol/l. We observed no linear association between 25OHD concentrations and MLD or DS ($P > 0.05$) overall nor when investigating the relationship in patients with SAP and ACS separately. We concluded that there was no association between vitamin D status and atherosclerosis progression in our study population.

4.2.2 Paper II

Cosinor modelling of seasonal variation in 25-hydroxyvitamin D concentrations in cardiovascular patients in Norway.

The aims of the second paper were to use cosinor models to study vitamin D status by identifying correlates of vitamin D status and the seasonal variation, as well as to evaluate cosinor modelling as an approach to adjust for seasonal variation. We fitted cosinor models to baseline measurements of the combined sum of 25OHD2 and 25OHD3 for 4116 subjects with suspected SAP. The annual mean and the total seasonal variation of 25OHD were estimated to 59.6 nmol/l and 15.8 nmol/l, respectively. Hence, seasonal variation equalled to 26.5% of the annual mean and resulted in variation in vitamin D status throughout the calendar year. The curve trough occurred between January and February and the peak between July and August.

With $P < 0.05$ as level of significance and adjustments for age, gender, study site, BMI and smoking (n=4006), we observed that a higher annual mean 25OHD concentration associated with older age (>62 years), physical activity and vitamin D supplement consumption, while lower annual mean associated with female gender, study site, smoking, adiposity and diabetes mellitus. The most profound differences were observed when comparing those who reported consuming vitamin D supplements regularly or daily (33.6% of the study sample) with non-supplement users (mean difference (95% CI): 8.4 (7.1, 9.6) nmol/l) and when comparing obese with normal weight subjects (-8.6 (-10.3, -6.9) nmol/l). Patients aged 62 years or older displayed less seasonal variation than patients younger than 62 years, 12.3 nmol/l and 19 nmol/l (P for difference = 0.025), respectively.

To evaluate cosinor modelling as an approach to adjust for seasonal variation, we used predictive performance as a surrogate measurement. Predictive performance was assessed from estimating how accurate cosinor models fitted to baseline measurements of 25OHD predicted follow-up measurements from 271 nested subjects. The nested subjects had either one or two follow-up measurements

available, thus 528 predictions were possible. For reference, we compared predictive performance with a linear regression model adjusted for season with a dummy variable, which is common in epidemiology, and the approach we used to adjust for seasonality in the first paper. Performance was also compared to "doing nothing", and we simply carried forward the baseline value as a prediction. We observed that cosinor models predicted most accurately, while no difference was observed between linear regression and simply carrying forward baseline values. In conclusion, we observed that seasonal variation in 25OHD was pronounced in Western-Norway, resulted in a higher frequency of vitamin insufficiency during the winter, associated with age, and was more accurately adjusted for by cosinor models. Consumption of vitamin D supplements, mainly cod liver oil, and obesity most strongly associated with 25OHD concentrations.

4.2.3 Paper III

Plasma 25-hydroxyvitamin D concentrations and all-cause and cardiovascular disease mortality among Caucasian patients with suspected stable angina pectoris.

In the third paper, the aim was to assess the relationships of 25OHD with all-cause and CVD mortality. The combined concentration of 25OHD2 and 25OHD3 were adjusted for seasonality with an unadjusted cosinor model and used to reflect exposure to vitamin D for 4114 participants with suspected SAP. 25OHD was modelled as a continuous variable and as quartiles in Cox models adjusted for age, gender, smoking, BMI, eGFR and systolic blood pressure.

During a mean follow-up time of 11.9±3.0 years, 895 (21.8%) deaths occurred in total and 407 (9.9%) due to CVD causes. For each 10 nmol/l incremental increase in 25OHD, adjusted HRs and 95% confidence interval (CI) were 0.91 (0.88-0.95) and 0.90 (0.85, 0.95) for all-cause and CVD mortality, respectively. When the lowest 25OHD quartile was used as reference, HRs in the second, third and fourth quartiles

were 0.64 (0.54, 0.77), 0.56 (0.46, 0.67) and 0.56 (0.46, 0.67) for all-cause and 0.70 (0.53, 0.91), 0.60 (0.45, 0.79) and 0.57 (0.43, 0.75) for CVD mortality, respectively.

We investigated the functional relationship of 25OHD with all-cause and CVD mortality and analysed for candidate thresholds, defined as the most statistical significant division of individuals into categories of higher and lower risk of the endpoint. The suggested thresholds for both endpoints were similar, with 42.5 and 40.9 nmol/l for all-cause and CVD mortality, respectively. A second threshold at 100 nmol/l was also indicated by a visual inspection of the functional relationships, which appeared U or J-shaped, and the threshold analysis. In comparison to those with concentrations 42.5-100 nmol/l (n=3214), adjusted HRs (95% CI) of all-cause and CVD mortality were 1.79 (1.31, 2.46) and 1.44 (0.87, 2.37) among subjects with 25OHD >100 nmol/l (n=121) and 1.94 (1.66, 2.27) and 1.87 (1.49, 2.36) among subjects <42.5 nmol (n=779), respectively. From these findings, we concluded that 25OHD associated with risks of all-cause and CVD mortality in patients with suspected SAP and that a U-shaped or J-shaped relationship was present between 25OHD and all-cause mortality.

