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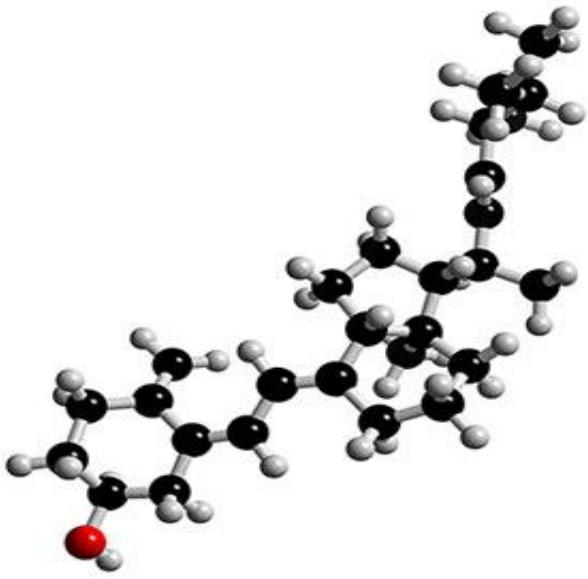
Factors affecting the development of type 2 diabetes and cardiovascular disease, with special reference to vitamin D



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ABSTRACT

There is increasing evidence that vitamin D may influence several non-skeletal conditions, including cardiovascular disease (CVD), diabetes, cancer, autoimmune disorders and infectious diseases. Vitamin D is among the few vitamins that can be produced by the skin in response to ultraviolet B radiation. Vitamin D is also a prohormone that is converted to 25-hydroxyvitamin D (25(OH)D) in the liver and 1,25-dihydroxyvitamin D (a hormone) in the kidneys. In addition, vitamin D receptors are present in most tissues and cells in the body. Many tissues and cells, including, colon, prostate, pancreatic β-cells and macrophages, express the enzyme 1α-hydroxylase to locally produce 1,25-dihydroxyvitamin D, which has the potential to regulate a number of genes. The pleiotropic effect of vitamin D may favorably influence diabetes and cardiovascular health through multiple mechanisms, including downregulation of the renin-angiotensin system, enhancement of insulin secretion and insulin sensitivity, protection against angiogenesis and modulation of inflammatory processes.

Epidemiological evidence suggests that vitamin D may reduce the risk of developing type 2 diabetes (T2D) and CVD. However, so far, studies found mixed results and data have been inconclusive. We aimed to investigate: 1) Whether low serum 25(OH)D concentrations predict the development of prediabetes and T2D; 2) The relationships between serum 25(OH)D concentration and established or emerging cardiovascular risk factors and risk of myocardial infarction (MI); 3) Serum 25(OH)D in relation to baseline severity and rate of progression of carotid intima-media thickness (cIMT); and 4) Whether vitamin D is causally implicated in CVD using vitamin D-associated genetic variants, serum 25(OH)D concentration and progression of subclinical carotid atherosclerosis.

In **Paper I**, subjects aged 35-56 years, without known T2D, underwent a health examination, including measurements of weight, height and blood pressure (BP), an oral glucose tolerance test (OGTT) was performed, and questionnaires covering life-style factors were answered at baseline and at follow-up. Serum 25(OH)D and serum insulin growth factor peptides were measured at baseline. Participants having prediabetes or T2D at follow-up 8-10 years later were selected as cases, age- and sex-matched to controls with normal glucose tolerance at both baseline and follow-up, in total 980 women and 1398 men. We found that high serum 25(OH)D concentrations predict reduced T2D risk in subjects having prediabetes but not in subjects with normal glucose tolerance. In **Paper II**, a total of 387 survivors of a first MI before the age of 60 and 387 sex- and age-matched controls were examined. Fasting blood samples, drawn three months after MI in cases and at the same time in matched controls, were used for biochemical analyses. Low 25(OH)D levels were associated with a range of cardiovascular risk factors but were not related to MI. Both **Paper III and IV** are based on the IMPROVE study, which is a European, multicentre, longitudinal cohort study that enrolled individuals aged 54 to 80 years, who had at least three cardiovascular risk factors and no history of CVD, from 7 centers in Finland, Sweden, the Netherlands, France, and Italy. Participants underwent carotid ultrasound examinations at baseline, month 15 and month 30. Blood samples, clinical data and information about life-style factors were collected at baseline from a total of 3,711 subjects, upwards of 900 of whom had diabetes. The results reported in **Paper III** demonstrated that levels of 25(OH)D differed across Europe and were not consistently, independently related to measures of cIMT. In **Paper IV**, we found one genetic variant (rs3829251) in the *DHCR7* (7-dehydrocholesterol reductase) gene which influenced progression of cIMT in a manner dependent on T2D status but independent of 25(OH)D levels.

Key words: *Type 2 diabetes, prediabetes, Vitamin D, 25-hydroxyvitamin D, Cardiovascular disease, Carotid intima-media thickness, Subclinical atherosclerosis, Single nucleotide polymorphisms.*

SUMMARY IN SWEDISH/SAMMANFATTNING PÅ SVENSKA

Det finns allt fler studier som visar att vitamin D har betydelse inte bara för skelettet utan även för hjärtkärl-sjukdom, diabetes, cancer, autoimmuna sjukdomar och infektionssjukdomar. Vitamin D är bland de få vitaminer som kroppen själv kan producera i huden när den utsätts för solens UV-strålning. Vitamin D är även ett prohormon och omvandlas till 25-hydroxyvitamin D (25(OH)D) i levern och 1,25-dihydroxyvitamin D (ett hormon) i njuren. Dessutom finns vitamin D-receptorer i de flesta vävnader och celler i kroppen. Många vävnader och celler, såsom bröst, tjocktarm, prostata, β-celler i bukspottkörteln och makrofager (vita blodkroppar), uttrycker enzymet 1α-hydroxylas för att lokalt producera 1,25-dihydroxyvitamin D, vilket ger möjlighet till genreglering. Denna pleiotropiska effekt hos vitamin D kan motverka utveckling av diabetes och hjärt-sjukdomar genom olika mekanismer såsom nedreglering av renin-angiotensin systemet, förbättrad insulinutsöndring och insulinkänslighet, skydd mot angiogenes och modulering av inflammatoriska processer.

Epidemiologiska studier tyder på att vitamin D kan minska risken för uppkomst av typ 2 diabetes (T2D) och hjärtkärlsjukdom, men hittills har resultaten varit varierande och motsägelsefulla. Vårt syfte var att undersöka: 1) Om låga serumkoncentrationer av 25(OH)D förutspår utveckling av prediabetes (förhöjt fasteglukos och/eller nedsatt glukostolerans) och T2D; 2) Relationen mellan serumkoncentrationen av 25(OH)D och kardiovaskulära riskfaktorer och risk för att insjukna i hjärtinfarkt; 3) Huruvida serumnivåer av 25(OH)D är associerade med svårighetsgrad och tillväxthastighet (progression) för intima-media-tjockleken i halspulsådern, som är ett surrogatmått för tidig ateroskleros; och 4) Om vitamin D har en kausal relation till hjärt-kärlsjukdom genom kombinerad analys av vitamin D-associerade genetiska varianter, serumkoncentrationen av 25(OH)D och tillväxthastigheten för intima-media-tjockleken i halspulsådern.

I **Artikel I** fick personer i åldrarna 35-56 år, utan känd T2D, genomgå en hälsoundersökning inkluderande blodprover, mätning av vikt, längd och blodtryck, och oral glukostoleranstest samt besvarade de frågeformulär om livsstilsfaktorer vid studiestarten och senare uppföljning. Deltagare som hade prediabetes eller T2D vid uppföljningen 8-10 år senare kategoriserades som fall och till dem utvaldes ålders och köns-matchade kontrollpersoner med normal glukostolerans vid både baslinjen och uppföljningen, totalt 980 kvinnor och 1398 män. Vi fann att höga serumkoncentrationer av 25(OH)D minskade risken att utveckla T2D hos individer med prediabetes men inte hos personer med normalt glukostolerans. I **Artikel II** studerades totalt 387 patienter som överlevt en första hjärtinfarkt och 387 köns- och åldersmatchade kontrollpersoner. Fasteblodprover togs tre månader efter hjärtinfarkten och patienter och matchade kontroller undersöktes vid samma tillfälle. Resultaten visade att låga D-vitaminnivåer var associerade med en rad olika kardiovaskulära riskfaktorer men inte med ökad risk för tidig hjärtinfarkt. Både **Artikel III och IV** baseras på IMPROVE-studien, som är en europeisk, multicenter, longitudinell kohortstudie. Personer i åldern 54-80 år, med minst tre kardiovaskulära riskfaktorer och utan känd hjärtkärlsjukdom, rekryterades vid 7 centra i Finland, Sverige, Holland, Frankrike och Italien. Deltagarna genomgick ultraljudsundersökning av halspulsådern vid baslinjen, månad 15 och månad 30 för mätning av intima-media-tjockleken. Blodprover, kliniska data och information om livsstilsfaktorer samlades in vid baslinjen från totalt 3711 individer, varav 900 hade diabetes. Resultaten som rapporteras i **Artikel III** visade att serumnivåerna av 25(OH)D varierar i Europa och att låga 25(OH)D-nivåer inte är relaterade till de använda surrogatmåtten på aterosklerotiska förändringar eller tillväxthastighet av ateroskleros i halspulsådern. I det arbete som beskrivs i **Artikel IV** fann vi en 25(OH)D-associerad genetisk variant (rs3829251) inom *DHCR7*-genen, som påverkade intima-media-tjocklekens tillväxthastighet i halspulsådern. Denna effekt var beroende av om individen var diabetiker eller inte men oberoende av 25(OH)D-nivåerna.

LIST OF PUBLICATIONS

I. **Deleskog A**, Hilding A, Brismar K, Hamsten A, Efendic S, Östenson C-G. Low serum 25-hydroxyvitamin D level predicts progression to type 2 diabetes in individuals with prediabetes but not with normal glucose tolerance. *Diabetologia. 2012;55:1668-1678.*

II. **Deleskog A**, Piksasova O, Silveira A, Samnegård A, Tornvall P, Eriksson P, Gustafsson S, Östenson C-G, Öhrvik J, Hamsten A. Serum 25-hydroxyvitamin D concentration, established and emerging risk cardiovascular factors and risk of myocardial infarction before the age of 60 years. *Atherosclerosis. 2012;223:223-229.*

III. **Deleskog A**, Piksasova O, Silveira A, Gertow K, Baldassarre D, Veglia F, Sennblad B, Strawbridge RJ, Larsson M, Leander K, Gigante B, Kauhanen J, Rauramaa R, Smit AJ, Mannarino E, Giral P, Gustafsson S, Östenson C-G, Humphries SE, Tremoli E, de Faire U, Öhrvik J, Hamsten A. Serum 25-hydroxyvitamin D concentration in subclinical carotid atherosclerosis. *Arterioscler Thromb Vasc Biol. 2013;33:2633-2638.*

IV. Strawbridge RJ*, **Deleskog A***[1], Piksasova O, Folkersen L, Kavousi M, Gertow K, Baldassarre D, Veglia F, Leander K, Gigante B, Kauhanen J, Rauramaa R, Smit AJ, Mannarino E, Giral P, Dehgan A, Hofman A, Franco OH, Humphries SE, Tremoli E, de Faire U, Gustafsson S, Östenson C-G, Eriksson P, Öhrvik J, Hamsten A. A serum 25-hydroxyvitamin D concentration-associated genetic variant in 7-dehydrocholesterol reductase (*DHCR7*) interacts with type 2 diabetes status to influence subclinical atherosclerosis. *Equal contribution. *Manuscript.*

CONTENTS

LIST OF ABBREVATIONS

25(OH)D	25-hydroxyvitamin D, calcidiol
$1,25(OH)_2D$	1,25-dihydroxyvitamin D, calcitriol
Apo	Apolipoprotein
Bif-IMT	Bifurcation intima-media thickness
BP	Blood pressure
BMI	Body mass index
cIMT	Carotid intima-media thickness
CAC	Coronary artery calcification
CAD	Coronary artery disease
CVD	Cardiovascular disease
CC-IMT	Common carotid intima-media thickness
CHD	Coronary heart disease
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CYP2R1	Enzyme responsible for the hydroxylation of vitamin D
DEQAS	Vitamin D External Quality Assessment Scheme
DHCR7	7-dehydrocholesterol reductase
DNA	Deoxyribonucleic Acid
FFA	Free fatty acids
FHD	Family history of diabetes
FPG	Fasting plasma glucose

GC	Group-specific component (vitamin D binding protein)
GWA	Genome-wide association
HDL	High-density lipoprotein
HR	Hazard ratio
ICA-IMT	Internal carotid intima-media thickness
IGF-1	Insulin-growth factor-1
IGFBP-1	Insulin-growth factor binding protein-1
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IGT and/or IFG	Prediabetes
IOM	Institute of Medicine
IMT	Intima-media thickness
IMPROVE	Carotid **I**ntima-**M**edia Thickness (cIMT) and cIMT-**PR**ogression as Predictors of **V**ascular **E**vents in a High-Risk European Population study
IU	International unit
LD	Linkage disequilibrium
LDL	Low-density lipoprotein
MI	Myocardial infarction
mRNA	messenger- Ribonucleic Acid
NGT	Normal glucose tolerance
NADSYN1	NAD synthetase-1
ng/mL	nanogram per milliliter (multiply by 2.496 to convert to nmol/L)
nmol/L	nanomol per liter (divide by 2.496 to convert to ng/mL)

OGTT	Oral glucose tolerance test
PAI-1	Plasminogen activator inhibitor-1
PTH	Parathyroid hormone
RAS	Renin angiotensin system
RCT	Randomized clinical studies
RR	Relative risk
RXR	Retinoic X receptor
SCARF	Stockholm Coronary Atherosclerosis Risk Factor study
SDPP	Stockholm Diabetes Preventive Program
SMC	Smooth muscle cell
SNP	Single-nucleotide polymorphism
TNF-α	Tumor necrosis factor alpha
T2D	Type 2 diabetes
UVB	Ultraviolet B
VDR	Vitamin D receptor
Vitamin D_2	Ergocalciferol
Vitamin D_3	Cholecalciferol

1. BACKGROUND

1.1 Type 2 diabetes

1.1.1 Epidemiology

The number of adults with diabetes worldwide has more than doubled over the past three decades[1], and it is estimated that 366 million people, or 8.3% of adults worldwide, have diabetes[2]. In addition, 183 million people (50%) with diabetes are undiagnosed[2]. Type 2 diabetes (T2D) is the most common form, accounting for 85-95% of all cases. The global prevalence is expected to increase to 552 million by 2030 which represents 9.9% of the total adult population of the world[2].

1.1.2 Aetiology and pathogenesis

Diabetes mellitus is a metabolic disease characterized by chronic hyperglycemia. It may cause long-term damage of the kidneys, eyes and nerves (microvascular complications) as well as cardiovascular disease (CVD) (macrovascular complication)[3-5]. The deficient insulin action does not only influence carbohydrate metabolism but also fat and protein metabolism. The development of T2D involves multiple pathophysiological mechanisms where deterioration of insulin secretion and impaired insulin sensitivity in liver, skeleton muscle and adipose tissue are the main factors[3]. Genetic and environmental risk factors are involved in the pathogenesis of T2D, leading to development of insulin resistance in muscle and liver and β-cell failure[3].

1.1.3 Type 2 diabetes

T2D is characterized by reduced response to insulin (insulin resistance) and/or insufficient insulin secretion[3]. Since the initial symptoms are nonspecific and mild, the disease often remains undiagnosed for several years[6]. Early signs of T2D are insulin resistance and impaired first-phase insulin secretion, resulting in postprandial hyperglycaemia, which is followed by deterioration of the second-phase insulin response and persistent hyperglycaemia in the fasting state[3, 4]. The progression from normal glucose tolerance (NGT) to T2D involves a stage of intermediate hyperglycaemia designated impaired fasting glucose (IFG) and impaired glucose tolerance (IGT), also called prediabetes[7].

1.1.4 β-cell function

T2D is a progressive disease caused by continued decline in β-cell function due to prolonged demand on the β-cells to increase insulin secretion[8]. Prediabetes and T2D do not develop until the β-cells fail to compensate for decreased insulin sensitivity[6]. β-cells have a genetically determined risk, and the combination of increased secretory demand and detrimental environmental factors result in β-cell dysfunction and decreased β-cell mass, and progression to IGT and T2D[6]. The mechanism by which insulin resistance leads to β-cell failure is not completely clarified. A possible explanation is that the cause of insulin resistance is directly responsible for β-cell failure through lipotoxicity due to increased exposure of free fatty acids (FFA)[3].

1.1.5 Insulin resistance

It is evident that insulin resistance has an important role in the pathophysiology of T2D[3]. Insulin resistance is defined as a reduced response to insulin of target tissues such as skeletal muscle, liver and adipocytes[3]. Insulin resistance occurs 10–20 years before the onset of the diagnosed disease and constitutes an important predictor of whether an individual will later develop T2D[9]. The majority of subjects with T2D are obese, have elevated FFA concentrations, increased circulating levels of proinflammatory cytokines and plasminogen activator inhibitor-1 (PAI-1)[6, 10]. Because high plasma glucose, FFA and cytokines concentrations can all induce insulin resistance, it is difficult to separate the contribution of each in the pathogenesis of T2D[11]. In obesity, the intra-abdominal fat accumulation is the critical determinant of insulin sensitivity[7]. The skeletal muscle is the major site of glucose uptake in the postprandial state, which provides strong evidence that insulin resistance in muscle is the earliest demonstrable defect resulting in hyperglycemia[11]. In the liver, insulin resistance leads to increased glucose production after a night of fasting and reduced suppression of the production of glucose after meals[3]. Insulin resistance in adipocytes suppresses the effect of insulin on lipolysis and causes elevated plasma FFA[11]. Of note, development of overt T2D occurs only in those individuals with insulin resistance whose β-cells are unable to compensate for the defect in insulin action[11]. Ectopic fat deposition in the liver is an important marker of insulin resistance and glucose dysregulation in adults[12]. The presence of steatosis is a sign of multiorgan insulin resistance, and the degree of insulin resistance is directly related to percent liver fat[13]. The severity of fatty liver was an independent predictor of prediabetes in obese adolescents[14]. In addition, the severity of hepatic steatosis is associated with significant decrease in insulin sensitivity and impairment of β-cell function[14].