5. General discussion

5.1 Methodological considerations

5.1.1 Study design

Cross-sectional and longitudinal observational data were used in this thesis. Cross-sectional studies provide valuable data for identifying novel relationships; however, it is not possible to establish whether the exposure preceded the outcome in time. This is known as the temporality criterion for evaluating whether relationships are causal [220]. Temporality can be ensured with longitudinal study designs by measuring the exposure before the outcome occurs. Observational studies have the advantage that large representative samples may be obtained and many clinical events recorded. Consequently, they have statistical power to identify and estimate the magnitude of novel relationships throughout the length of the conditional probability distribution, relationships that might be the results of beneficial or adverse effects of the exposure [221]. True relationships in nature may be accurately quantified in longitudinal observational studies and a strong case made in favour of causality [220].

However, errors and bias related to study design may reduce the ability to identify true relationships and increase the risk of observing spurious relationships. For instance, when categorising the study population according to the exposure of interest, the groups may differ for relevant prognostic factors. If associated with the exposure and causally related to the outcome, they are referred to as third variables or confounders of the relationship between the exposure and outcome. Confounding is not present if the third variable is causally related to both the exposure and the outcome, as then it is in the causal pathway. In order to elucidate the true relationship between the exposure of interest and the outcome, and reduce the risk of spurious observations, the effects of confounders needs to be adjusted for by statistical methods [222]. However, dietary intake is associated with a host of social and behavioural factors which are difficult or impossible to accurately assess and thus

difficult to adjust for [221]. As a result, observed relationships between diet and health outcomes are often subjected to residual confounding.

In order to obtain an equal distribution of confounding factors between exposure groups and thereby limit the risk of residual confounding, researchers can randomise individuals into groups by chance and then administrate the exposure of interest in varying doses. This is known as a randomised trial. In order for the treatment to be allocated without bias, the treatment must be concealed from the participants in order to prevent them from behaving differently. This is known as blinding. In comparison to foods which are nearby impossible to blindly allocate, micronutrients and antioxidants may be distributed as indistinguishable pills and are suitable for randomised controlled trials.

On several occasions during the last 30 years, strong and robust relationships between micronutrients and health outcomes have been consistently reported from longitudinal observational studies [223]. In order to acquire necessary data for evaluating causality, large RCTs were subsequently conducted. Findings in trials were not consistent with previous findings in observational studies. Consequently, the result from observational studies were discredited as wrong and believed to be the result of residual confounding [222]. When performing observational research, it is therefore important to interpret results in light of the strengths and limitations of the available data.

The use of RCT data as observational data

In the observational studies included in this thesis, we used data from a prospective observational cohort performed at HUS in Bergen (BECAC). The majority in BECAC also participated in WENBIT, a two-centre RCT. WENBIT data from patients recruited at SUH in Stavanger was also used. Trial data was considered eligible for observational research because the homocysteine-lowering effect of the B-vitamin intervention did neither affect the risk of CVD events or mortality in comparison to participants who received placebo [224] nor the progression of

atherosclerosis [225]. Another assumption underlying the use of trial data is that no interaction between vitamin D metabolism and one-carbon metabolism could influence the results, for which a short summary of available evidence is provided below.

Cystathionine is an intermediate metabolite in the transsulfuration pathway which generates cysteine from homocysteine. Cystathionine beta synthase (CBS) is the enzyme responsible for converting homocysteine to cystathionine. Global CBS knockout effectively prevents transsulfuration of homocysteine and results in hyperhomocysteinemia [226]. Hypervitaminosis D was reported to result in secondary cystathioninuria, indicating that vitamin D activity may increase transsulfuration of homocysteine [227]. This effect may be mediated by an intragenic VDRE on the CBS gene, as demonstrated by induced CBS transcription upon calcitriol stimulation in various cell lines *in vitro* and abolished transcription of CBS in murine osteoblast with VDR knockout [228]. Hypothetically, a reduction in vitamin D status could therefore contribute to elevated homocysteine by reducing clearance through the transsulfuration pathway. However, VDR knockout mice did not display any difference in circulating homocysteine concentrations [228] and no inverse correlation was observed with 25OHD concentrations in the study population (data not shown). If the transcriptional regulation of VDR on CBS is physiologically relevant, it is probably not through a systemic influence on homocysteine, but occurring locally within specific cells.

5.1.2 Study population

The source population were patients in Western-Norway referred to coronary angiography for suspected CAD and the target population might be considered as comparable to patients in other countries around the world. The degree in which the results in the study population may be generalised to the target population is referred to as external validity [229]. To evaluate external validity, it is relevant to assess how representative the study population is of the source and target population. There was

not sufficient capacity to screen all eligible (n=10241) patients in the source population and we are thus unable to compare it to the study population. Participants in WENBIT were, however, comparable to contemporary samples of patients with verified CAD in Europe concerning age, gender distribution and smoking habits [224]. Individuals with heavy alcohol consumption and mental illnesses, which were exclusion criteria, are not represented in the study population.

Agreeing to participate in a study could reflect personal traits such as willingness to follow doctor's orders and dietary guidelines, both relevant to the patient's health and risk of future illness. This is known as the *healthy volunteer effect* [229]. If present, it may result in the study sample differing from the source population and reduce external validity. As this was a prospective observational study with data collection only at baseline for all participants, and in conjunction with elective coronary angiography, participation did not require too much effort for the participants. The *healthy volunteer effect* is probably small in the data from BECAC, but could be more present in the data from patients recruited in Stavanger, as they agreed to participate in an RCT, which requires more effort. The effect could also be more present in data from participants in WENBIT-RA, who willingly agreed to undergo repeat angiography. WEBIT-RA patients with SAP had a 25OHD concentration of 64.1 nmol/l, while SAP patients overall had 59.7 nmol/l. Minor difference in the timing of enrolment could explain part of this difference, although we did not observe any patterns. We did observe some differences in physical activity and cod liver oil consumption, hence, a small *healthy volunteer effect* could be present in this substudy data.

An alternative explanation to the higher 25OHD concentrations in WENBIT-RA, was the timing of inclusion to the substudy. Inclusion to WENBIT-RA occurred primarily at the baseline WENBIT exam, but some patients were also included at the 1-month and 1-year WENBIT follow-up exams. Although this resulted in a larger sample size, patients recruited to the substudy at follow-up may have been recruited from a pool of patients with higher 25OHD concentration than at baseline. This could be the result if those who died or experienced events in the intermittent period had

lower 25OHD than survivors. This is known as the *survivor phenomenon* [229]. This phenomenon is relevant, as we observed relationships with all-cause and CVD mortality during follow-up.

5.1.3 Data collection

Random and systematic errors can occur during data collection. In general, systematic errors bias the available information and result in misclassification of subjects according to exposures, covariates and outcomes [229]. Measurements are seldom perfect and too much random error may cause sample distributions that are more extreme than the true distribution. Depending on the research question, a certain degree of inaccuracy from random error is acceptable. As we ranked our individuals according to 25OHD concentration, a higher accuracy was required than if we were to compare the mean of two groups.

A concept known as *regression to the mean*, is that a single measurement is more extreme estimate of the average or true value, while repeated measurements jointly provide a more accurate estimate [229]. For example, a study where repeat measurements of 25OHD spaced 12 months apart were available, follow-up measurements were ~8 nmol/l higher than baseline concentrations among subjects with baseline concentrations <40 nmol/ and ~8 nmol/l lower for those with baseline >75 nmol/l [230]. When the assessment of exposures in longitudinal studies is limited to single inaccurate measurements that may change during follow-up, the consequent loss of ability to detect a true association with an outcome, also known as internal validity, is referred to as *regression dilution bias* [231]. As we relied on single measurements of 25OHD to reflect vitamin D status in the studies of mortality and atherosclerosis progression, a potential *regression dilution bias* may have influenced the internal validity of these studies.

Measurement of 25OHD

Plasma samples for biobanking were immediately frozen at -80°C in the time period between 1999 and 2004 and analysed for 25OHD between 2011 and 2012. The stability of deep-frozen plasma samples of 25OHD have been demonstrated after repeated freeze and thawing cycles [232]. Analysis was performed by Bevital, a laboratory which participates and is certified by the Vitamin D External Quality Assessment Scheme (www.deqas.org). Certification requires reliable measurements of 25OHD2 and 25OHD3 from 5 quarterly distributed samples of unprocessed human serum with unknown concentrations. Hence, we argue that there is little evidence for random errors in the measurement of 25OHD.

Blood sampling

Blood sampling was conducted differently at the two study sites. In Bergen, blood samples were taken non-fasting and prior to coronary angiography. In Stavanger, samples were taken after angiography and thereby also after at least 8 hours of fasting. In patients from Stavanger, we observe ~5 nmol/l lower 25OHD concentration than in participants from Bergen, which could be explained by somewhat lower intake of vitamin D supplements and physical activity in patients from Stavanger (data not shown), although there was no *a priori* reason to assume that participants from the two study sites should differ in this matter. Circadian variation in 25OHD concentrations in healthy and diseased populations has been described, with a nadir at early morning and peak around noon [233, 234]. This could be a potential source of systematic error if blood sampling is not standardised.