1.1.6 Genetic and environmental risk factors for diabetes

The genetic component of T2D is strong. The majority of T2D patients have polygenic inheritance (70-85%)[3] ***(Figure 1)***. The number of known genetic loci in which variants exist that influence T2D is over 60[15-17]. Most of these are associated with β-cell function or have so far unknown impact. Each individual risk gene variant increases the risk of diabetes by a factor of only 1.05 to 1.4[15]. The occurrence of diabetes in persons with afflicted close relatives is increased 2-4 fold[18]. Twin and family studies show that T2D has an inherited component estimated to >50%[19]. In addition, studies support the existence of a genetic predisposition to develop diabetes in the presence of "diabetogenic" environmental factors such as high-calorie nutrition, lack of exercise and tobacco use[3, 5].

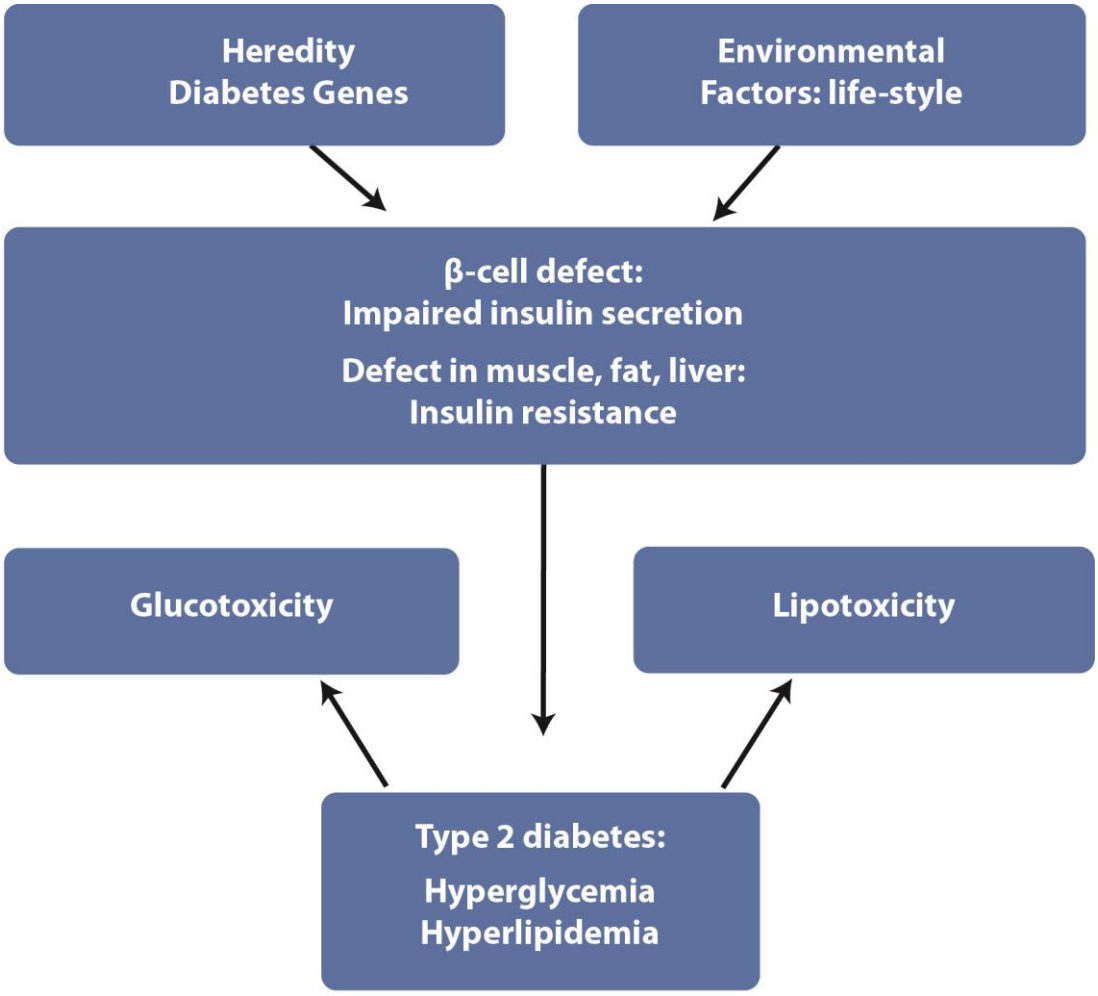


Figure 1. *Proposed pathogenesis of T2D. Adapted from Östenson[3]*.

Environmental factors, mainly obesity and physical inactivity, are strongly related to the occurrence of T2D[10, 12]. Other life-style factors such as smoking and alcohol consumption are also of importance[20-22] . For example, high consumption of alcohol increased the risk of abnormal glucose regulation in men in a graded manner, but in women this was only seen among women with high intake while in women with low or medium intake the risk was decreased[23]. In addition, high consumption of coffee has shown a positive effect on glucose regulation, resulting in reduced the risk of IGT and T2D[24]. Recently, it was found that high consumption of smokeless tobacco ("snuff") was associated with increased risk of T2D[25]. Moreover, behavioral factors, such as psychological distress including anxiety, apathy, depression, fatigue and insomnia, and work stress, such as high work demands, low decision latitude and shift work, increased the risk of prediabetes and T2D[26, 27].

1.1.7 Diagnosis of T2D

The oral glucose tolerance test (OGTT) has been considered to be the gold standard for the diagnosis of T2D. For an OGTT, the person fasts overnight[28]. Fasting plasma

glucose (FPG) is tested in the early morning next day, whereafter the person receives 75 grams of glucose. Blood samples are drawn at different time points most commonly 2 hours after ingestion of the glucose load to monitor blood glucose. Diabetes is present if FPG ≥7.0 mmol/L and/or 2-hours postload glucose ≥11.0 mmol/L. IFG is diagnosed by FPG 5.6-6.9 mmol/L and IGT by 2-hours postload glucose 7.8-11.0 mmol/L (*Table 1*). Another way of diagnosing T2D is by measuring HbA1c (glycated hemoglobin), which serves as a marker of the average blood glucose levels over the previous 4-6 weeks. This method is easier and does not require a fasting sample, thus allowing more people to be investigated[29]. The euglycemic insulin clamp technique is considered to be the gold standard for measuring insulin action[11]. By this technique, whole-body insulin action is quantified as the rate of exogenous glucose infusion required to maintain the plasma glucose concentration at euglycemic levels (normal blood sugar level maintained) in response to a fixed increment in the plasma insulin concentration[11].

1.1.8 Prevention of T2D

Individuals with IGT and/or IFG (prediabetes) run a high risk of future T2D[5]. Prediabetes is not only related to an increased risk of T2D but may also cause damage to kidney and nerves[5]. Intensive life-style interventions, specifically aimed at weight loss and increased physical activity in high-risk individuals, can prevent or at least delay the progression to overt T2D by more than 50%[5]. Individuals with both genetic risk variants and a diabetes-promoting life-style could be predicted on the basis of risk scores for being at risk of developing T2D[4].

FPG <5.6 mmol/L	Normal fasting glucose
FPG 5.6-6.9 mmol/L	Impaired fasting glucose
FPG ≥7.0 mmol/L	Provisional diagnosis of diabetes
2-h postload glucose <7.8 mmol/L	Normal glucose tolerance
2-h postload glucose 7.8-11.0 mmol/L	Impaired glucose tolerance
2-h postload glucose ≥11.0 mmol/L	Provisional diagnosis of diabetes

Table 1. *Diagnosis and classification of diabetes mellitus (Diabetes Care, volume 27, suppl 1, 2004)[28]*

1.2 Cardiovascular disease

1.2.1 Epidemiology

CVD remains the leading global cause of death, accounting for 17.3 million deaths per year, a number that is expected to grow to >23.6 million by 2030[30, 31]. Atherosclerosis and thrombosis are the two primary processes leading to clinically manifest coronary artery disease (CAD)[32]. Atherosclerotic CVD is a chronic disorder developing throughout life and progressing to an advanced level at the time symptoms appear. The death rates for CVD have fallen over recent decades in the Western World, and it is estimated that >80% of all CVD mortality now occurs in developing countries[31].

1.2.2 Aetiology and pathogenesis of atherosclerosis

The progression of atherosclerosis involves a series of pathological stages, such as intimal thickening and successive formation of fatty streaks, intermediate lesions, fibrous plaques and complicated plaques, processes including many cell types such as endothelial cells, monocytes/macrophages, smooth muscle cells (SMCs) and lymphocytes[33].

The normal artery wall is composed of three layers; the intima, the innermost layer which includes the endothelium and is separated from the other two by the internal elastic lamina, the media is the middle layer, consisting of SMCs, and the adventitia is the outermost connective tissue-containing layer *(Figure 2)*[34, 35]. The first morphological change seen in early atherosclerosis is the fatty streak, caused by extracellular deposition of lipids in the intima[35]. Low density-lipoprotein (LDL) particles are retained in the extracellular matrix, where they are oxidised. Oxidised LDL cholesterol then releases phospholipids which activate the endothelial cells into expressing leukocyte adhesion molecules[35]. Leukocytes adhere to the endothelium and migrate into the intima in response to cytokines. Scavenger receptors mediate the uptake of oxidised LDL particles by macrophages (monocytes), turning them into foam cells[34]. SMCs are recruited from the media to the cap surrounding the lipid core, which apart from SMCs, also contains a collagen-rich matrix.

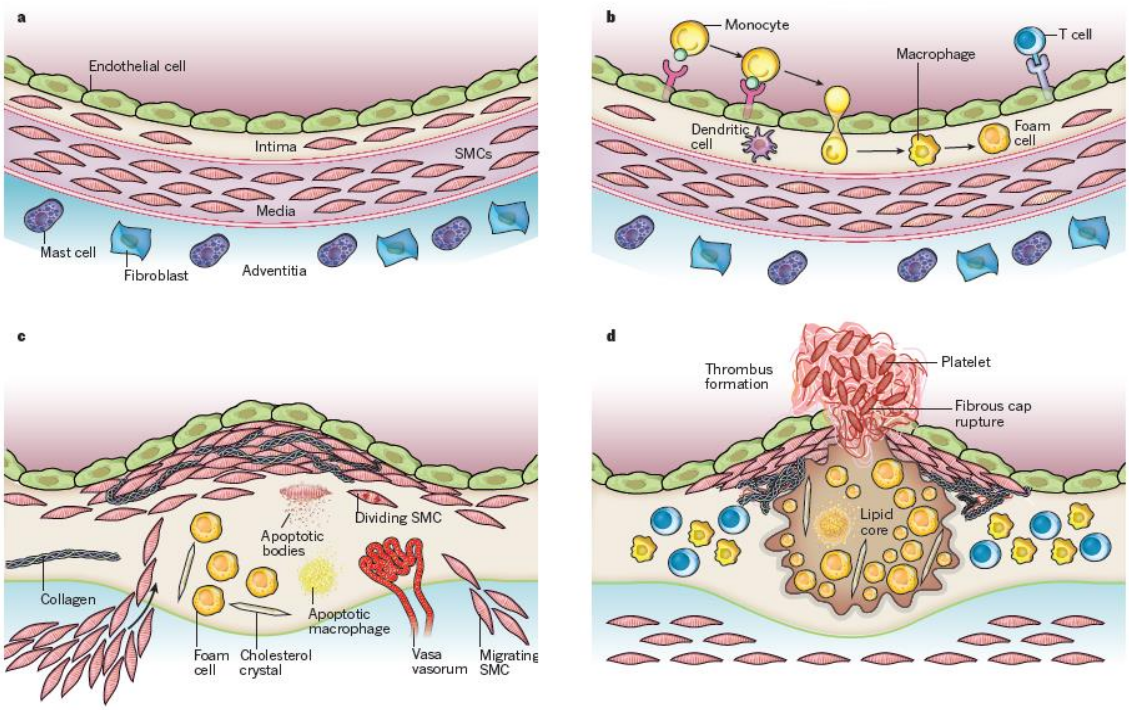


Figure 2*. The evolution of atherosclerotic lesions (atheromata)*
a*, The three layers of the normal artery* ***b****, Proinflammatory conditions of hyperlipidemia (infiltration and retention of LDL) leads to platelet aggregation and leukocyte adhesion, monocytes (the most numerous of the leukocytes) accumulate lipids and transform into macrophages or foam cells resulting in fatty streak.* ***c****, Lesion progression involves the migration of SMCs from the media to the intima (the proliferation), and the synthesis of extracellular matrix such as collagen and elastin. Apoptosis of macrophages and SMCs creates a necrotic core.* ***d****, Plaque rupture of the fibrous cap releases tissue factors from the plaque into the blood and trigger coagulation components forming a thrombosis, resulting in arterial occlusion and MI or stroke. Reprinted with permission P Libby, 2011[34].*

As the person ages and the disease becomes more advanced, fibrotic and calcific layers add to the plaque. Inside the plaque, the accumulating immune cells are predominantly macrophages and T-cells[34]. During plaque formation macrophages and vascular cells produce cytokines which attract T-cells. By activating T-cells and macrophages, intimal lipid accumulation leads to the chronic inflammation that is characteristic of atherosclerosis.

1.2.3 Atherosclerosis and Intima-Media Thickness

Atherosclerosis is the main underlying cause of MI and ischaemic stroke[34]. Ultrasound examination of carotid arteries provides estimates of their intima-media thickness (cIMT) and presence and severity of plaques, both widely used as surrogate measures of preclinical atherosclerosis and predictors of CVD[36, 37] (including subclinical atherosclerosis and coronary/cerebrovascular events[36, 38, 39]). The cIMT is defined as the distance between the blood–intima and media–adventitia interfaces of the carotid wall[38]. Even though cIMT and plaques are highly inter-correlated, the role of cIMT

measures as a marker of atherosclerosis has been questioned[40]. Most often, cIMT has been measured in the common carotid artery because measurements are easily acquired from this segment. However, plaques in this arterial segment are uncommon[40]. Characteristically, plaques occur at the level of the bifurcation and the internal carotid artery, sites of non-laminar turbulent flow, which suggests that cIMT measurements at these sites better reflect true atherosclerosis than measures in the common carotid segment[41]. However, common carotid intima-media thickness (CC-IMT) is related to changes in local shear stress and lumen diameter as part of arterial remodelling, the latter reflecting early stages of atherosclerosis[40]. In contrast, plaques represent a later stage of atherogenesis related to inflammation, oxidation, endothelial dysfunction, and SMC proliferation. Therefore, cIMT expansion and plaque formation should perhaps not be equated, since they might reflect different biological processes. Carotid plaques can be further evaluated by considering plaque echogenicity, plaque heterogeneity, plaque numbers, plaque area and plaque volyme[40]. Notably, presence of echolucent plaques (lipid-rich plaques) seems to increase the risk of MI and stroke[40].

Interestingly, an independent association has been demonstrated between CC-IMT and fasting glucose concentration[42]. In addition, normal weight, overweight, and obese glucose-tolerant first-degree relatives to subjects with T2D have a larger CC-IMT than age- and body mass index (BMI)-matched control subjects with no family history of diabetes. These results suggest that genetic predisposition to T2D may accelerate development of atherosclerosis[42]. It has also been demonstrated that subjects with manifest T2D have an 0.13 mm increase in cIMT compared with subjects without T2D[43].

1.2.4 Coronary artery calcification

Coronary artery calcification (CAC) is an important risk factor for clinical coronary heart disease (CHD)[44]. Vascular calcification is a complex process that is dependent on pH, calcium, phosphate and smooth muscle factors that may be regulated by these ions as well as by parathyroid hormone (PTH) and calcitriol[45]. Comparing vessel layers, intimal calcification is associated with atherosclerosis[45]. Previous studies report associations between vascular calcification and osteoporosis and indicate an inverse relationship between the amount of vascular and skeletal calcium[46].

1.2.5 Genetic and environmental risk factors for CVD

CVD is a multifactorial disease and involves genetic factors alone or in combination with environmental factors[47, 48]. Family history of early CAD is in itself an independent risk factor for CAD[49]. Heritability explains 40-60% of the risk of CAD[32] . Young people with a family history of CHD have an increased cIMT, independently of traditional risk factors for CVD, suggesting that genetic predisposition to CHD influences arterial structure before occurrence of clinical manifestations of CHD[50].
Estimating the relative contribution of genetic and environmental factors for a particular trait in family studies has shown that genetic variation has a significant impact on the plasma levels of cholesterol-containing lipoproteins (low-density and high-density lipoproteins) as well as the plasma concentrations of apolipoprotein (apo) B and apoAI[51, 52]. Other traits, such as fasting plasma levels of triglycerides and fibrinogen,

have shown less consistent measures of heritability[52, 53]. Genetic variants that influence CAD risk are relatively common and each exert small effects, but rare variants may also contribute to CAD[32]. Genome-wide association (GWA) studies have identified a multitude of common variants associated with systolic and diastolic blood pressure (BP), lipid traits and CAD[54, 55]. Each individual risk variant typically increases the risk by 1.05 to 1.30[56]. Most single nucleotide polymorphisms (SNPs) therefore explain only a very small proportion of the population variance in a phenotype. CVD is a multifactorial disease, involving many genetic and non-genetic factors,that probably interact with each other, resulting in heterogeneity in populations studied[57]. The small contribution to the risk of the individual conferred by SNPs explains why they are not useful risk indicators or biomarkers for CVD. In contrast, blood biomarkers such as LDL cholesterol, glucose, BP, C-reactive protein (CRP) et cetera integrate a large number of genetic and non-genetic influences and hence are much more informative[57]. An association of genetic variants with both the circulating levels of the gene products and disease risk speaks for a causal relationship[57].

The major risk factors for the development of CHD are male gender, increasing age, elevated levels of plasma LDL cholesterol, elevated BP, obesity, diabetes and life-style factors such as smoking, a high fat diet and lack of exercise. The Interheart study showed that nine modifiable risk factors could explain more than 90% of the risk of suffering myocardial infarction (MI)[58]. Smoking and dyslipidemia were found to be the most important risk factors. Hypertension, diabetes, physical inactivity and no regular alcohol consumption were more closely associated with MI in women than in men. There is a significant difference in CVD risk between sexes[59]. Middle-aged men have 2- to 5-fold higher CVD mortality rates than women[60]. However, the relative risk (RR) of CVD is higher in women with diabetes than in men. Also, results from a European cohort study showed that women with newly diagnosed diabetes had a higher RR of death from CVD than men with manifest diabetes[61]. The risk of developing CVD in the subject with T2D is increased 2- to 4-fold, independently of other concomitant risk factors[62]. In fact, blood glucose predicts increased cardiovascular morbidity and mortality even at levels below the threshold for established diabetes[63], i.e. in prediabetic states (IGT and/or IFG)). Both fasting and 2-hours postload plasma glucose concentrations are independent risk factors for all-cause and cardiovascular morbidity and mortality even in people without diabetes[64, 65]. Persons with T2D but without prior MI run the same risk of death from CAD as non-diabetic persons with previous MI[66].

CVD is the leading cause of morbidity and mortality in individuals with diabetes, 65% of which is attributable to heart disease or stroke[66, 67]. Moreover, it is estimated that 80% of patients with diabetes mellitus die a thrombotic death[68, 69]. The primary defense against thrombosis is the vascular endothelium, which is abnormal in diabetes. Several pathways have been proposed by which hyperglycaemia may induce endothelial injury and chronic vascular inflammation. Acute hyperglycaemia is associated with endothelial dysfunction, platelet hyper-reactivity, impaired microcirculatory function, increased cytokine activation, increased FFA levels, and increased oxidative stress, all of which adversely affect outcome in acute MI. In addition, elevations of insulin are associated with increases in PAI-1levels in subjects with and without diabetes[68]. In vitro

experiments with hepatocytes reveal that elevated levels of very low density lipoprotein, triglycerides and fatty acids increase the ability of insulin to induce PAI-1 production[68].

1.2.6 Prevention of CVD

The Framingham Risk Score is a gender-specific algorithm used to estimate the 10-year cardiovascular risk of an individual. Cardiovascular risk scoring estimates the probability to develop CVD within a specified period of time[70]. cIMT reflects the severity of atherosclerosis in coronary arteries and constitutes a reliable surrogate marker of subclinical atherosclerosis[38]. CC-IMT and the internal carotid intima-media thickness (ICA-IMT) have been strongly associated with the risk of MI and stroke in asymptomatic subjects[36]. Individuals with cIMT wider than 1 mm had higher incidence of CHD than those with IMT below 0.60 mm[71]. Besides predicting coronary and cerebral events, cIMT is also associated with CVD risk factors[36, 71]. There is a gradual increase in cardiovascular risk with rising cIMT; a value >0.9 is considered abnormal[36]. The relationship between mean cIMT and incident CHD persists even after adjustment for traditional risk factors, indicating that part of the risk for developing atherosclerosis is still unexplained[41]. Imaging of arteries has proved to identify prognostic important subclinical atherosclerosis even in subjects classified as "low-risk" according to traditional risk stratification, where family history, abdominal adiposity, inflammation and other factors shown to predict cardiovascular are not incorporated[37, 72].

1.3 Vitamin D

The past several years have seen a growing interest in the role of vitamin D in the aetiology of T2D and CVD. Evidence exists that individuals deficient in vitamin D are more likely to have (or be at risk of developing) T2D and CVD[73-76]. In addition, established risk factors, such as ageing, smoking, hypertension, diabetes, obesity and dyslipidemia, do not fully explain the high prevalence of CVD, suggesting the presence of other pathophysiological pathways[77]. Overall, the available results from epidemiological studies conducted to date are insufficient to support the notion that T2D and CVD can be prevented by raising 25-hydroxyvitamin D (25(OH)D) concentrations. Thus, more research is needed.

1.3.1 Biological function

In the 17th century, Whistler (in 1645) and Glisson (in 1650) described rickets as a bone disease in children characterized by bone pain and skeletal deformities[74]. Vitamin D (the fourth vitamin to be discovered and hence alphabetically named ‘D’) was discovered by McCollum et al. in 1922 as the substance that cured rickets[78]. During 1970s, people started believing that vitamin D deficiency may also cause CVD, and the hypothesis was launched that the increased incidence of CVD observed during winter may be a consequence of low UV-B irradiation, resulting in a poor vitamin D status[79]. In the 1980s, it was shown that vitamin D deficiency in rodents and rabbits inhibits pancreatic insulin secretion, indicating that vitamin D is essential for the function of the endocrine pancreas[80]. Vitamin D (calciferol) is the generic name for a group of fat-soluble steroids, of which the two major ones are vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol)[81, 82]. The main source of vitamin D is endogenous generation of

cholecalciferol (vitamin D_3) from 7-dehydrocholesterol in skin through ultraviolet light exposure from the sun[83, 84]. Some vitamin D also derives from dietary intake, e.g. intake of vitamin D_3 from animal sources/products like oily fish, egg yolk and fortified products (milk and margarines) and vitamin D_2 from plants, fungi, and invertebrates produced by ultraviolet light exposure[84, 85].

1.3.2 Physiology of vitamin D

Vitamin D is a precursor compound and has no significant biological activity. Two hydroxylation steps are required to produce the most active vitamin D metabolite, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) or calcitriol[74] (*Figure 3)*).

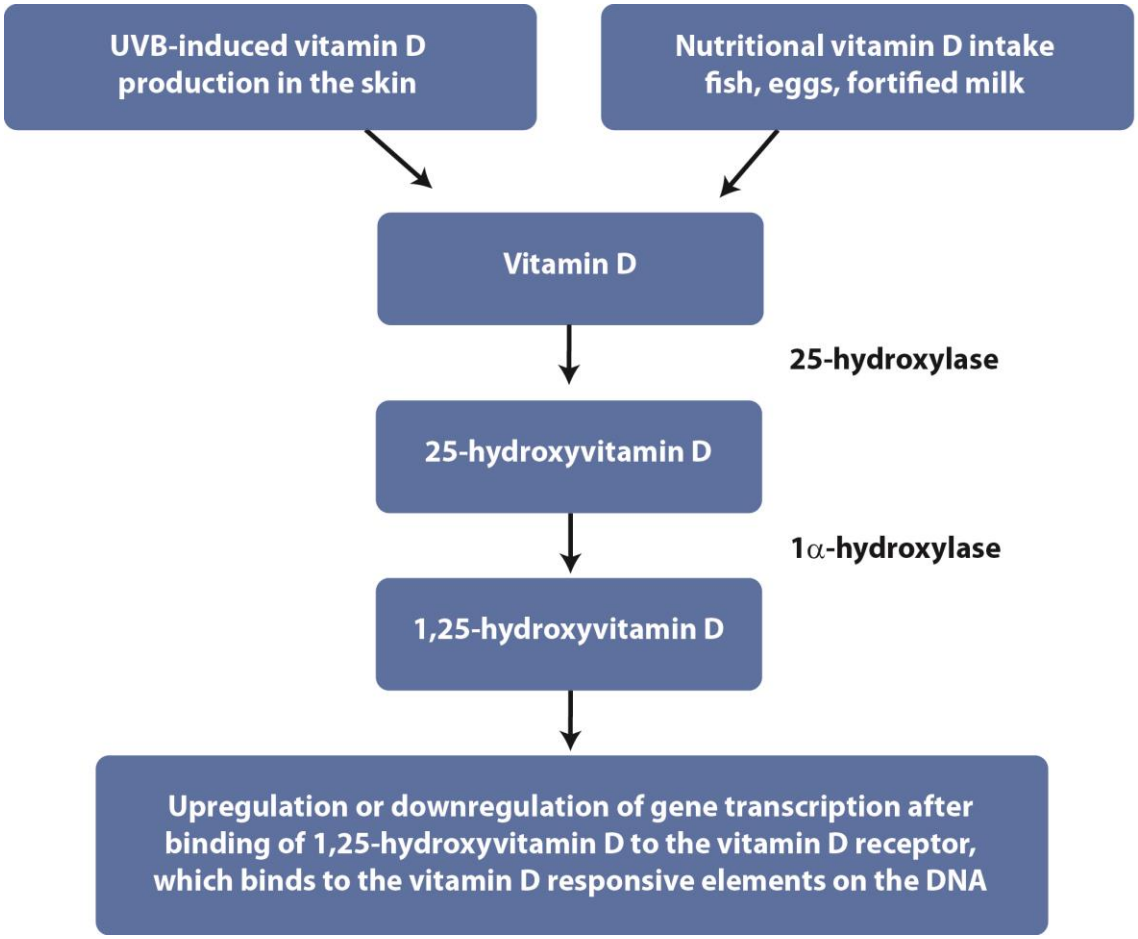


Figure 3*. Metabolism of vitamin D. Adapted from Pilz et al[83].*

In the circulation, all metabolites of vitamin D are bound to a carrier protein known as the vitamin D-binding protein[86]. Liver 25-hydroxylases and kidney 1α-hydroxylase belong to the large family of cytochrome P450-dependent steroid hydroxylases[74]. Vitamin D (vitamin D2 or vitamin D3) is hydroxylated to 25(OH)D or calcidiol by 25-hydroxylases in the liver *(Figure 4)*. In the kidney, 25(OH)D is further hydroxylated by 1α-hydroxylase

to $1,25(OH)_2D$, which is the active form of vitamin D and functions as a steroid hormone[87]. Many extra-renal tissues also express 1α-hydroxylase (breast, colon, prostate, pancreas β-cells and macrophages), which can produce significant tissue levels of $1,25(OH)_2D$ [88-90]. This local production of $1,25(OH)_2D$ is dependent on the substrate availability of 25(OH)D, but is also regulated by other factors such as cytokines or growth factors[88]. Circulating 25(OH)D levels are a main determinant of the extra-renal tissue levels of $1,25(OH)_2D$ and thus the best indicator of whole body vitamin D status[85]. In addition, 25(OH)D is the major circulating form of vitamin D and reflects the total production of vitamin D from both endogenous and exogenous sources, plus has the longest half-life (3-6 weeks), and thus constitutes the best clinical measure of vitamin D stores[81, 85]. Serum levels of $1,25(OH)_2D$ are largely determined by renal 1-α-hydroxylase activity, which is tightly regulated by factors related to calcium and phosphor metabolism (stimulation by PTH or inhibition by fibroblast-growth factor 23)[74, 79]. 24-hydroxylation of 25(OH)D or $1,25(OH)_2D$ is considered to be the main degradation process and produces vitamin D metabolites, which are converted to water-soluble inactive calciotroic acid. Vitamin D receptor (VDR) activation induces this 24-hydroxylase, resulting in regulatory loop[85, 88].

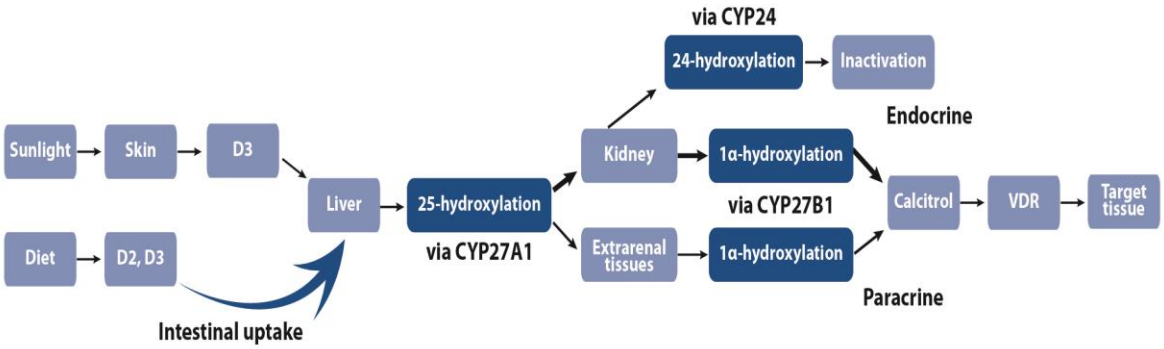


Figure 4. *Vitamin D activation via liver and kidney hydroxylation to calcitriol, and vitamin D activation in extra-renal tissue via VDR to calcitriol (called pleiotropic effects). Adapted from Brandenburg et al[91].*

1.3.3 Molecular action of 1,25(OH)$_2$D

The $1,25(OH)_2D$ is the only form of vitamin D that is metabolically active, and this molecule exerts its effects by activating the nuclear VDR[74]. The VDR is present in over 30 tissues and cells, among them pancreatic β-cells, skeletal muscle cells, cardiomyocytes, SMCs, vascular endothelial cells, neurons and immune cells[81, 92] [93]. The VDR is a member of the nuclear receptor superfamily of ligand-activated transcription factors[74]. The binding of $1,25(OH)_2D$ to the VDR leads to transcription of genes regulated by $1,25(OH)_2D$. The vitamin D response element (specific DNA sequence) to which the VDR binds consists of a hexanucleotide. The VDR binds as a heterodimer to the retinoic X receptor (RXR), and the effects of $1,25(OH)_2D$ are the result of interactions with this nuclear receptor[83]. The DNA sequences are located in the promoter regions of various vitamin D-dependent genes that are either up-regulated or down-regulated by the RXR-VDR complex[83]. Besides the effect on calcium and bone

metabolism, the $1,25(OH)_2D$ effects on tissues (tissue-specific autocrine and paracrine roles) can only be observed at concentrations exceeding the physiological levels needed for maintenance of calcium and bone homeostasis[74, 83, 94]. More than 200 genes have been estimated to be directly or indirectly influenced by $1,25(OH)_2D$[85]. These genes control cellular proliferation, differentiation, and apoptosis, as well as insulin, rennin and cathelicidin production[89]. About 3% of the human genome is directly or indirectly regulated by the vitamin D endocrine system[85].

1.4 Vitamin D and health

An inadequate vitamin D status is a global issue, and around 1 billion people worldwide are considered to suffer from vitamin D insufficiency (defined as <75 nmol/L, (divide by 2.496 to convert to nanograms per liter)[85]. In some studies, 40% to 100% of the elderly have been diagnosed with an insufficient 25(OH)D status[85]. Individuals at risk for vitamin D deficiency are infants, young children, veiled women, persons with coloured skin, older adults and persons who live at high latitudes[85, 95].

1.4.1 Optimal levels of 25(OH)D

There is no consensus on the optimal levels of serum 25(OH)D. Based on relationships to several clinical outcomes, target concentrations between 75 and 100 nmol/L serum 25(OH)D have been proposed[96]. This is the level associated with maximal suppression of PTH and reduced fracture rates[97]. Sufficient levels are generally defined as ≥ 75 nmol/L, insufficient levels as 25-74 nmol/L and deficient levels as < 25 nmol/L[85], but other cut-off points for 25(OH)D levels are also considered[81, 83] [98]. The Institute of Medicine (IOM) has recently declared that circulating concentrations of only 50 nmol/L are sufficient for the general population[99].

1.4.2 Sources of vitamin D

Vitamin D is synthesised in the skin from 7-dehydrocholesterol following exposure to ultraviolet B (UVB) radiation with a wavelength of 290-320 nm[85]. The synthesis is influenced by a number of factors, including age, season, skin pigmentation, latitude, use of sun screen, clothing and amount of skin exposed[84]. Vitamin D production by sunlight exposure is particularly efficient in individuals with low levels of melanin. Therefore, individuals migrating to northern regions developed a fair skin to efficiently synthesize vitamin D under conditions of less UV-B exposure, whereas those individuals residing in sunny regions have a high melanin content of the skin, which protects against sunlight-induced damage[79]. The dietary sources of vitamin D include food, e.g. fatty fish, fish liver oil and egg yolk, and dietary supplements[84, 85]. Of note, some food is fortified with vitamin D. Excess intake of vitamin D can lead to a state of vitamin D intoxication or hypervitaminosis (through diet and supplementation). Toxic levels of vitamin D do not occur from prolonged sun exposure, due to regulation by a mechanism which leads to photo-degradation at excessive levels[85, 100].

1.4.3 Vitamin D requirements

Recommendations from IOM for adequate daily intake of vitamin D are 600 international units (IU) (15µg) for children and adults up to 70 years of age and for adults 71 years or older 800 IU (20 µg), amounts which are based on the vitamin D required for bone

health[99]. For each 100 IU of vitamin D, the serum 25(OH)D concentration rises by approximately 2 nmol/L. Sun exposure can provide an adequate amount of vitamin D. Exposure of arms and legs twice a week between 10 a.m. and 3 p.m. for 15-30 minutes is adequate[85, 100].

1.4.4 Assessment of vitamin D

Serum 25(OH)D comprises the sum of $25(OH)D_2$ and $25(OH)D_3$. To determine the total 25(OH)D level in serum both must be measured. There is no standardization of assays to measure 25(OH)D; hence comparisons across studies are intricate[101]. However, most assays used are able to distinguish between high and low vitamin levels, but there may be some variation when looking at absolute levels. The International Vitamin D External Quality Assessment Scheme (DEQAS) monitores 25(OH)D analyses. DEQAS requires at least 80% of all values to be within +/- 30% of the method mean.

1.5 Potential mechanisms for the effects of vitamin D in T2D

There are several potential mechanisms for how vitamin D might influence the progression from prediabetes to T2D (*Figure 5)*. VDRs are present in many tissues and cells including pancreatic islet and skeletal muscle cells, and 25(OH)D concentrations may exert modulating effects on both insulin secretion and insulin action[102-105]. Studies in both animals and humans have suggested that vitamin D may exert positive effects on insulin secretion and insulin sensitivity, either directly or indirectly through modulation of calcium metabolism[102-105]. Vitamin D deficiency correlated with β-cell dysfunction and insulin resistance in a study of normoglycemic healthy adults, who were examined with OGTT and hyperglycemic clamp[106].