Lastly, the difference may be explained by the timing of blood sampling in relation to cardiac catheterisation. After knee arthroplasty, a post-operative reduction of 25OHD of up to 40% in comparison to preoperative concentrations has been observed [235]. A marked reduction was observed after 6 to 12 hours and a lasting reduction of up to several months. The reduction was not fully explained by reduction of vitamin D binding proteins (DBP and albumin) [235]. Other mechanisms include

increased uptake and metabolism of 25OHD by macrophages [52] or redistribution of fluids due to increased endothelial permeability [236]. The systemic inflammatory response, as measured by CRP, is strongly associated with 25OHD concentrations in observational studies [237]. However, there was no correlation between 25OHD and CRP after knee arthroplasty [235] and we did not observed any difference in CRP between participants sampled in Bergen and Stavanger (data not shown). However, it is possible that a reduction in 25OHD after cardiac catheterisation and related procedures could have occurred prior to or without an increase in CRP and thus resulted in the observed difference.

Seasonality

This thesis is in part dedicated to the issue of how to deal with seasonality, a source of systematic error and misclassification when assessing vitamin D status, as well as a confounder of the relationship with CVD and mortality. In paper II, we evaluated three possible approaches; not adjusting for seasonality, linear regression with dummy variables for season, and cosinor modelling. We observed that cosinor modelling performed more accurately than the other two, who were indistinguishable in terms of accuracy. When assessing atherosclerosis progression in paper I, we used linear regression adjusted with dummy variable for season to deal with seasonality. After conducting the second paper, we logically applied cosinor modelling in the third paper when assessing clinical events. If we assume that the findings in the second paper are valid, we must concede that the probability of misclassification due to seasonality was higher in the assessment of atherosclerosis progression than for all-cause and CVD mortality.

Covariates

In this study we used only observational data and thus blinding was not an issue. All participants who underwent cardiac catheterisation at the two hospital sites in the study period were required to fill out a self-administrated questionnaire (**Appendix**).

From this questionnaire, we used data regarding dietary supplements, physical activity levels, smoking, previous and current medical conditions, medications and alcohol consumption. As these are based on self-report, they are subjected to several sources of bias. First of all, recalling is difficult by itself. Therefore, information regarding medical history was controlled against hospital records by trained personnel. Due to symptoms and visits to the general practitioner, they may also have made lifestyle changes recently that could distort or make recall more complicated. A common phenomenon for humans is to underreport or overreport in order to appear more healthy or less unhealthy to others and self. We may speculate that patients referred to coronary angiography are even more aware of the fact that the questionnaire assesses CVD risk factors. The information regarding dietary supplement data of vitamin D and physical activity are likely to contain some overreport, as it is socially desirable to take care of your body, and it can therefore be more difficult to accurately adjust for their confounding effects. However, there was much less focus on potential health effects of vitamin D at the time of recruitment (1999-2004) compared to now. To reduce bias in the assessment of smoking, we complemented the subjective measurement with an objective measurement based on cotinine concentrations, which has a good correlation with nicotine intake from tobacco [205]. In order to prevent underreporting of weight, anthropometrical measurements were performed by trained study personnel and not based on self-report.

Atherosclerosis progression

From invasive imaging of atherosclerosis by coronary angiography, stenosis size and the extent of luminal intrusion may be assessed. Other invasive and non-invasive methods to visualise atherosclerosis are available [238]. Non-invasive measurements require fewer resources and have therefore more often been used as endpoints in observational studies of 25OHD. A common non-invasive approach is to use ultrasound on the carotid artery to visualise plaque volume, plaque thickness and intima-media thickness, and another to measure coronary artery calcium by electron

beam computed tomography, a correlate of plaque burden. In general, non-invasive imaging does not provide the same accuracy and information as invasive techniques [238]. Even more accurate than coronary angiography is intravascular ultrasound, an invasive imaging technique that is performed in conjunction with cardiac catheterisation. It enables the assessment of lesion size and characteristics within the arterial wall, such as the thickness of the fibrous cap, which aforementioned is more predictive of plaque vulnerable than lesion size alone [140]. A limitation of our study is that, apart from assessing stenosis progression, we did not assess additional plaque characteristics relevant to vulnerability of rupture.

Registry data

Information about the time and cause of death was gathered from the Cause of Death registry at Statistics Norway and the Western Norway Cardiovascular Registry and controlled for by medical records at the hospitals in Western Norway. Based on this information, two members of the endpoint committee who were blinded to baseline data considered whether the information was sufficient to be registered as an endpoint. All registry data share the potential error of missing data, however, most data should be missing at random. The strength of all-cause mortality as an endpoint is that it does not suffer from misclassification, which is a major source of information bias when using registry data. Of 90% of deaths occurring in Norway, information on cause of death is limited to certificates filled out by on-site medical doctors, and only 10% are supplemented by autopsies [239]. When autopsies are performed, the underlying cause of death is changed in 61% of the cases, including a 32% reclassification between major ICD-10 chapters [239]. A net influx of cases to the chapter *Diseases of the circulatory system (IX)* was observed. If we apply their findings to our data and adjust for a 10% autopsy rate correction, 52 of 407 CVD deaths (12.9%) was not caused by CVDs while 127 of 588 non-CVD deaths (21.5%) were actually caused by CVDs. As we observed a relationship with all-cause mortality and 25OHD, reclassifying more deaths as CVD would probably not weaken the association between CVD mortality and 25OHD.

5.1.4 Confounding

The categorisation of the study population according to 25OHD concentrations revealed an extensive list of potential confounding factors that were distributed unequally. Those with lower 25OHD concentrations were to a larger extent older, males, smokers, obese and diabetic, reported performing less exercise, had a more proatherogenic lipid profile and reduced heart function. Although we did not assess dietary patterns in this thesis, it is reasonable to assume that they also follow less healthy diets. Kidney function, as assessed by eGFR, was higher among individuals with low vitamin D status in our study sample, but we do not propose an explanation for this.

In order to identify and adjust for the effect of possible confounders, we evaluated the relationships of 25OHD with study outcomes in paper I and III with crude and multivariate models. Although there is a rational for adding many potential confounders, this also increases the probability of fitting random noise in the data. When assessing the relationship with atherosclerosis progression in paper I, the multivariate model confirmed the null-finding observed in the crude model, indicating a robust relationship. CRP is as a measure of inflammation and both a source of systemic error and confounding, thus we included CRP in multivariate models when assessing the relationship with atherosclerosis progression. In the analysis of clinical events in paper III, we decided *a priori* to include age, gender, study site, smoking, body composition, seasonality, blood pressure and kidney function. We did not include other potential confounders in the final model, such as CRP, as they neither improved the performance of the model nor significantly changed the magnitude of the relationship between our exposure of interest and the outcome. When modelling the relationship with CVD mortality using 25OHD thresholds, a difference in risk was observed between the medium and high 25OHD group in a crude model, but no difference was observed when confounding effects were adjusted for.

A weakness of our study was that we did not assess all potential confounders, such as socioeconomic position, which is, among other health behaviours, associated with dietary quality and the intake of vitamin D [240] and strongly associated with coronary heart disease and all-cause mortality [241]. The frequency of outdoor activities, which is a surrogate marker for sun exposure, was not assessed directly, but may be indicated by physical activity levels. However, physical activity was incompletely assessed and, in order to keep statistical power, we did not include it in final multivariate models when assessing subclinical and clinical outcomes. We performed a sensitivity analysis in paper III and observed no confounding effects on the relationships with all-cause and CVD mortality; nevertheless, it is questionable whether the adjustment itself was accurate as the measurement of physical activity was based on self-report which may overestimate physical activity and cause misclassification [242]. Consequently, results in paper I and III are limited by the risk of residual confounding and we may not conclude that variation in 25OHD in the study population was causally related to CVD and all-cause mortality.

5.1.5 Reverse causality

A cause must always precede the effect in time. In the situation where an outcome precedes and causes the exposure, known as reverse causality, biased associations may be observed [243]. The majority of the study population had significant CAD at baseline. More severely diseased participants could have experienced malaise and reduced appetite prior to study enrolment and reduced their dietary intake, including the intake of vitamin D. Consequently, associations of low 25OHD with higher risk of all-cause and CVD mortality could be the result of reverse causation.

As the majority of our study population had significant CAD at baseline, the frequency of CVD risk factors should, by definition, differ from the general population. A cluster of participants within our study population may have, despite a low frequency of risk factors and healthy lifestyle, developed CAD as result of

unfavourable genotypes. Upon symptoms or suspicion of CAD, it is possible that these subjects further increased intake of fatty fish and cod liver oil in comparison to individuals with high risk from lifestyle. Theoretically, individuals with an unfavourable CVD genotype and healthy lifestyle may have been overrepresented among those with a high 25OHD concentrations (>100 nmol/l) and the observed association with all-cause mortality within this group the result of reverse causality. However, as we do not observe a statistical significant relationship with CVD-mortality at high 25OHD concentrations, this bias appears less likely.

5.2 Discussion of main findings

5.2.1 Vitamin D status and seasonal variation

Vitamin D status

In the study population overall, 6% were vitamin D deficient (<30 nmol/l), 28% insufficient (30-50 nmol/l) and 66% sufficient (>50 nmol/l). The mean 25OHD concentration (59.4 nmol/l) was slightly higher than reported in various populations in Europe, lower than North America, similar to Asia, and higher than the Middle East and Africa [244]. As a result of seasonal variation in vitamin D status (15.8 nmol/l, 26.5% of mean), the proportion sufficient was 53% from January through March and 83% from July through September.