Evidence supports that vitamin D has a direct effect on pancreatic β-cell function by binding of its circulating active form, $1,25(OH)_2D$, to the pancreatic β-cell VDR, or by activating vitamin D within the β-cell through the 1α-hydroxylase enzyme[73]. In addition, vitamin D may have effects on insulin action by stimulating the expression of the insulin receptor and enhancing insulin responsiveness for glucose transport. The indirect effects can be mediated via regulation of extracellular calcium and calcium flux through the β-cell. Vitamin D may also be involved in systemic inflammation processes and improve insulin sensitivity and promote β-cell survival by directly modulating the effect of cytokines[73].

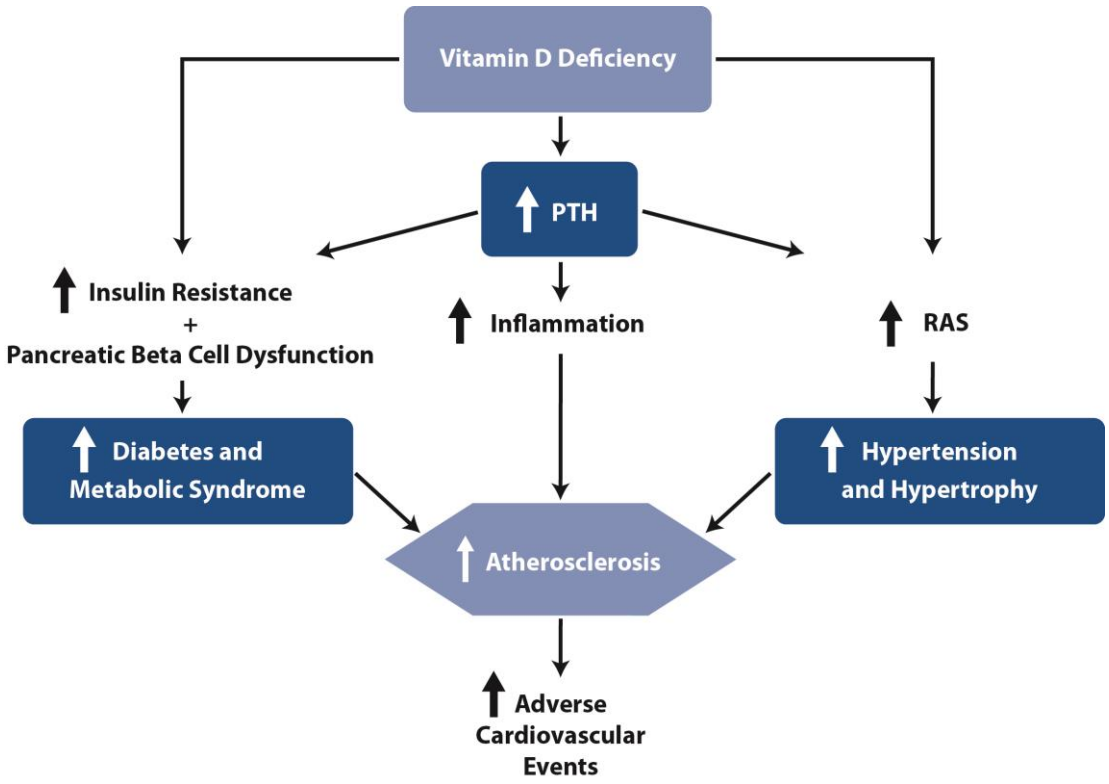


Figure 5. *Potential mechanisms of increased diabetes and cardiovascular risks from vitamin D deficiency. Through the VDR and 1-α hydroxylase expressed in the pancreas, circulating 25(OH)D can be converted to 1,25(OH)$_2$D to work as a paracrine or autocrine hormone. The black arrows show suggested links between vitamin D deficiency and diabetes as well as atherosclerosis. Adapted from Lee at al[81].*

1.5.1 Vitamin D and insulin growth factor-1 axis

Insulin-growth factor 1 (IGF-1) is similar in structure and function to insulin, and both have multiple roles in the regulation of metabolic processes[82]. Much of the metabolic effects of the IGFs are attributable to particular IGF-binding proteins to which they normally bind[82]. The role of the IGF-1 system peptides in relation to development of T2D is not completely clarified. Interactions between the IGF system and vitamin D have been suggested[82, 107]. Thus, serum 25(OH)D may regulate IGF-1 activity, and, conversely, IGF-1 may increase vitamin D concentrations. Furthermore, vitamin D has been shown to stimulate insulin-growth factor binding protein-1 (IGFBP-1) production, probably via binding to the VDR[108].

1.5.2 Evidence from observational and interventional studies

Several observational studies have shown that low concentrations of vitamin D, measured as 25(OH)D, are associated with T2D and metabolic syndrome, although the relationships have not been consistent[73, 74, 82, 85, 89, 109] . A meta-analysis of 11 prospective studies, involving a total of 3,612 cases and 55,713 non-case participants, assessed the association between circulating 25(OH)D levels and T2D. A strong inverse association between serum 25(OH)D concentration and incident T2D was observed, with a combined RR of 0.59 when comparing the highest quartile of 25(OH)D with the

lowest[101]. Similarly, another meta-analysis, which included the most recent prospective studies (measuring both 25(OH)D or self-reported dietary intake of vitamin D), reported a 19% lower risk of developing T2D among those with highest vs. lowest score of vitamin D[110]. However, there were some limitations. For example, surrogate markers of vitamin D status were used by assessment of dietary vitamin D intake, which does not include the major non-dietary component of vitamin D generated from sun exposure[111]. Also, self-reported T2D and incomplete identification of incident T2D[112-115], and a single measurement of serum 25(OH)D concentration to define vitamin D status, may not reflect vitamin D status over long periods[101, 116]. Other weaknesses were small sample sizes or limited number of cases [112]. In addition, 25(OH)D was measured by a number of methods, which may reduce the ability to directly compare studies.

A total of 8 observational cohort studies and 11 randomized controlled trials (RCTs) were included in the meta-analysis examining the association between vitamin D status and incident T2D, and the effect of vitamin D supplementation (with or without calcium) on glycemic outcomes[116]. Doses of vitamin D ranged from 400 to 8571 IU/day. Vitamin D supplementation did not show any effects on glycemic or incident diabetes outcomes among persons with NGT but beneficial effects of vitamin D and calcium supplementation on fasting blood glucose and insulin resistance in the group that had IFG at baseline[116, 117]. Study limitations included post-hoc analyses of trials that were not designed to assess diabetes or glucose metabolism as a primary outcome, small sample size, inadequate dose of supplement, and use of combination supplementation with calcium such that individual vitamin D effects could not be evaluated[116, 118-120]. For example, the Women's Health Initiative Trial, which is the largest trial on vitamin D and calcium supplementation, reported no statistically significant effect on T2D[119]. Participants were randomly assigned to receive 1,000 mg elemental calcium plus 400 IU vitamin D3 daily, or placebo, and followed for 7 years. The result showed that calcium plus vitamin D3 supplementation did not reduce the risk of developing diabetes. Potential explanations for the null finding include the low dose of vitamin D, the fact that only 60 % of the participants were compliant by the end of the trial, and both treatment arms were allowed to take supplements in addition to the study medication[119].

1.6 Potential mechanism for the effects of vitamin D in CVD

There are several lines of evidence for a number of potential mechanisms whereby vitamin D status may influence CVD risk *(Figures 5 and 6)*[46]. Many tissues not only express the VDR but also possess 1α-hydroxylase activity, thus have potential for autocrine and paracrine production of $1,25(OH)_2D$[46]. This process may be involved in a wide range of physiological functions, including regulation of cytokines, inflammatory and or fibrotic pathways, renin-angiotensin system, vascular and cardiac cell function, immune response modulation, cell growth and differentiation[93]. Vascular SMCs express VDRs, and calcitriol inhibits proliferation of these cells by influx of calcium into the cells. Matrix Gla protein (MGP) is synthesized by SMCs and increased by calcitriol and inhibits vascular calcification[46]. PTH levels increase in response to low levels of calcitriol and excess PTH levels may promote atherosclerosis via insulin resistance plus calcium and phosphate deposition in vessel walls, and may lead to myocardial

calcification[46]. Inflammatory processes play an important role in atherosclerosis. Cytokines, such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and other stimuli of CRP production, are suppressed by calcitriol, whereas the anti-inflammatory interleukin-10 (IL-10) is up-regulated. The renin-angiotensin system (RAS) is modulated by calcitriol via the reduction in plasma renin activity and angiotensin II concentrations[46]. Moreover, low vitamin 25(OH)D may influence the expression of macrophages and lymphocytes in atherosclerotic plaques, thus promoting chronic inflammation in the artery wall[79]. In addition, low levels of vitamin D have been associated with endothelial dysfunction[121]. Apart from a direct impact on cardiomyocytes and CVD, it has been suggested that 25(OH)D indirectly influences CVD by modifying cardiovascular risk factors like diabetes, obesity, cholesterol concentration, hypertension, and effects of smoking[73, 122, 123]. Thus, in the National Health and Nutrition Examination III survey, including 15,088 women and men, 20 years or older, the prevalences of hypertension, diabetes and hypertriglyceridemia were significantly higher in those with the lowest concentration of 25(OH)D[124].

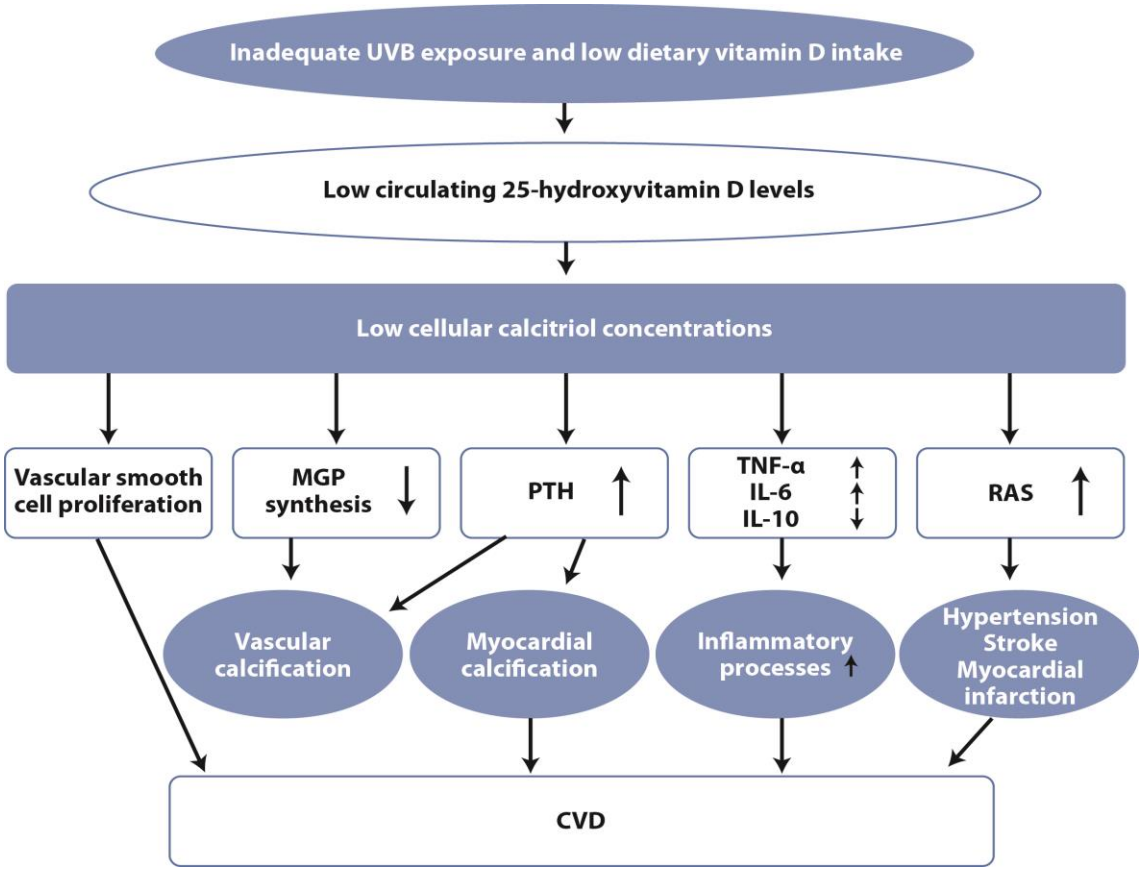


Figure 6. *Potential mechanisms for the effects of insufficient vitamin D levels on CVD. MGP indicates matrix Gla protein; PTH, parathyroid hormone; and RAS, renin angiotensin system. Adapted from Zittermann et al[46].*

1.6.1 Vitamin D and cardiovascular risk factors

1.6.1.1 Physical inactivity

In physically inactive individuals, the excess of absorbed calcium may in part be deposited in the vasculature, whereas in physically active individuals, the overflow of absorbed calcium is partly deposited in the skeleton and large amounts are excreted via sweat[125]. Compared with sedentary individuals, physically active subjects have higher 25(OH)D levels, higher intestinal calcium absorption rate and higher dietary calcium intakes.

1.6.1.2 Obesity

Obesity, particularly visceral obesity, is inversely associated with the serum 25(OH)D concentration[82, 126]. Uptake into adipose tissue and skeletal muscle accounts for the rapid postprandial disappearance of vitamin D from the circulation and probably also explains that increased adiposity causes sequestering of vitamin D and lower levels of 25(OH)D[99]. Vitamin D deposited in fat tissue is not easily available, and obese individual may require larger than usual doses of vitamin D supplements to achieve a serum 25(OH)D level comparable to individuals with normal weight. Serum 25(OH)D levels increase when obese individuals lose body fat[127]. Candidate gene studies examining the association of SNPs in genes implicated in vitamin D metabolism with obesity traits found that vitamin D pathway genes are unlikely to have an important impact on obesity, suggesting that obesity promotes a poor vitamin D status[128].

1.6.1.3 Smoking

Smoking has been shown to have a significant effect on calcium and vitamin D metabolism[123]. Smokers had on average an approximately 10% decrease in circulating levels of 25OHD and $1,25(OH)_2D$, and a 20% decrease in PTH. The mechanisms whereby smoking could decrease circulating levels of PTH and vitamin D metabolites remain to be defined[123].

1.6.1.4 Dyslipidemia

Results regarding the role of vitamin D in blood lipid regulation are inconsistent. In one study, levels of 25(OH)D were inversely associated with presence of multiple metabolic risk factors including the plasma LDL cholesterol concentration, irrespective of BMI, but only in male subjects[129]. Conversely, in the Women´s Health Initiative study, 5-year treatment with vitamin D and calcium did not induce any change in lipid parameters[130]. In overweight subjects, vitamin D supplementation resulted in decrease in triglycerides whereas LDL cholesterol levels were either decreased or unchanged[130, 131].

1.6.1.5 High BP

Studies examining the association between circulating 25(OH)D and risk of future hypertension found that individuals with the lowest levels of 25(OH)D had a RR for hypertension of 6.13 (95% confidence interval (CI), 1.00-37.8) for men and 2.67 (95% CI, 1.05-6.79) for women after adjustment[132]. Also, the Third National Health and Nutrition Examination Survey found that 25(OH)D levels are inversely associated with systolic and diastolic BP after adjustment[133]. A meta-analysis including 18 studies

found a pooled odds ratio of hypertension of 0.73 (95% CI 0.63-0.84) when comparing the highest with the lowest quartiles of 25(OH)D[134]. However, results from three, large independent prospective cohort studies concluded that higher intake of vitamin D (diet and supplementation) is not associated with lower risk of contracting hypertension[135]. A randomized study where patients with hypertension were allocated to either exposure to vitamin D-producing UVB spectrum of light or placebo showed a significant fall in BP of 6 mmHg and a mean increase in 25(OH)D concentrations of 162%[136]. Furthermore, vitamin D supplementation reduced systolic BP in elderly women by 9.3%[137]. In contrast, meta-analysis of 10 trials of vitamin D supplementation (doses from 400 to more than 5000 IU/day) found no significant effect on systolic BP[118].

1.6.2 Evidence from observational and interventional studies

In a meta-analysis of 9 prospective studies of incident cardiovascular events, a pooled hazard ratio (HR) of 1.64 (95% CI (95% CI): 1.27-2.11) was reported for subjects with low 25(OH)D[75]. The two largest studies of serum 25(OH)D concentration in relation to incident cardiovascular events (based on the Framingham Offspring Study and the Health Professionals Follow-up Study) showed an approximately 2-fold increased risk in subjects with levels under 37.5 nmol/L[122, 138]. However, only 5 out of the 9 individual studies included in the meta-analysis reported a significantly increased risk in subjects with low serum 25(OH)D concentration[75, 79]. Another meta-analysis comprising 19 prospective studies with 6,123 CVD cases and 65,994 participants reported a pooled RR of 1.52 (95% CI: 1.30-1.77) for total CVD and 1.38 (95% CI: 1.21-1.57) for CHD, when comparing the lowest with the highest quartile of 25(OH)D concentrations[76]. Differences between studies with respect to age, outcome measures and exposure definitions as well as different adjustments for confounders and seasonal variation of 25(OH)D might have contributed to the discrepant findings.

A RCT of vitamin D supplementation reported a statistically non-significant reduction in CVD events[139]. Similarly, the Women´s Health Initiative Calcium-Vitamin D trials, in which 36,282 women were randomized to calcium 1000 g/d plus vitamin D 400 IU/day or placebo for 7 years, found no reduction in CHD[140]. It has been suggested that the vitamin D dose used was too low[140]. It has also been speculated that combined vitamin D and calcium supplementation modestly increases the risk of cardiovascular events[140, 141].

There is evidence suggesting an association between low vitamin D concentrations and subclinical atherosclerosis. Cross-sectional studies on the relationship between serum 25(OH)D and cIMT, have shown conflicting results[142-146]. Inconsistent results have also been found in studies examining relationships of vitamin D metabolites and the vitamin D metabolism gene *CYP24A1* to CAC[126, 144, 147-149].