The daily recommended intake of vitamin D in Norway is a flat 10 µg and assumes a contribution from sun exposure. In the absence of sun exposure, 15 µg is required if ~97.5% of Norwegians are to be sufficient [119]. Norwegians have access to affordable sources of fatty fish and share a culture for consuming cod liver oil, nevertheless, every second participant in our study had inadequate vitamin D status during the winter. By reversing the dose-response relationship, this finding indicative that the mean intake of vitamin D during the winter was closer to the average requirement of 7.5 µg [119]. Consequently, vitamin D status and dietary intakes of vitamin D in our study population was not adequate.

Correlates of 25OHD

Vitamin D status is determined by many factors, and in the second paper, we assessed the influence from both known and potential determinants. As expected, consumption of vitamin D supplements and physical activity, a surrogate marker of outdoor activity and sun exposure, were associated with higher 25OHD concentrations. A strong inverse relationship with adiposity was observed independently of supplement intake and physical activity. Adipose tissue sequestration of calciferols has been suggested as the cause of this relationship, potentially the result of rapid uptake or a larger volume for distribution, and an attenuated response to both UVB irradiation [245] and dietary supplementation studies [230] has been demonstrated. Whether this phenomenon is adaptive or detrimental is unknown, but obese individuals do require more vitamin D than normal weight individuals in order to reach the target 25OHD concentration for sufficiency.

Variation in the abundance and activity of converting and catabolising enzymes, cofactors and plasma carrier proteins can also determine 25OHD concentrations, which is most profoundly illustrated from animal knockout models and humans with inborn errors in metabolism. Organ function is relevant in this respect, as a loss of kidney function i.e. may reduce abundance and activity of CYP enzymes and delivery of substrate to proximal tubule cells, impairing production and catabolism of calcitriol and catabolism of 25OHD [246, 247]. Although we observed that moderately reduced glomerular filtration associated with lower 25OHD concentrations, severely or completely loss of kidney function does not strongly associate with 25OHD concentrations, potentially due to extra-renal catabolism of 25OHD [247, 248]. Genetic variation is also highly relevant and single nucleotide polymorphisms (SNPs) identified from genome wide association studies (GWAS) have been shown to determine the response in 25OHD concentration after 12 months with vitamin D supplementation [230]. The meaning of SNPs for circulating 25OHD concentrations in the general population is moderate, with single variants associated with ~5 nmol/l lower mean concentrations and the sum of several variants associated with ~13 nmol/l lower mean concentrations [249].

The potential influence of age on vitamin D status is interesting, as we observe both a higher mean 25OHD concentration and less seasonal variation among individuals above 62 years of age. We hypothesised that retirement provides the opportunity to follow higher quality diets and perform outdoor activities when sunlight is available during late spring and early autumn, thus resulting in a more stable vitamin D status. Retirement could also open up for traveling during the winter-time to warmer countries. This behavioural pattern may be in contrast to indoor workers who have less time for outdoor activity during the day and compensate from the lack of sun exposure by "binging" during the summer holidays.

5.2.2 Cosinor models and adjustement for seasonality

The performance of cosinor models in predicting future vitamin D status was demonstrated previously in healthy individuals from North-America [215]. In comparison to our study population, they did not have symptoms of CVD, slightly higher 25OHD concentrations and displayed more seasonal variation. We argued that our findings in cardiovascular patients, combined with previous results, strengthened the external validity of the use of cosinor models to adjust for seasonal variation.

Cosinor models were fitted using linear regression that fits the conditional mean of 25OHD given covariate values and transformations of the time variable. Month was chosen to represent time. The model fits a sine curve through the observed means within each month. When adjusting person specific measurements for seasonal variation, individual deviations from the fitted curve are added to the annual mean of the study population according to the model. As an example, a person may have a measured 25OHD concentration of 70 nmol/l in January, and the sine curve in January is at 50 nmol/l. Further, the cosinor model estimates the annual mean of the study population to be 60 nmol/l. The resulting equation is 60 + (70 - 50) = 80 nmol/l - an estimate of that person's yearly average.

A comparable and direct approach, which avoids modelling, may be performed by first calculating the difference between a person's observed value and the empirical mean of observations taken the same month [250]. Then, add this value to the empirical mean of the entire population. Hence, if the person's concentration is measured at 70 nmol/l in January, the mean in January is 50 nmol/l, and the population mean is 60 nmol/l, the equation becomes exactly the same as before, 60 + (70 - 50) = 80 nmol/l. Hence, in a hypothetical scenario where the sine curve is perfectly fitted to the empirical monthly means, the adjustments would be identical. A potential advantage of cosinor models, when it comes to adjusting for seasonality, is that it combines the information from all the months. Hence, it may compensate for few observations within one month, which could be a problem when using the empirical approach.

As seasonal variation associated with age, we also evaluated whether adjusting for age in cosinor models resulted in more accurate prediction than a simple cosinor model. Although we did not observe a difference, the possibility to include covariates in the adjustment for seasonal variation is a unique feature of cosinor models that is not available for the direct approach.

5.2.3 Atherosclerosis and clinical events

When assessing the relationship with CVD, we observed that 25OHD associated with fatal clinical events, but not progression of subclinical disease. Events were registered during 12 years of follow-up and subclinical progression after approximately 1 year. Although atherosclerosis progression occurred in this period, as measured by repeat measurement of stenosis size by coronary angiography, a longer follow-up may have been required to observe an association with 25OHD. Vitamin D activity may have a regulatory role in stenosis development, progression, as well as plaque vulnerability. Aforementioned, coronary angiography is not the most sensitive measurement for plaque vulnerability and prediction of future clinical events [140]. It is possible that a

relationship with other relevant characteristics of atherosclerosis may have been observed with a more sensitive method.

Patients received risk-reducing medications during follow-up in various extents. These medications target mechanisms suggested to be relevant to vitamin D activity, including LDL-cholesterol, inflammation, platelet aggregation, hypertension and vasodilatation. It is therefore relevant to distinguish between development of incident disease in subjects without CAD at baseline from disease progression in subjects with CAD at baseline. Although we also studied progression in lesions which did not fulfil CAD diagnosis criteria (>50% luminal narrowing), lesions in other coronary vessels fulfilled this criteria. Our findings are perhaps not generalizable to individuals without CAD.

The relationships with all-cause and CVD mortality were assessed in a large cohort with 25OHD concentrations spanning a wide interval. This combination provides statistical power to identify true relationships throughout the continuum of the 25OHD distribution and increases both the internal and external validity of the results. Despite an accurate measurement of atherosclerosis by coronary angiography and the use of multiple lesions to increase the relative sample size, fewer observations were available for the assessment of atherosclerosis progression, especially in the lower and higher end of the 25OHD distribution. Hence, we might not have had similar statistical power to identify relationships throughout the continuum of the 25OHD distribution and somewhat lower internal and external validity.

5.2.4 Increased mortality with high 25OHD concentrations

Inclusion of the upper threshold

A comment is required for the assessment and use of the upper threshold at 100 nmol/l. Despite statistically significant linear tendencies in the relationships between 25OHD and all-cause and CVD mortality, risk curves indicated non-linearity, with increased risk at the lower and higher end of the 25OHD distribution. From the

subsequent threshold analyses, limited to observations between 15 nmol/l and 150 nmol/l, small peaks around 100 nmol/l were observed. This was less statistically significant than candidate thresholds around the peaks at ~40 nmol/l, but we considered that the inclusion of an upper candidate threshold was justified. This decision was based on the visual interpretation of the risk curves, the threshold analyses and previous findings in other cohorts [251, 252].

Characteristics of subjects with 25OHD above the upper threshold

While patients below the lower threshold (~42.5 nmol/l) were younger, smoked more, were more obese, exercised less, were more diabetic, consumed less supplements, had better kidney function and a worse blood lipid profile than individuals in the medium distribution (42.5-100 nmol/l), patients above the upper threshold (100 nmol/l) were older, leaner, exercised more, consumed more supplements, had lower kidney function and were to a larger extent enrolled at HUS. Hence, we observed that these patients tend to follow unhealthier and healthier lifestyles, respectively. The distributions of blood measurements throughout the year were comparable between groups and consumption of vitamin D supplements other than cod liver oil did not differ. In light of available data, we suggested that differences in cod liver oil consumption, sun exposure from physical activity and BMI are responsible for the differences in 25OHD concentration.

Potential mechanisms

Why should healthier individuals with high 25OHD concentrations be at higher risk of mortality than individuals who are less healthy with medium 25OHD concentrations? They could be compensation for diseases or risk factors we did not account for. The relationship could also be biased by reverse causality, as mentioned previously. Assuming that the observation reflects a detrimental effect of vitamin D, it may be mediated by several mechanisms.

Elevated levels of calcium may cause vascular calcification [253] and arrhythmia [254]. Consumption of ionised calcium from supplements are believed to

result in a rapid and long-lasting increase in serum calcium which differs from the pattern observed when dietary sources of calcium are consumed [253]. Calcium supplements may therefore have a negative effect on cardiovascular risk and it is questionable whether a beneficial effect on fracture risk outweighs a potential negative effect [255]. Chronically high 25OHD concentrations may be relevant as absorption from calcium supplements may further increase. Hence, vitamin D status may act as an effect modifier on the relationship with calcium and CVD risk and all-cause mortality. Unfortunately, we did not have access to information on dietary data and calcium supplements specifically and thus were unable to investigate whether this differed for threshold groups. According to trial data, vitamin D3 supplements alone may reduce mortality in individuals of older age [126], but when co-administrated with calcium, increase the risk of kidney stones [126], AMI and stroke [256]. However, not all meta-analyses of trial data find negative effects on CVD endpoints from calcium supplements alone or co-administrated with vitamin D [257].