1.7 Other diseases and vitamin D

Vitamin D deficiency has been associated with increased risk of common cancers[150-154] and autoimmune diseases[155], such as multiple sclerosis[156, 157], rheumatoid arthritis[158], and type 1 diabetes[159]. Epidemiological studies have provided support for an association between peri-natal vitamin D supplementation and risk of later type 1

diabetes[160], recent genetic studies suggesting a causal relationship[161]. Cohort studies suggest a link between 25(OH)D status and a wide range of outcomes, including the metabolic syndrome[162], lung function and respiratory infections[163], but causal associations have not yet been demonstrated. In a RCT, patients with chronic obstructive pulmonary disease (COPD) were largely unaffected by vitamin D supplementation, but in patients with baseline 25(OH)D levels below 25 nmol/L, COPD exacerbations were significantly reduced[164].

1.8 Vitamin D and genetics

Vitamin D exerts its actions on target tissues through its binding to the nuclear VDR, a member of the steroid/thyroid hormone receptor family that functions as a transcriptional activator of many genes[165]. VDR is expressed in a large number of tissues and cells, including those involved in the regulation of glucose metabolism (muscle and pancreas) and atherosclerosis (macrophages, SMC and endothelial cells)[79].
VDR polymorphisms are generally located in intronic regions and have unknown effects, thus hindering understanding of the results of most VDR association studies[165]. Genome wide association studies report three genes contributing to the variability of serum concentrations of 25(OH)D[166, 167] *(Figure 7)*. Two of them encode enzymes involved in vitamin D metabolism; the *DHCR7* gene which encodes a reductase catalyzing the conversion of 7-DHC to cholesterol and the *CYP2R1* gene, which codes for a member of the cytochrome P450 superfamily of enzymes that catalyze many reactions involved in the synthesis of cholesterol, other lipids, steroids and the enzyme primarily responsible for the hydroxylation of vitamin D to 25(OH)D. The third locus maps to the gene region *GC* (group-specific component or Gc globulin), which encodes the vitamin D binding protein[166, 167].

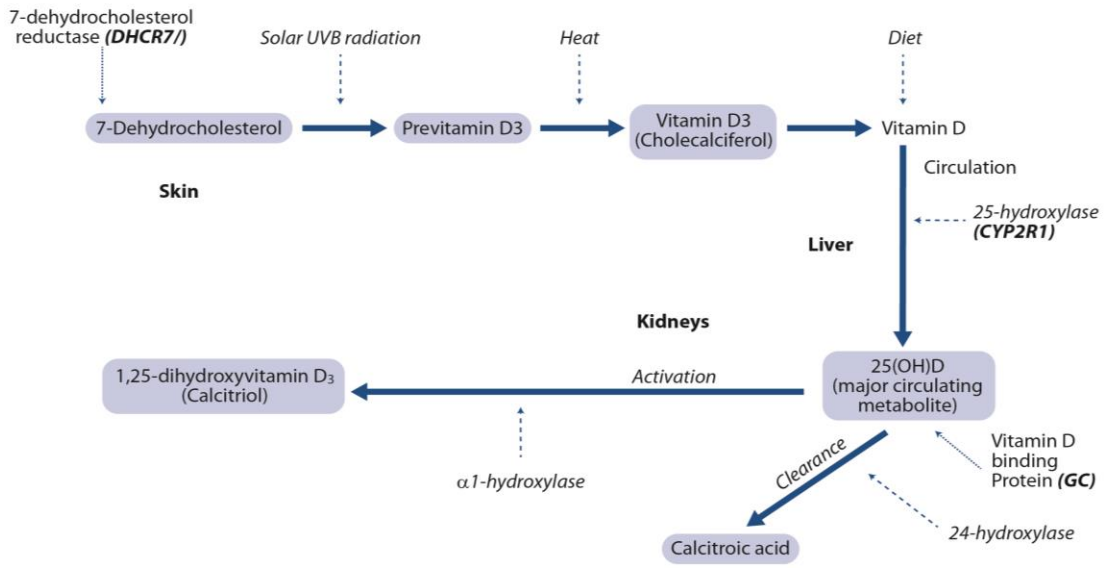


Figure 7*. Vitamin D synthesis and metabolism. Genetic variants in three loci are implicated in vitamin D metabolism; DHCR7, CYP2R1 and GC.*
Adapted from Berry et al[168].

2 AIMS

Overarching hypotheses

Deficiency of serum vitamin D increases the risk of CVD by predisposing T2D and/or accelerating coronary and carotid artery atherosclerosis

I. To investigate whether low serum 25-hydroxyvitamin D concentrations predict the development of prediabetes (impaired fasting glucose and/or impaired glucose tolerance) and type 2 diabetes
II. To examine the relationships of serum 25-hydroxyvitamin D concentration to established and emerging cardiovascular risk factors and risk of myocardial infarction
III. To determine whether the serum 25-hydroxyvitamin D concentration is associated with the severity and rate of progression of cIMT
IV. To elucidate whether vitamin D is causally implicated in CVD in persons with or without diabetes, using vitamin D-associated genetic variants, serum 25-hydroxyvitamin D concentration and measures of cIMT severity and rate of progression

3 MATERIAL AND METHODS

The work presented in this thesis is based on three epidemiological studies:

- The population-based cohort of the Stockholm Diabetes Prevention Programme (SDPP)
- The population-based case-control study of the Stockholm Coronary Atherosclerosis Risk Factor study (SCARF)
- A prospective cohort study of European high-risk individuals (acronym: IMPROVE, Carotid Intima-Media Thickness (IMT) and IMT-Progression as predictors of Vascular Events

3.1 The Stockholm Diabetes Prevention Program (SDPP)

SDPP is a prospective population-based cohort study[12, 26]. The study population consists of residents from five suburban municipalities in the Stockholm county: Sigtuna, Tyresö, Upplands-Bro, Upplands Väsby (only women), and Värmdö. The enrolment started in 1992 and ended in 1998. The sample was collected in two steps to obtain a sample where about half of the participants had diabetes in the family. First, a questionnaire was sent to all men and women in the age group of 35-55, asking about presence of close relatives with diabetes, i.e. a family history of diabetes (FHD), defined as at least one first-degree or at least two second-degree relatives with T2D. Persons with diabetes and unclear FHD were excluded. In a second step, subjects with FHD together with subjects randomly selected among those without FHD, matched for age and municipality, were invited to a health examination. Participants underwent blood sampling, a standardized OGTT, body measurements and answered a detailed

questionnaire about life-style factors. The final baseline group comprised 7,949 middle aged subjects (3,128 men and 4821 women). After 8-10 years, the baseline study group, (excluding those with newly diagnosed diabetes at baseline), was invited again to a health examination. The total follow-up study comprised of 2,383 men and 3,329 women, representing 76.2% and 69.1% respectively, of the baseline study population and they were reinvestigated with a similar procedure as at baseline. In the vitamin D substudy, associations between 25(OH)D concentration and abnormal glucose regulation were evaluated in a nested case-control setting. The study population consisted of women and men aged 35-56 years at baseline. The follow-up examination included individuals who at baseline had either NGT or prediabetes (IFG and IGT), but not those who had newly diagnosed T2D. Serum concentrations of 25(OH)D were measured in samples drawn at baseline in all participants with normal glucose regulation, or prediabetes, who developed either prediabetes or T2D at follow-up, and in an equal number of participants, randomly selected within sex but pair-matched by exact age, from the group who had NGT at both baseline and follow-up. Analyses were performed in three separated groups; two prospective and one cross-sectional. In prospective study 1, cases were individuals who had progressed from NGT at baseline to either prediabetes (304 women, 428 men) or type T2D (47 women, 87 men) at follow-up, or from prediabetes at baseline to T2D (53 women, 92 men) at follow-up. Controls were 404 women and 607 men with NGT at both baseline and follow-up. In the prospective study 2, which included a subset of the participants in prospective study 1, all cases had NGT at baseline and progressed to either prediabetes (304 women 428 men) or T2D (47 women, 87 men) at follow-up; the controls were 351 women and 515 men with NGT at both baseline and follow-up. A cross-sectional study was performed, in which the cases were all individuals who had prediabetes at baseline, including not only those who progressed to T2D, but also those who had prediabetes at follow-up, a total of 139 women and 184 men. In this analysis, the controls were all participants with NGT at baseline, irrespective of glucose tolerance at follow-up, giving a total of 841 women and 1,214 men.

3.1.1.Measurements

3.1.1.1 Seasonal variation in serum 25(OH)D concentration

Serum 25(OH)D levels were measured at baseline in all studies and determined by chemiluminescence immunoassay (LIAISON 25 OH vitamin D TOTAL, DiaSorin). This assay was used in all four studies (paper I-IV). Because there is a seasonal variation in concentrations of 25(OH)D and to adjust for variations across seasons, the months were categorised into four quarters: November–January, February–April, May–July and August–October. Linear regression analyses were performed with 25(OH)D as the dependent variable and the four quarters coded as dummy variables, using quarter November-January as the reference (the sunlight exposure in Sweden has the fewest sun hours in the course of these months) and independent variables. Estimated β-coefficients were then used to adjust all individual 25(OH)D values for seasonal variation. Seasonal adjusted 25(OH)D levels were calculated in the same way in paper I-IV.

3.1.1.2 Measurement of IGFBP-1 and IGF-1
IGFBP-1 was measured in serum using an in-house RIA with intra- and inter-assay (CV) values of 3% and 10%, respectively[169]. Serum IGF-1 was measured by RIA after acid-ethanol extraction and cryoprecipitation, using des(1–3)IGF-1 as a tracer to minimise interference by IGFBPs, intra- and interassay CVs were 4% and 11%, respectively[169].

3.1.1.3 Classification of glucose tolerance
The 75 g OGTT was performed at baseline and at follow-up examination. Glucose regulation was defined according to American Diabetes Association guidelines[28], using a fasting serum glucose value of ≥5.6 mmol/l as the cut-off value for IFG *(Table 1)*.

3.1.1.4 Classification of risk factors and potential confounders
Physical activity during leisure hours was categorised into three groups: low, middle and high. BMI was also divided into three groups (<25.0, 25.0–29.9 and ≥30.0 kg/m2). Waist circumference was categorised into three groups (<80, 80–87 >87 cm for women, and <94, 94–101 and >101 cm for men). Tobacco use was described as either never/former use or current tobacco use. FHD was positive when at least one first-degree (parents or siblings) or two second-degree relatives (grandparents, uncles or aunts) had been diagnosed with diabetes, or negative when no close relative had diabetes. Socioeconomic position was categorised into four groups (high, middle, low and self-employed). Finally, education was categorized into low, middle and high. High BP was defined as a systolic BP ≥140 mmHg and/or a diastolic BP ≥90 mmHg and/or the use of antihypertensive treatment.

3.1.2 Statistics
We calculated ORs with 95% CIs in the multiple logistic regression analyses, to evaluate the association between serum 25(OH)D concentrations and abnormal glucose regulation. In all analyses, seasonal adjusted 25(OH)D levels were used categorised into quartiles or used as a continuous variable and reported per increment of 10 nmol/l. Categorisation into quartiles was performed according to the distribution between all participants. Potential confounders were physical activity during leisure time, BMI, waist, tobacco use, FHD, socioeconomic position, education and BP. The logistic regression models used were: model 1 adjusted for age (and sex when combining male and female participants), and model 2 adjusted for age, BMI, FHD, physical activity and BP (and sex if applicable). Combinations of 25(OH) D and IGF-I or IGFBP-1 were analysed with dichotomised variables at their medians. Only those with data on all variables were included in the study. The analyses were performed using SAS statistical package version 9.2 for Windows (SAS Institute, Cary, NC, USA).

3.2 Stockholm Coronary Atherosclerosis Risk Factor Study (SCARF)

The SCARF study is a population-based case control study of MI conducted in three hospitals in the northern part of Stockholm (Danderyd hospital, Karolinska hospital and Norrtälje hospital)[170]. Patients below 60 years of age who were admitted for an acute MI to any of the three hospitals during the period of January 1996 to December 2000 were screened for inclusion in the study of biochemical risk markers for CAD. A total of

755 MI patients were screened, 433 entered and 387 completed the study. Age-matched control subjects were recruited from the general population of the same county. Three months after the index cardiac event, patients and matched control subjects were interviewed about background facts, such as ethnicity, social situation, life-style characteristics, medical history and medication, and a medical examination was performed. Blood samples were drawn on the same occasion under fasting conditions. Patients included at the Danderyd and Norrtälje hospitals were also offered routine coronary angiography, of whom 243 accepted to be included in the coronary angiography substudy.

From 57 patients with MI participating in a cohort study of the long-term outcome after thrombolysis, serum samples of 25(OH)D concentrations were assessed to evaluate consistency over time[171]. Patients were selected at random (out of samples from the 222 patients enrolled) for examination of serum 25(OH)D concentration at two time-points after the onset of MI (at <4 h and 3 months).

3.2.1 Measurements

3.2.1.1 Established and emerging cardiovascular risk factors
Evaluation of risk indicators was performed and included evaluation of plasma concentrations of total-, LDL- and high-density lipoprotein (HDL), triglycerides, glucose, insulin, proinsulin, haemostatic factors and inflammation markers. Measurement on BP, BMI, tobacco use and diabetes were collected. Hypertension was defined as a previous diagnosis of hypertension or as BP above 160 mmHg systolic and/or 95 mmHg diastolic, smokers being categorized into current smokers vs. former/never smokers. All participants were categorized into Nordic vs. non-Nordic ethnicity.

3.2.1.2 Coronary angiography
Angiograms were analyzed by quantitative coronary angiography (QCA; Medis QCA-CMS system) performed if needed for clinical reasons during the initial hospital stay or routinely 3 months later[170]. The coronary artery tree was divided into 15 segments according to American Heart Association (AHA) guidelines[170]. Minimum lumen diameter (MLD), reference diameter, percentage diameter stenosis, mean segment diameter (MSD), segment length, plaque area, segment area and number of significant (>50%) stenosis were measured in each segment[170].

3.2.2 Statistics
25(OH)D was adjusted for seasonal variation by allocating the sample date to one of four seasons (February-April, May-July, August-October and November-January, as described above), using only the control subjects as the basis for adjustment to avoid any confounders of disease in relation to the seasonal pattern. All exploratory variables were standardized to facilitate comparisons of effect size across variables. Univariable and multivariable regression analyses were performed to investigate the relationships of 25(OH)D to clinical and biochemical risk indicators. Best subset analysis was used to select variables to be tested for inclusion in the multivariable model. For assessing the association between serum 25(OH)D concentration and MI (calculation of odds ratio

(OR), logistic regression models were used, adjusted for established and emerging risk indicators, instead of conditional logistic regression models, since a few of the initially selected controls had to be replaced due to incomplete data. LDL cholesterol was not included in the multivariable analyses since no differences between cases and controls were observed after exclusion of patients on lipid-lowering treatment (n=135, 35% of the group). In the subset of patients examined by QCA, the relationships of serum 25(OH)D level to mean MLD, mean MSD, mean percentage stenosis and plaque area were examined using the same linear regression approach as for clinical and biochemical risk indicators. Serum 25(OH)D determinations in samples drawn at the time of the MI event and three months after the MI in the same patients were compared with respect to representativeness using a BlandeAltman plot[172]. All statistical analyses were performed using SAS statistical package version 9.2 for Windows (SAS Institute Inc., Cary, NC, USA).

3.3 The Carotid Intima-Media Thickness (cIMT) and cIMT-progression as predictors of vascular events in a high-risk European population (IMPROVE) study

The IMPROVE study is a multicentre, longitudinal cohort study, investigating cIMT in a high-risk European population[77]. The study involves seven centres in five European countries (Finland, France, Italy, the Netherlands and Sweden). A total of 3,711 individuals were recruited (48% men and 52% women), aged 55 to 79 years, with presence of at least three vascular risk factors and no history of CVD, of whom 3,430 were included in the present study[77, 173]. Participants underwent carotid ultrasound examination at baseline, month 15 and month 30. Blood samples, clinical data and information about life-style factors were collected at baseline.

3.3.1 Measurements

3.3.1.1 Classification of 25(OH)D and risk factors for CVD

The serum concentration of 25(OH)D was determined at baseline and classified as deficient (<25 nmol/L), insufficient (25-75 nmol/L) and sufficient (>75 nmol/L)[46, 81, 85]. Hypertension was defined as a systolic BP ≥140 mmHg and/or diastolic BP ≥90 mmHg and/or the use of antihypertensive treatment. Diabetes was defined as FPG concentration >7mmol/L or treatment with insulin or oral glucose-lowering treatment. Information about smoking habits were collected, including duration of smoking and average number of cigarettes smoked per day. Cumulative life-time smoking was expressed as a 5-level categorical variable according to never-smoker status and quartiles of pack-years. Physical activity was categorized as low, medium or high level[77]. Estimated glomerular filtration rate (eGFR) was calculated according to the CKD-EPI creatinin equation[174].

3.3.1.2 Carotid ultrasonography

Measurements of cIMT were acquired by using a Technos system (Esaote, Genoa, Italy), equipped with a 5-10Mhz linear array probe and a dedicated software (M´Ath, Metris SRL France[175]), and recorded on VHS videotape. Both sonographers and readers had been trained and certified prior to the start of the study. The ultrasonographic measurements included the mean and maximum IMT of the common carotid arteries (CC-IMT_{mean} and CC-IMT_{max}), the mean and maximum IMT of the bifurcations (Bif-IMT_{mean} and Bif-IMT_{max}), the mean and maximum IMT of the internal carotid arteries (ICA-IMT_{mean} and ICA-IMT_{max}), the mean and maximum IMT of the whole carotid tree (IMT_{mean} and IMT_{max}) and the average of the maximum IMT values across the whole carotid tree ($IMT_{mean-max}$) (*Figure 8 and 9)*. In addition, a measure of total plaque area in the whole carotid tree, both left and right side, was obtained from measurements based on 3 projections per each cm of the CC, Bif and ICA segments. The projection associated with the thickest profile was identified in each carotid segment.

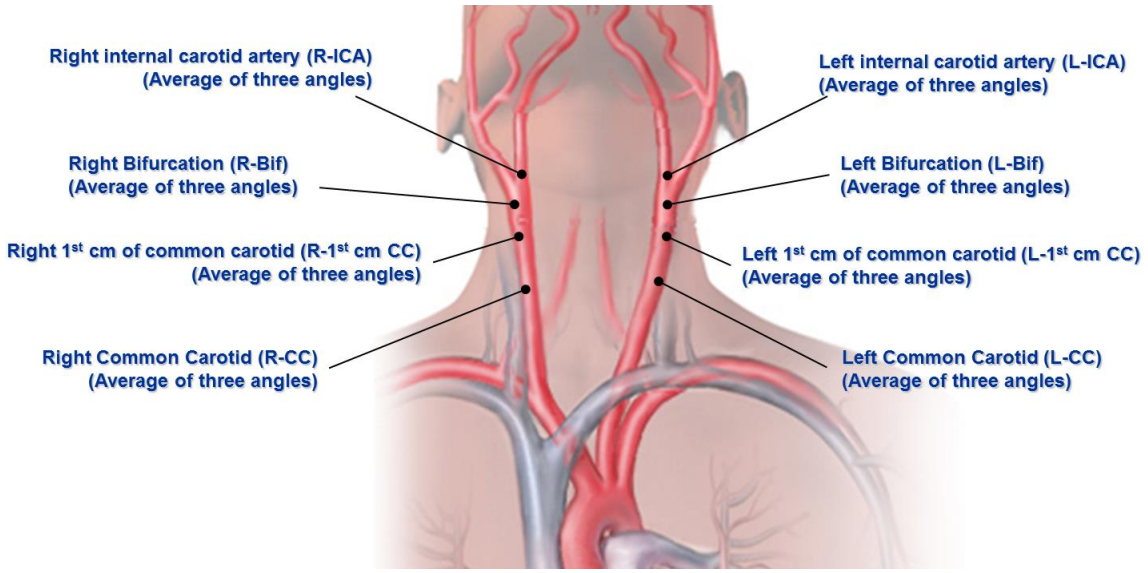
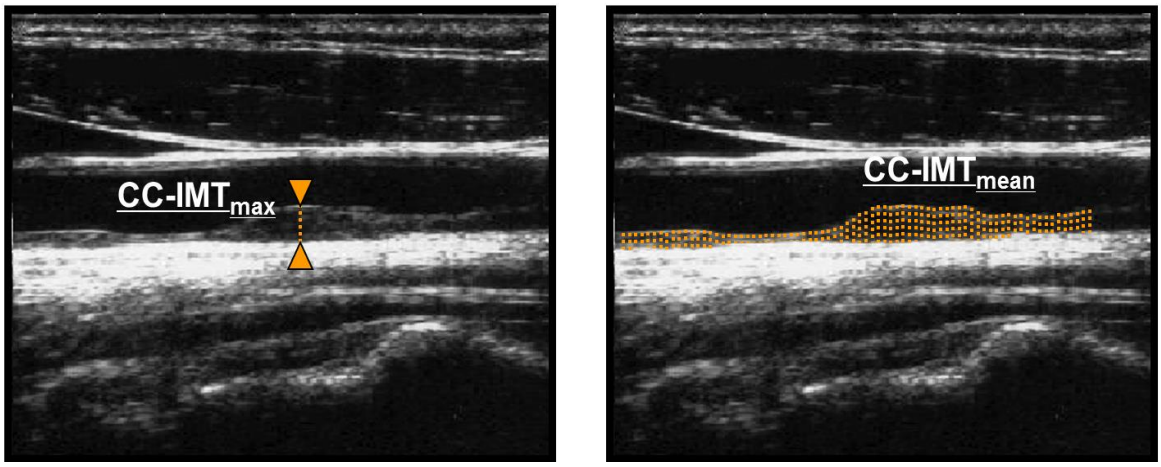


Figure 8*. Carotid artery ultrasound examination. Four segments were investigated for each carotid artery (common carotid, 1st cm of common carotid, bifurcation and internal carotid artery. Each one of these variables represents the mean value of measurements acquired from three projections. Figure obtained by courtesy of Dr.Damiano Baldassarre,Dipartimento di Scienze Farmacologiche e Biomoleculari, Universita di Milano, Milan, Italy.*

If the local maximum IMT exceeded 1.5 mm, the corresponding local mean IMT multiplied by 14 mm (standard length of each segment measured) was used as an approximation of the plaque area. In subjects with >1 plaque, total plaque area was calculated as the sum of all plaque areas. Change in ultrasonographic measurements over time (progression; in mm/year for cIMT and mm^2/year for total plaque area) was calculated by linear regression considering all three time-points (0, 15 and 30 months, with the time-points of the repeat measurements expressed in days from the baseline evaluation).

***Figure 9**. Measurements and calculations of cIMT. For each segment, two values were generated: the mean and the maximum. For example CC-IMT$_{max}$ and CC-IMT$_{mean}$ Figure produced by Dr. Damiano Baldassarre.*



3.3.2 SNP selection and genotyping

Based on GWA studies, SNPs associated with serum 25(OH)D levels from three loci, *GC*, *CYP2R1* and *DHCR7*, were selected[167] [166]. Two SNPs (*Figure 10*) in each locus were included to provide better coverage of the region. From the HapMap CEU population, 2 haplotype blocks were identified in the *GC* and *CYP2R1* loci, which were only covered by the reported lead SNPs; thus, a further SNP in each locus was included to cover the region. The reported lead SNPs in *DHCR7* were located in independent haplotype blocks. Accordingly, both SNPs were included (*DHCR7* is located close to the *NADSYN1* gene (NAD synthetase-1 gene).

SNPs in *CYP2R1*and rs7041 in *GC* were included in the Illumina Immunochip 200K genotyping platform[176]. Genotyping and calling of the Immunochip were carried out at the Uppsala SNP Genotyping platform (Uppsala, Sweden) and quality control measures included exclusions for low SNP call rate (<95%), deviation from Hardy Weinberg equilibrium ($p \leq 0.001$), cryptic relatedness or failure of sex checks. Taqman SNP genotyping assays were used for genotyping genetic variants in the *DHCR7* locus and rs2282679 in the *GC* locus and called using the SDS 2.2.1 software (both from Applied Biosystems, California). Quality control was for the Taqman-genotyped data was consistent with that applied to the Immunochip SNPs. After quality control, 3,418 subjects with both genetic data and 25(OH)D measurements were available for analysis.

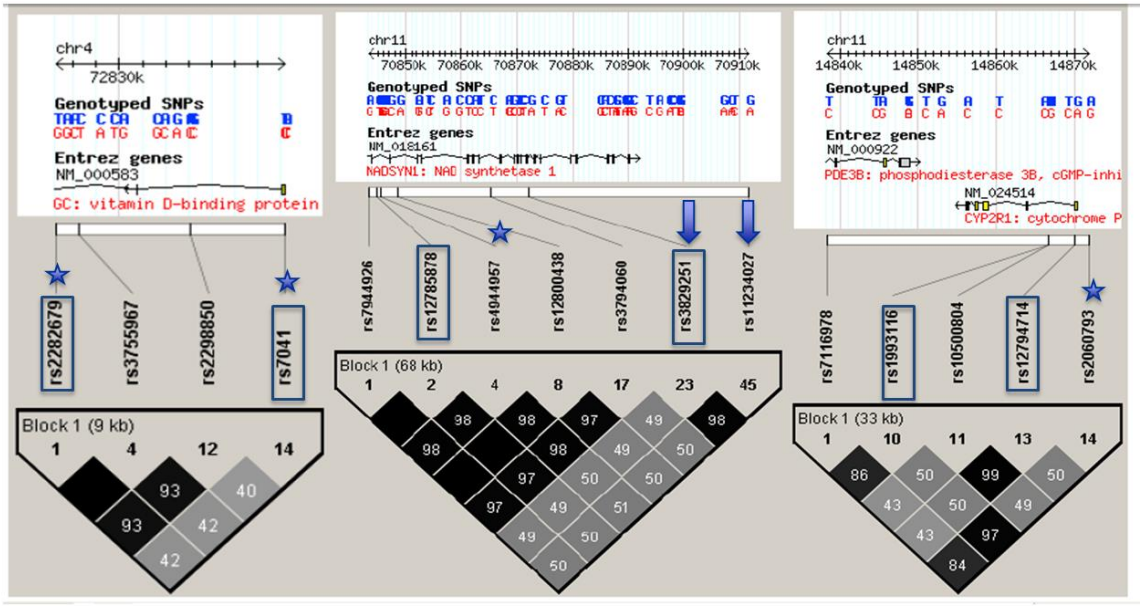


Figure 10. Linkage disequilibrium between SNPs robustly associated with vitamin D levels in Wang et al. (Common genetic determinants of vitamin D insufficiency. Lancet 2010[166]). Stars indicate SNPs reported by Ahn et al. (Genome-wide association study of circulating vitamin D levels. Human Molecular Genetics 2010[167]) and arrows SNPs unique to Ahn et al. Colours and values are r2. Boxes indicate SNPs included in this study. Figure prepared by Dr. Rona Strawbridge, Atherosclerosis Research Unit, Department of Medicine Solna, Karolinska Institutet, Stockholm, Sweden.

3.3.3 Statistics

3.3.3.1 Serum 25(OH)D and IMT

The serum 25(OH)D concentration was adjusted for season and latitude to account for differences in time of blood sampling and sunlight hours, in analyses with 25(OH)D as dependent variable, and for categorization of individuals into groups with sufficient, insufficient and deficient 25(OH)D status. Associations between 25(OH)D levels and latitude were analyzed by the Jonckheere-Terpstra test for ordered alternatives[177]. Stratified analyses were performed by gender, diabetes and statin treatment. IMT measurements used in the analyses were CC-IMT_{mean}, CC-IMT_{max}, (not including the first centimetre closest to the bifurcation), Bif-IMT_{mean}, Bif-IMT_{max}, ICA-IMT_{mean}, ICA-IMT_{max}, IMT_{mean}, IMT_{max} and $IMT_{mean-max}$. In regression analyses of 25(OH)D against baseline and progression measures of carotid IMT, adjustments were made for age and gender in a basic model, and potential confounders not believed to be in the causal pathway (latitude, waist (as marker of adiposity), high physical activity, current smoking, and eGFR) were added in an extended model. The number of subjects with non-missing data available for regression analyses using the extended model ranged from 3,375 to 3,407 for baseline cIMT measures (out of the total n=3,430), and from 3,001 to 3,022 for cIMT progression measures (out of the n=3,042 subjects with cIMT progression data). No adjustment for multiple testing was performed since baseline cIMT and cIMT progression measurements were strongly correlated. A two-sided p-value <0.05 was

considered significant for all analyses. STATA 11.1 (StataCorp LP) and SAS 9.2 for Windows were used for the statistical analyses (SAS Institute Inc., Cary, NC, USA).

3.3.3.2 Vitamin D-associated genetic variants and IMT

Linear regression analysis was used to assess the impact of 25(OH)D-associated SNPs on serum 25(OH)D and cIMT levels, assuming an additive genetic model. IMT measurements used in the analyses were CC-IMT_{mean}, CC-IMT_{max}, (not including the first centimetre closest to the bifurcation), Bif-IMT_{mean}, Bif-IMT_{max}, $IMT_{mea,}$ IMT_{max}, and $IMT_{mean-max}$. Since vitamin D levels differ between subjects with and without T2D and to evaluate whether the potentially causal role of vitamin D in CVD differs between subjects with and without T2D, analyses were stratified by T2D status. Thereafter, the two strata were combined and a SNP-by-T2D interaction term was included. The basic model adjusted for population substructure (by multi-dimensional scaling), age and gender. The extended model included other established risk factors (waist-hip ratio, levels of LDL cholesterol, HDL cholesterol and triglycerides, CRP, smoking (quartiles of pack years) and lipid-lowering therapy. Statistical analysis was carried out using PLINK[178] and STATA (STATcorp, Texas, USA). A two-sided p-value $<0.05/6 = 0.0083$ was taken as statistically significant (Bonferroni-correction for 6 uncorrelated SNPs).

3.3.4 Gene expression analysis

The ASAP study[179] was investigated to analyse differential expression of mRNA according to genotype in liver and aorta tissues. Tissue-extracted RNA was hybridised to Affymetrix ST 1.0 exon arrays, and RMA-normalised before log2 transformation. DNA was extracted from whole blood and genotyped on the Illumina 610w-Quad bead array platform. SNPs with >95% call rate were used for imputation, and quality scores of MACH <0.3 was used to filter out poorly imputed SNPs. Analysis assumed an additive model for association between SNPs and gene expression. A two-sided p-value < 0.0028 was considered statistically significant (Bonferroni correction for testing 6 SNPs in 3 tissues).

3.4 Ethics

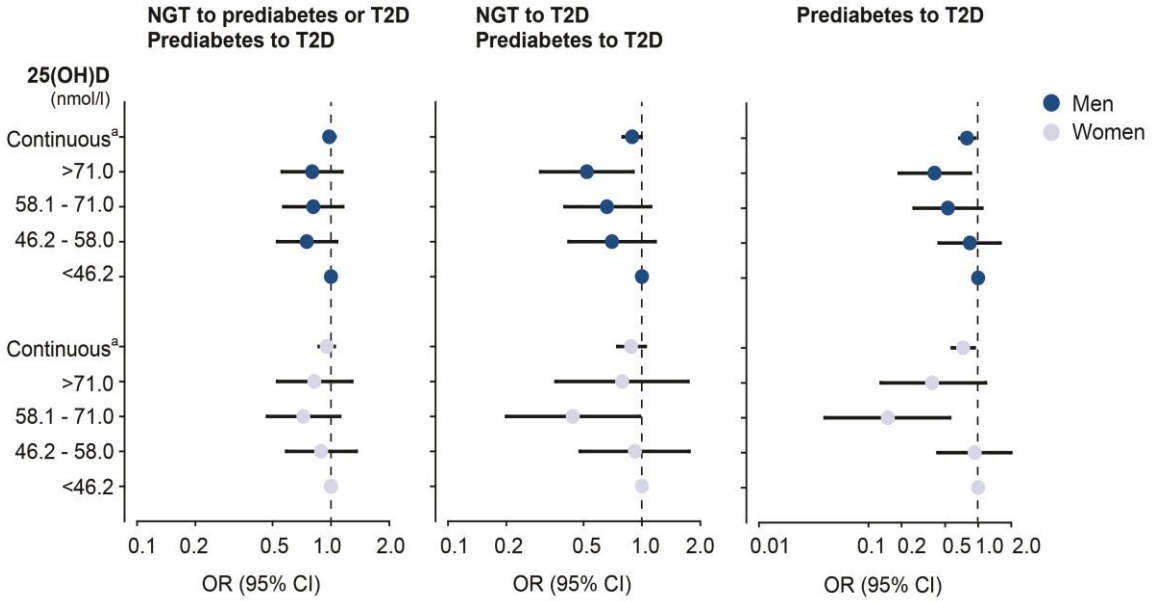
All studies were conducted according to the Declaration of Helsinki and the protocols were approved by the local ethics committees. All patients gave their informed consent to participation.

4 RESULTS

Study I
The Stockholm Diabetes Prevention Program (SDPP)
(Published in Deleskog A et al. *Diabetologia.*2012;55:1668-1678)

Subjects in the highest quartile of 25(OH)D levels were at baseline more physically active, had lower 2-h glucose in OGTT and lower BMI and waist circumference compared to subjects in lower quartiles. There was a seasonal variation in serum 25(OH)D, which we adjusted for in our evaluation. In the prospective study, men, but not women, in the highest quartile of serum 25(OH)D had a decreased OR of developing T2D after adjustments (OR=0.52; 95% CI 0.30-0.90) *(Figure 11).* This effect was mainly accounted for by subjects with prediabetes at baseline. However, there was a decreased risk in women when serum 25(OH)D was used a continuous variable, and the risk reduction was almost the same as in men, being 27% vs 21% reduction for each 10 nmol/L increase in 25(OH)D. Development of prediabetes was not associated with 25(OD) D levels.

Figure 11. Prospective study

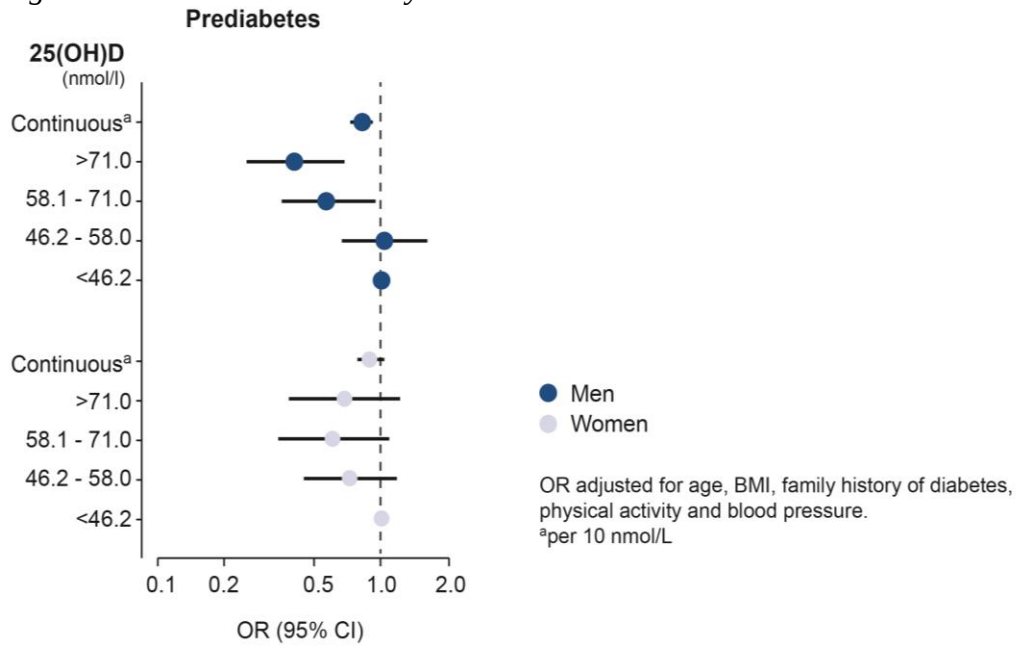


OR adjusted for age, BMI, family history of diabetes, physical activity and blood pressure.
[a]per 10 nmol/L

At baseline, subjects in the highest quartile of serum 25(OH)D had a decreased risk of having prediabetes, however significant only in men after adjustments (OR=0.41, 95% CI 0.25-0.68) *(Figure 12).* Conversely, prediabetes subjects had lower mean concentrations of 25(OH)D compared to subjects with NGT. High IGFBP-1 was a better predictor of reduced T2D risk than high 25(OH)D in both sexes, while high IGF-1 concentrations

predicted decreased risk only in men. There were no interactions between 25(OH)D and IGF system peptides with regard to diabetes prediction.