Participants almost exclusively consumed vitamin D supplements in the form of cod liver oil, which has a high content of omega-3 fatty acids. Previous findings using dietary data from a subset of WENBIT patients showed increased risk of fatal AMI with higher dietary intake of omega-3 long-chain polyunsaturated fatty acids in patients with normal glucose tolerance [258]. The findings could describe the same underlying pattern and are connected by the consumption of cod liver oil and fatty fish. However, it is not possible to elucidate whether increased mortality was caused by a high intake of omega-3 fatty acids, vitamin D, serum calcium or a third variable.

An interesting area relevant to nutrient interactions is residing within the cell nucleus. RXR is a partner for several ligand-bound receptors, including vitamin A, D and fatty acids. Provided that RXR availability is limited, competition over this partner could result in functional insufficiency. Furthermore, the cistrome for RXR heterodimers overlap and may be relevant to nutrient interaction. Does the cistrome for VDR-RXR increase, decrease or become altered upon the activity of other ligands such as vitamin A and fatty acids, and vice versa? However, this is highly theoretical and difficult to elucidate at the moment.

6. Conclusions

We aimed to investigate the vitamin D status of patients referred to coronary angiography with suspected CAD in Western-Norway. In conclusion, we observe that the status is not satisfactory. Seasonal variation contributes to a variation in 25OHD concentration of ~25%, and in combination with insufficient dietary intakes, every second patient has inadequate vitamin D status during the winter. Adiposity was strongly associated with lower 25OHD concentrations, while consumption of vitamin D supplements and higher physical activity associated with higher concentrations.

Seasonality may cause a systematic error and misclassification when assessing vitamin D status and bias observational research. Cosinor modelling provides a flexible approach to adjust for seasonality, which is preferable to not dealing with season, and more accurate than an alternative method commonly used in epidemiology.

Concentrations of 25OHD was not associated with progression of atherosclerosis, as measured by stenosis size from coronary angiography, but was inversely associated with higher all-cause and cardiovascular disease mortality. The association was reflected by a threshold located at ~42.5 nmol/l, defined as the most optimal division of individuals into high and low risk. This threshold is within the current threshold of sufficiency for optimal skeletal health.

All-cause mortality was increased in individuals with 25OHD concentrations above 100 nmol/l and we conclude that there is reason to be suspicious to chronic excessive intakes.

7. Further perspectives

Fortification of foods with vitamin D in Norway is static throughout the year, but we observe a high prevalence of vitamin D insufficiency during winter. A Danish RCT demonstrated that bread and milk fortification from September to April prevented seasonal-dependent reduction in 25OHD concentrations in the fortification group, resulting in only 16% insufficiency in April compared to 65% in the control group [259]. Hence, a more dynamic approach to food fortification could prevent winter-time insufficiencies without contributing to very high concentrations during the summer.

In addition to well-designed observational studies using 25OHD as a measure of vitamin D exposure, SNPs and common allele variants that influence concentrations of vitamin D binding proteins or activity of metabolising enzymes may be used. As the relationship between DNA sequences and endpoints are not confounded, they overcome some of the challenges with residual confounding. However, it also requires the identification of potent genotypes that causes a distinct difference in vitamin D exposure. So far, candidate genotypes have been discovered and used in studies of hard endpoints. No associations with genotypes were observed in the comparable LURIC cohort, despite an inverse relationship of 25OHD with higher risks of all-cause and CVD mortality [260]. Results from less comparable cohorts are mixed for all-cause and null for CVD mortality [261-263], while another study found an association with calcification scores [264]. Genotypes may be detrimental or beneficial depending on the patient's vitamin D status and researchers should maintain a two-sided hypothesis.

Several large RCTs assessing the effect of vitamin D on CVD or mortality are planned or currently underway. These trials are designed to overcome suggested fallacies of previous trials that were either to small, administrated doses that did not raise 25OHD concentrations sufficiently or co-administrated calcium or omega-3 fatty acids. The US multi-ethnic VITAL trial finished recruiting over 25000 participants in 2014 [265] and the Australian D-Health reported in 2015 that they had

finished recruiting over 21000 participants [266]. The Finnish FIND trial reported in 2015 that they stopped recruitment at 2500 participants and would not obtain the planned sample size of 18000 due to problems with recruitment and funding [267]. The UK VIDAL trial finished a two-year feasibility trial of 1600 patients in 2015 [268] and is planning to recruit 20000 participants in the main trial [269]. Besides the study of hard endpoints, nested sub studies may provide information regarding potential mechanisms, such as repeat measurements of blood pressure and cardiac output using echocardiography in VITAL [265]. Measurement of genotypes in vitamin D metabolism is also planned in the VITAL and may reveal novel gene-nutrient interactions. Considering the resources and time invested in vitamin D, we may hope that these trials provide convincing and unidirectional data for drawing conclusions about vitamin D and its effect on cardiovascular disease and survival. However, as illustrated by the promiscuous nature of nuclear receptors and the complex regulation of gene expression, even well-designed randomised controlled trials will not fully elucidate the intricate role of vitamin D in human health.

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