Figure 12. Cross-sectional study



Conclusions
High levels of serum 25(OH)D predict reduced T2D risk in subjects having prediabetes, but not in subjects with NGT at baseline. We found no significant interactions between 25(OH)D and IGFBP-1 or IGF-1 for diabetes risk.

Study II
Stockholm Coronary Atherosclerosis Risk Factor Study (SCARF)
(Published in Deleskog A et al. *Atherosclerosis.* 2012;223:223-229)

Serum concentrations of 25(OH)D, adjusted for seasonal variation, were lower in cases than controls (55.0 (40.0-71.0) nmol/L vs 60.5 (47.0-75.0) nmol/L). Only 30% of the healthy population-based control subjects and 20% of the cases had sufficient serum levels (≥ 75 nmol/L). High 25(OH) D levels were associated with a decreased risk of MI (OR 0.80, CI 0.69-0.93; p=0.003) (*Table 2)*. However, the association with MI disappeared entirely after adjustment for established and emerging risk factors (OR: 1.09 (0.90-1.33) (*Table 2*). There were two main factors, plasma glucose and HDL cholesterol, that particularly attenuated the effect of 25(OH)D seen in the univariable analyses.

Table 2. Odds ratios for the risk of MI

Unvariable analysis			
Variable	**OR**	**95%CI**	**p-value**
Season-adjusted 25(OH)D	0.80	0.69-0.93	0.003
Waist	1.35	1.16-1.56	<0.001
BMI	1.54	1.32-1.79	<0.001
Smoking	3.15	2.31-4.27	<0.001
Triglycerides	2.16	1.76-2.67	<0.001
LDL-cholesterol*	1.15	0.97-1.40	0.106
HDL-cholesterol	0.33	0.27-0.41	<0.001
Insulin	2.16	1.64-2.84	<0.001
Proinsulin	2.37	1.77-3.18	<0.001
Glucose	4.09	2.92-5.73	<0.001
CRP	2.15	1.60-2.89	<0.001
Fibrinogen	1.79	1.52-2.11	<0.001
IL-6	1.27	0.95-1.70	0.114
Cystatin-C	0.95	0.82-1.09	0.462
PAI-1	1.34	1.15-1.58	0.001
Hypertension	8.22	5.13-13.15	<0.001
Non-Nordic ethnicity**	1.86	1.39-2.49	<0.001

Multivariable analysis			
Variable	**OR**	**95%CI**	**p-value**
Season-adjusted 25(OH)D	1.09	0.90-1.33	0.389
Smoking	2.47	1.65-3.69	<0.001
HDL-cholesterol	0.36	0.27-0.46	<0.001
Glucose	3.51	2.27-5.45	<0.001
Fibrinogen	1.26	1.03-1.53	0.026
Hypertension	5.50	3.15-9.60	<0.001

Univariable and multivariable logistic regression analyses with MI as the outcome variable, in which the risk associated with low 25(OH)D and other risk markers of CHD were evaluated. Variables to be included in the multivariable logistic regression analysis were selected by best subset analysis. Age, sex and season-adjusted 25(OH)D were forced into the model. Results were expressed as standardized odds ratios (ORs) with confidence interval (CI). 25(OH)D, serum 25-hydroxyvitamin D concentration; BMI, body mass index; LDL, low-density lipoprotein, HDL, high-density lipoprotein; CRP, C-reactive protein, PAI-1, plasminogen activator inhibitor-1; IL-6, interleukin-6.
** Patient on lipid-lowering therapy were excluded.*
*** Non-nordic ethnicity (subjects originating from countries outside of Scandinavia).*

Univariable and multivariable analyses of serum 25(OH)D association to cardiovascular risk factors were performed separately in cases and controls (*Table 3*). Significant inverse relationships were found in both patients and controls between 25(OH)D and waist circumference, BMI, current smoking and plasma levels of triglycerides, glucose and PAI-1 activity. In controls, 25(OH)D was inversely correlated with plasma LDL

cholesterol, insulin and proinsulin. Conversely, significant associations with 25(OH)D were encountered for HDL cholesterol (positive), cystastin C and non-nordic ethnicity (inverse) in cases only. Current smoking and plasma levels of proinsulin and PAI-1 activity were independently associated with 25(OH)D in controls. The corresponding variables in patients were waist circumference, plasma triglycerides and PAI-1 activity, cystatin C and non-Nordic ethnicity.

Table 3. Relationships of serum 25(OH)D concentration to clinical and biochemical risk indicators

	Controls		Cases	
Variable	**Beta-coefficient***	**p-value**	**Beta-coefficient***	**p-value**
Waist	-	-	-3.17	0.008
Smoking	-8.09	0.001	-	-
LDL-cholesterol**	-1.72	0.114	-	-
Triglycerides	-	-	-4.16	<0.001
Proinsulin	-4.51	0.029	2.04	0.049
Glucose	-	-	-1.50	0.125
PAI-1	-5.92	<0.001	-2.46	0.032
Cystatin-C	-	-	-2.06	0.042
Non-Nordic ethnicity***	-	-	-18.42	<0.001

Multivariable linear regression analysis with 25(OH)D used as a continuous variable. Established and emerging risk factors with p<0.20 in univariable analysis were considered for inclusion in multivariable analysis. Variables to be included in the multivariable analysis were selected separately in cases and controls by best subset analysis, using the Akaike information criterion. Age and sex were forced into the model. BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; CRP, C-reactive protein; PAI-1, plasminogen activator inhibitor-1; IL-6, interleukin-6.

** Continuous exploratory variables have been standardized as effect per standard deviation unit.*

*** Patients on lipid-lowering therapy were excluded, and LDL cholesterol only considered for inclusion in the multivariable analysis in controls.*

**** Non-Nordic ethnicity (subjects originating from countries outside of Scandinavia).*

Conclusion

Vitamin D insufficiency was associated with a range of cardiovascular risk factors. In the univariable analyses, serum 25(OH)D appeared to be protective, but after having taken other risk factors into account, serum 25(OH)D concentrations were not independently related to premature MI.

Study III

Serum 25(OH)D concentration in subclinical carotid atherosclerosis

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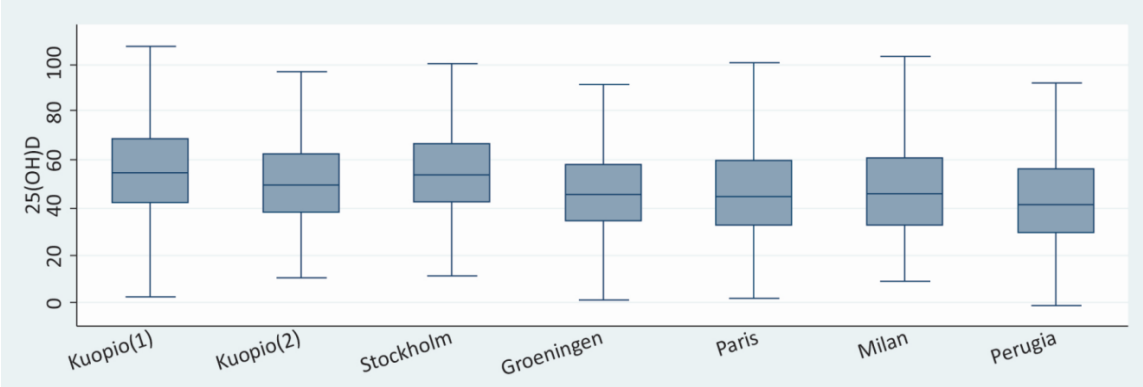
The serum 25(OH)D concentration adjusted for season and latitude was 7 % higher in men than in women (49.6 (37.2-62.7) vs. 46.2 (34.1-60.2) nmol/L, p<0.001).
Of the entire study population, 8% had deficient, 82% had insufficient and 10% sufficient 25(OH)D levels. Subjects having deficient levels of 25(OH)D (<25 nmol/L) were more often women, obese and smokers, had more frequently diabetes, higher BP, triglycerides, CRP and eGFR, had lower HDL cholesterol and were less physically active. Furthermore, in the multiple robust regression analyses of risk indicators related to 25(OH)D concentration, waist circumference, diastolic BP, triglycerides, HDL cholesterol, LDL cholesterol, current smoking and high physical activity showed significant and independent associations with 25(OH)D.

Serum 25(OH)D levels differed significantly across centers and were positively correlated with latitude (*Figure 13*). To evaluate the extent to which various risk factors explained the variability of 25(OH)D levels between different centers, we included variables independently related to 25(OH)D in a robust regression model. The included variables accounted for a total of 10% of the variation in serum 25(OH)D concentrations. More than a third of the cohort had diabetes (n=900). Subjects having diabetes had lower serum 25(OH)D levels than subjects without diabetes (47.8 nmol/L vs. 50.8 nmol/L).

Figure 13. Serum 25(OH)D across the European centers



Serum 25(OH)D levels differed across centers and were positively correlated with latitude (Jonckheere Terpstra χ = 166.643, p<0.001).

In the linear regression analyses investigating the relationships of serum 25(OH)D levels to baseline cIMT with adjustment for age and sex, significant positive associations were found with IMT_{mean}, $IMT_{mean\text{-}max}$, $CC\text{-}IMT_{mean}$, $CC\text{-}IMT_{max}$, bifurcation IMT_{mean} and $bif\text{-}IMT_{max}$. After additional adjustment for latitude, waist, high physical activity, current smoking, and eGFR, all significant positive associations disappeared (*Table 4*).

Table 4. Relationships of serum vitamin D concentration to baseline severity and rate of progression of cIMT

Males and Females		Baseline severity			Progression	
	r	β (95% CI)	p-value	r	β (95% CI)	p-value
IMT mean	0.019	0.78 (-0.058; 0.215)	0.261	-0.003	-0.005 (-0.061; 0.050)	0.856
IMT max	0.010	0.075 (-0.183; 0.333)	0.567	-0.019	-0.148 (-0.430; 0.133)	0.302
IMT mean-max	0.011	0.047 (-0.098; 0.192)	0.526	-0.001	-0.003 (-0.093; 0.088)	0.950
CC-IMT mean	0.033	0.111 (-0.004; 0.227)	0.058	-0.035	-0.047 (-0.094; 0.005)	0.052
CC-IMT max	0.018	0.101 (-0.084; 0.286)	0.283	-0.019	-0.086 (-0.247; 0.074)	0.292
ICA-IMT mean	0.016	0.106 (-0.116; 0.329)	0.349	-0.003	-0.011 (-0.142; 0.120)	0.869
ICA IMT max	0.015	0.120 (-0.146; 0.386)	0.375	-0.014	-0.111 (-0.386; 0.165)	0.430
Bif-IMT mean	0.007	0.048 (-0.183; 0.278)	0.684	0.017	0.059 (-0.066; 0.184)	0.356
Bif-IMT max	-0.009	-0.079 (-0.380; 0.221)	0.605	0.016	0.124 (-0.154; 0.401)	0.382

Linear regression adjusting for age, sex, latitude, waist, high physical activity, current smoking, and eGFR. β and CI limits should be multiplied by 0.001. Because baseline IMT measures were log-transformed but progression measures were not, a tabulated β=0.1 means that a change in 25(OH)D of 1000 nmol/L corresponds to a baseline IMT change of 10% and an IMT progression of 0.1 mm/y, respectively. Measurements of baseline IMT were log-transformed to achieve a more symmetrical distribution. 25(OH)D indicates 25-hydroxyvitamin D adjusted for season; Bif-IMT, bifurcation intima-media thickness; CC-IMT, common carotid intima-media thickness; ICA-IMT, internal carotid artery intima-media thickness; IMT, intima-media thickness (whole carotid tree); r, partial correlation coefficient; β, regression coefficient and CI, confidence interval.

In the stratified analyses performed on baseline cIMT measurement, serum 25(OH)D levels were positively associated with CC-IMT_{mean} and CC-IMT_{max} in men, in subjects with diabetes and subjects not using statins with CC-IMT_{mean}. For progression measures, significant inverse associations were found between 25(OH)D and IMT_{mean} and CC-IMT_{mean} and IMT_{max} in subjects with diabetes, and an inverse association was seen with CC-IMT_{mean} in subjects without statin treatment. In addition, there was no association of 25(OH)D levels with baseline severity or progression measurements of plaque area.

Conclusions
Serum 25(OH)D levels differed across Europe and were highest in the North. Variation in established and emerging risk factors were significantly associated with serum 25(OH)D levels, but there were no consistent and independent associations between serum 25(OH)D levels and cIMT measures. Of note, patients having diabetes had an increased IMT progression.

Study IV
A serum 25(OH)D concentration-associated genetic variant in 7-dehydrocholesterol reductase (DHCR7) interacts with T2D status to influence subclinical atherosclerosis.
(Strawbridge RJ*, Deleskog A* et al. *Equal contribution. Manuscript)
In the linear regression assuming an additive model, investigating the influence of SNPs on vitamin D levels, SNPs in the genes encoding vitamin D binding protein *(GC*, rs2282679 and rs7041) and *DHCR7/NADSYN1* (rs12785878 and rs3829251) were

negatively associated with 25(OH)D levels. Effect sizes and significance of associations between SNPs and 25(OH)D levels differed between subjects with and without T2D *(Table 5)*. For the *DHCR7* locus, the effect size was 2-fold higher among persons with diabetes compared with subjects without diabetes. However, no significant interaction was detected.

Table 5. Effect of SNPs on season-adjusted 25(OH)D levels, stratified by T2D status, assuming an additive allele effect model

			Non Diabetic subjects					Diabetic subjects				
CHR	SNP	A1	MAF	β	L95	U95	P value	MAF	β	L95	U95	P value
4	rs2282679	G	0.25	-0.077	-0.106	-0.048	3.00E-07	0.24	-0.073	-0.120	-0.026	0.0024
4	rs7041	A	0.40	-0.062	-0.087	-0.037	1.47E-06	0.38	-0.053	-0.095	-0.012	0.0118
11	rs1993116	A	0.38	0.035	0.009	0.062	0.0084	0.41	0.008	-0.033	0.050	0.7010
11	rs12794714	A	0.46	-0.015	-0.040	0.010	0.2441	0.41	-0.010	-0.051	0.030	0.6280
11	rs12785878	G	0.33	-0.032	-0.059	-0.005	0.0201	0.31	-0.074	-0.119	-0.030	0.0011
11	rs3829251	A	0.21	-0.035	-0.066	-0.004	0.0290	0.20	-0.076	-0.126	-0.025	0.0035

CHR, chromosome; SNP, single nucleotide polymorphism; A1, minor allele; MAF, minor allele frequency; β, regression coefficient; L95, lower limit of 95% CI; U95, upper limit of 95% CI. All analyses were adjusted for population structure, age and gender. Statistical significance at p<=0.0083 (multiple testing for 6 SNPs).

Table 6. Effect of SNPs by T2D status interaction on progression of c IMT, assuming an additive allele effect model

				Basic model				Established model + 25(OH)D			
	CHR	SNP	A1	β	L95	U95	P value	β	L95	U95	P value
IMT mean	4	rs2282679	G	0.000	-0.005	0.004	0.8926	0.000	-0.004	0.004	0.9616
	4	rs7041	A	0.001	-0.002	0.005	0.4385	0.002	-0.002	0.005	0.4009
	11	rs1993116	A	0.003	-0.001	0.007	0.1285	0.004	0.000	0.008	0.0520
	11	rs12794714	A	-0.002	-0.006	0.002	0.2842	-0.002	-0.006	0.001	0.1990
	11	rs12785878	G	0.004	0.000	0.008	0.0664	0.003	-0.001	0.007	0.0935
	11	rs3829251	A	0.006	0.002	0.011	0.0080	0.006	0.002	0.011	0.0072
IMT mean-max	4	rs2282679	G	-0.003	-0.010	0.004	0.3613	-0.002	-0.009	0.005	0.4976
	4	rs7041	A	0.000	-0.006	0.006	0.9538	0.000	-0.006	0.006	0.9835
	11	rs1993116	A	0.004	-0.002	0.010	0.2163	0.005	-0.001	0.011	0.1230
	11	rs12794714	A	-0.003	-0.009	0.003	0.3757	-0.003	-0.009	0.003	0.2982
	11	rs12785878	G	0.005	-0.001	0.012	0.0941	0.005	-0.001	0.012	0.1092
	11	rs3829251	A	0.011	0.004	0.019	0.0022	0.012	0.004	0.019	0.0022

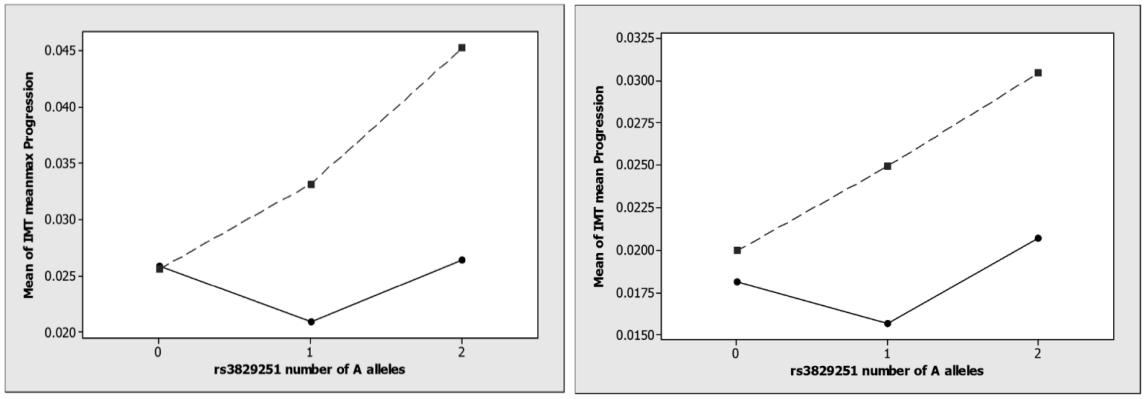
CHR, chromosome; SNP, single nucleotide polymorphism; A1, minor allele; β, regression coefficient; L95, lower limit of 95% CI; U95, upper limit of 95% CI. Basic model adjusting for age, gender, T2D and population structure; established model adjusting for age, gender, type 2 diabetes, population structure, WHR, LDL, HDL, TGs, CRP, lipid-lowering therapy and smoking. P-value for statistical significance p<0.0083.

In the linear regression analyses of the effect of SNP x diabetes interaction on cIMT, the 25(OH)D-decreasing allele, SNP rs 382951 in *DHCR7*, interacted with T2D to significantly influence progression of cIMT, resulting in increased IMT_{mean} and $IMT_{mean-max}$ measures (*Table 6*). These associations were independent of established CVD risk factors and serum 25(OH)D level. There was no association between any SNPs and cIMT at baseline. Figure 14 shows number of this allele (rs3829251) in relation to cIMT progression in T2D and non-T2D, clearly illustrating that there is an interaction.

The variation of 25(OH)D explained by the extended model was 9% in non-diabetic subjects and 12% in persons with diabetes, respectively. Addition of the 25(OH)D-associated SNPs (rs2282679, rs7041, rs12785878 and rs3829251) to the extended models explained an additional 2% and 3% of the variation of 25(OH)D levels in non-diabetic and diabetic subjects, respectively.

In addition, gene expression analyses showed that the 25(OH)D-lowering alleles of both rs12800438 (proxy for rs12785878) and rs3829251 were associated with lower levels of *DHCR7* mRNA.

Figure 14. Genetic variant in DHCR7 interacts with T2D status to influence progression of composite IMT measures (in mm)



Crude means for each number of minor alleles according to diabetes status category. Dashed line; subjects with T2D and solid line; subjects without T2D.

Conclusions

SNPs in the locus encoding the vitamin D-binding protein (*GC*) and the *DHCR7* gene were inversely associated with serum levels of 25(OH)D. Only rs3829251 in *DHCR7* influenced progression of subclinical atherosclerosis, depending on T2D status but independently of 25(OH)D levels.

5 DISCUSSION

Over the last decades, vitamin D deficiency has gained attention in the fields of prevention of CVD and T2D, primarily because of the extra-skeletal pleiotropic effects of this hormone and the association of low vitamin D with insulin resistance, T2D and increased cardiovascular risk[86, 101, 112]. However, epidemiological data has been conflicting. Therefore, to further elucidate the role of vitamin D, we explored the relationship between serum 25(OH)D concentration and outcomes in three independent studies in which participants differed with respect to age, gender, risk factors, diagnoses (T2D and MI) and severity of disease.

Study I
The Stockholm Diabetes Prevention Programme (SDPP)

We examined whether serum 25(OH)D concentration is associated with risk of developing prediabetes and T2D. For this purpose, serum 25(OH)D levels at baseline in individuals with NGT and prediabetes who 8-10 years later developed prediabetes or T2D was compared with the corresponding measures in age- and gender-matched controls who had NGT both at baseline and at follow-up. We found that high serum 25(OH)D concentrations were associated with reduced T2D risk in subjects having prediabetes, but not in subjects with NGT. In addition, there was a positive association between prediabetes and low serum 25(OH)D concentrations in the cross-sectional study, which is in agreement with data from a substudy of NHANES III[180]. In the latter study, prediabetes was defined as either IGT or IGF, using the same cut-off plasma glucose levels in OGTT as in our study. Significant and graded associations with prediabetes were found in the two lowest quartiles of the 25(OH)D distribution, below 24.5 ng/ml, corresponding to 61 nmol/L, as compared to the highest quartile with 25(OH)D levels above 32.4 ng/ml (81 nmol/L). It is of interest to note that this association was present only in non-Hispanic whites but not in non-Hispanic blacks and Mexican Americans. Furthermore, similar to our study, the association was independent of confounders, such as BMI and BP.

Meta-analysis of prospective studies support an inverse association between serum 25(OH)D levels and incident T2D[101, 110]. The pooled RRs for incident T2D in individuals with highest vs lowest categories of 25(OH)D levels were 0.59[101] and 0.81[110], respectively. The meta-analysis reported by Forouhi et al. included only prospective studies where serum 25(OH)D levels have been measured[101]. Other strengths were the possibility to examine impact of several potential confounders, and the analyses included a large sample with over 3,600 cases[101]. There were also some limitations, for example single baseline measurement of 25(OH)D not reflecting vitamin status over a longer period, and T2D was established by self-report in several included studies, which may have led to some undiagnosed cases. The other meta-analysis by Khan et al. included studies carried out in different geographical locations across Europe, Asia and North America, improving the generalizability[110]. However, the measure of exposure was limited by the use of vitamin D intake or a single measurement of 25(OH)D levels as a proxy for the "usual" levels of vitamin D[110]. Studies measuring

vitamin D status by dietary intake are unlikely to reflect the overall vitamin D status since sun exposure is the main source of vitamin D. Separate analyses were performed of serum 25(OH)D measurement vs dietary exposure. When only studies addressing the relationship of serum 25(OH)D levels to risk of T2D were included, the pooled RR, based on 2,637 cases in the meta-analysis, became 0.75[110]. Our study had several strengths, such as a prospective design, a rather large sample size and carefully characterized participants allowing for adjustment for potential confounders. In addition, the participants were not aware of having prediabetes or T2D when exposure data was recorded; hence, disease status was unlikely to affect their behavior or life-style.

It should also be noted that participants with the highest serum levels of 25(OH)D were more physically active, had lower 2h glucose levels, were less obese and had a smaller waist circumference compared with participants in the lower quartiles of the 25(OH)D distribution, which is consistent with previous studies where vitamin D deficiency has been associated with several cardiovascular risk factors[181]. In addition, the effect of 25(OH)D levels on risk of developing T2D was only present in subjects with prediabetes and not in subjects with NGT at baseline, suggesting that other established risk factors are more crucial for the progression from normal glucose tolerance to prediabetes. Intervention studies performed on insulin sensitivity and insulin secretion with various forms of vitamin D, with or without calcium supplementation, have shown inconsistent results[182-185]. The largest intervention study conducted by the Womens Health Initiative, where 33,951 postmenopausal women without diabetes at baseline were included and assigned to treatment with either 1000 mg calcium/day plus 400 IU (10 μg) vitamin D or placebo for 7 years, did not reduce the risk of developing diabetes[119]. Similarly, the RECORD study did not show any effect of intake of 800 IU vitamin D compared with placebo on incident T2D in elderly participants[120]. Of note, these two studies were originally designed for skeletal outcomes, and the doses might have been too low to increase serum 25(OH)D to an adequate level.

Studies investigating the role of vitamin D in prediabetes concluded that in a population with vitamin D insufficiency and prediabetes, vitamin D supplementation may have a beneficial effect[186], which is in concordance with our result, indicating that it is only when prediabetes is present that low serum 25(OH)D levels are of importance for the further deterioration in glucose tolerance to T2D. Other interventional studies support these findings. For example, vitamin D supplementation improved insulin sensitivity only in subjects with prediabetes[117, 187]. Discordance in results amongst interventional studies may be explained by heterogeneity with respect to subject characteristics, study design and outcome measures. Other contributing factors can be small effect size of vitamin D action, variable vitamin D absorption, storage in adipose tissue in obese subjects and altered vitamin D metabolism and pharmacokinetics in vitamin D-deficient populations. Further limitations include small sample size, short duration of follow-up, lack of control groups and inability to achieve vitamin D sufficiency.
The mechanisms for how vitamin D might influence progression from prediabetes to T2D might include stimulation of pancreatic β-cells or enhanced insulin action. Since diabetes is considered as a state of chronic inflammation, vitamin D might also improve the glucometabolic state by modulating the effect of cytokines.

High IGFBP-1 levels were associated with reduced risk of diabetes in men and women, irrespective of 25(OH)D levels. In contrast, the Ely study demonstrated that high serum 25(OH)D concentrations were associated with improved glucose tolerance in participants with low, but not high, serum IGFBP-1 concentrations[112]. Notably, SDPP reported previously that high IGFBP-1 predicts T2D[169, 188]. Furthermore, we found that high serum IGF-1 levels predicted a reduced risk of diabetes only in men, and there were no further effects of the 25(OH)D concentration. The British cohort study found that high but not low 25(OH)D levels, combined with high serum IGF-1 levels, are associated with a decreased risk of developing components of the metabolic syndrome[162]. However, we could not confirm an interaction between the IGF system and vitamin D in our cohort.

Study II

Serum 25(OH) concentration and MI

We elucidated the relationships of serum 25(OH)D concentration to established and emerging cardiovascular risk factors and risk of MI in a population-based case-control study of MI before the age of 60 years. Our results showed that low serum 25(OH)D levels were not independently associated with MI. Population-based studies of the relationship between serum 25(OH)D concentration and risk of CVD have provided discrepant results[75, 79, 86]. A meta-analysis comprising four studies of incident CVD and five independent mortality studies demonstrated a pooled HR of 1.54 and 1.83, respectively[75]. Combining the estimates from incidence and mortality studies generated a pooled HR of 1.64. Participants in the incidence studies were initially healthy in contrast to CVD mortality studies where the general population or patients with cardiovascular symptoms or samples enriched for diabetes were studied. The two largest incidence studies provided the strongest support for an association between vitamin D deficiency and incident CVD (fatal or non-fatal CHD or MI)[122, 138]. The Health Professionals Follow-up Study exhibited 2-fold increased risk of MI in vitamin D-deficient (<15 ng/ml or 37.5 nmol/L) subjects after 10 years follow-up[138]. In the Framingham Offspring Study, severe vitamin D deficiency (<10 ng/ml or 25 nmol/L) was associated with a HR of 1.8 for a first cardiovascular event after 5 years follow-up[122]. In another meta-analysis including 19 independent prospective studies, the pooled RRs were 1.42 for CVD mortality, 1.38 for CHD, and 1.64 for stroke, comparing the lowest with the highest part of the 25(OH)D distribution[76]. The combined RR for total CVD was 1.54. In addition, the relationship of CVD risk to decreasing 25(OH)D levels was generally linear over the range of 25(OH)D from 20 to 60 nmol/L, with a pooled RR for CVD of 1.07 per 25-nmol/L decrement in 25(OH)D, when the meta-analysis was limited to studies excluding participants with existing CVD at baseline[76].

The association between 25(OH)D levels and CVD risk may be nonlinear and reach a plateau between 50 to 75 nmol/L or U-shaped, with an increase in CVD risk at 25(OH)D levels both <50 nmol/L and >125 nmol/L[76, 189]. The meta-analysis by Grandi et al, found that cardiovascular risk was increased in individuals with 25(OH)D levels below a cut-off ranging from 25 to 50 nmol/L[75]. In addition, a meta-analysis of prospective studies investigating vitamin D deficiency in relation to mortality reported a nonlinear decrease in mortality risk as circulating 25(OH)D increases, with optimal concentrations

around 75-87.5 nmol/L[189]. The data demonstrated a 31% reduction in mortality risk at levels between 75-87.5 nmol/L compared with concentrations of 27.5 nmol/L. Some researchers along with the IOM consider 25(OH)D concentrations of 50 nmol/L to be adequate for bone and overall health in healthy individuals[99, 190]. Others have pointed out that advantageous serum concentrations start at the level of 75 nmol/L and that further improvement is achieved between 90 to 100 nmol/L[96]. The SCARF study indicated that the serum 25(OH)D concentration is not an independent predictor of MI when we categorized the vitamin D status as < or ≥50 nmol/L respectively < or ≥75 nmol/L.

Two RCTs of vitamin D supplementation report a slight but non-significant reduction in CVD events[139, 191]. A recent meta-analysis including six RCTs assessing the effect of vitamin D supplementation on risk of MI showed a non-significant pooled RR of 1.02[192]. Concerns have been raised about a possible increased risk of MI due to calcium supplementation[141]. Thus, the Auckland Calcium Study in healthy older women, in which a cardiovascular event was prespecified as secondary endpoint, showed an increased rate of cardiovascular events after 5 years[193]. Furthermore, in the Women's Health Initiative, calcium supplementation was found to modestly increase the risk of cardiovascular events, especially MI[141]. Of note, the Women´s Health Initiative Study showed no effect of combined vitamin D and calcium supplementation on CVD[140]. Participants were allocated to doses of 400 IU vitamin D daily, leading to an increase of median 25(OH)D levels from 42.3 nmol/l to only 54.1 nmol/L. Supplementation with at least 1,000 IU/day would be required to attain 25(OH)D concentrations above 75 nmol/L[96]. This suggests that the vitamin D supplementation used in the Women´s Health Initiative Study was too low to result in a preventive effect on CVD. It is notable in this context that only a few studies are available so far. Other limitations are poor adherence to study medication, permission to use own calcium or vitamin D supplements and study designs primarily targeting the effect of vitamin D on fracture and not CVD[141, 194].

Our study replicated the findings of earlier studies that several established risk factors are associated with the serum 25(OH)D concentration[124, 162, 181]. In addition, serum 25(OH)D concentrations were independently associated with PAI-1 activity and proinsulin levels. PAI-1 is an inhibitor of plasminogen activation and constitutes an important regulatory protein in fibrinolysis. High plasma levels of PAI-1 have been shown to be associated with increased risk of CVD[195, 196]. However, supplementation with vitamin D failed to support a cause-and-effect relationship between serum 25(OH)D and PAI-1 levels[197]. In contrast, vitamin D analogues were found to downregulate the expression of PAI-1 in human arterial SMCs[198]. High plasma proinsulin levels, on the other hand, indicate β-cell stress due to insulin resistance, impaired β-cell function and/or abnormal secretion. Deficient levels of 25(OH)D have been suggested to affect pancreatic β-cell dysfunction. In line with this, we found an inverse association with proinsulin, which suggests an extension to the proposed mechanism behind development of T2D and CVD. An elevated plasma proinsulin concentration also predicts CAD[199, 200].

Contrary to some other prospective studies, we could not demonstrate an independent association with MI, which may be explained by the fact that our cohort was well phenotyped. Accordingly, we were able to account for a multitude of cardiovascular risk factors associated with 25(OH)D and MI. Further explanations for discrepant findings may include differences in age distribution, sample size, follow-up time, distribution of 25(OH)D concentrations and outcome measures. Our result thus either suggests that low serum 25(OH)D in persons who suffer premature MI constitutes an innocent bystander or that vitamin D insufficiency increases the risk of MI by promoting aggregation of established risk factors that in turn predispose to atherothrombosis.

Study III

Serum 25(OH)D concentration in subclinical carotid atherosclerosis

We investigated changes in cIMT measures in relation to serum levels of 25(OH)D and other established risk indicators to evaluate the role of vitamin D status in progression of atherosclerosis. Our result showed that whereas the level of circulating 25(OH)D exhibited multiple associations with established cardiovascular risk factors, there were no consistent independent associations between 25(OH)D concentrations and segment-specific or composite cIMT measures. In addition, serum 25(OH)D levels differed significantly across centers and were positively correlated with latitude. Of note, the stratified analyses in subjects with and without T2D indicated that serum 25(OH)D levels were significantly and independently associated with composite cIMT in persons with diabetes, and the effect size was considerably (8-fold) higher in subjects with diabetes compared with those without diabetes . In addition, IMT progression was increased among subjects with diabetes. Established risk factors accounted for a minor proportion of the variation in vitamin D levels, around 10%. Furthermore, differences in serum 25(OH)D concentrations existed between centers, which were not accounted for by differences in risk factor profiles. Also, the serum 25(OH)D concentration adjusted for season and latitude was 7 % higher in men than in women (49.6 (37.2-62.7) vs 46.2 (34.1-60.2) nmol/L, $p<0.001$).

Individuals living at geographical latitudes between 40° N to 60° N, such as residents in North America and Europe, show seasonal fluctuation in circulating 25(OH)D levels, vitamin D concentrations being higher in summer than winter[46]. Moreover, the synthesis of vitamin D is only possible between 10 a.m. to 6 p.m., with maximal capacity at 12 a.m. to 2 p.m.[85]. We observed a positive relationship between serum 25(OH)D levels and latitude despite the fact that the Nordic countries are at the latitude of 60° N where ultraviolet light radiation is virtually absent from October to March, whereas ultraviolet light radiation is present throughout the year in the south of Europe. Notably, highest 25(OH)D levels were encountered in Finland during winter. However, low vitamin D levels in people living in sunny climates have been observed before. The SENECA study evaluated the serum 25(OH)D concentration of elderly people living in 19 towns in Europe, distributed across a total of 12 European countries, and reported that the lowest 25(OH)D levels were encountered in South European countries (Greece: 27 nmol/L in men vs. 21 nmol/L in women compared with Norway where the corresponding figures were 61 nmol/L in men and 48 nmol/L in women)[201]. Similarly, serum

concentrations among postmenopausal women in European countries were higher in northern European compared with southern European countries[202].
In addition, 90% of subjects examined in the city of Dehli, India, had as low 25(OH)D levels as 8 nmol/L in winter and 18 nmol/L in summer[203]. A possible explanation for these counterintuitive findings is pigmented skin[81], which is more common in the South, thus requiring more sun exposure to synthesize equivalent amounts of vitamin D compared with lighter colored skin. Other possible explanations include differences in diet, vitamin D supplementation, genetic and/or socioeconomic factors or habits of exposure to the sun. Notably, individuals with dark skin have proved to have higher levels of $1,25(OH)_2D$ and lower levels of 25(OH)D than individuals with pale skin, a result of diminished cutaneous synthesis of the precursor to 25(OH)D[148, 204].

Studies on the relationship between 25(OH)D levels and cIMT have shown contradictory results. A study of 390 patients with T2D and 390 non-T2D controls showed that subjects with low 25(OH)D levels had increased cIMT compared with the controls[142]. Also, inverse relationships between 25(OH)D and cIMT measures have been observed in a cohort of older adults, where low levels of 25(OH)D were associated with increased ICA-IMT[143]. Similarly, another study of 203 older people living in North America, the Manhattan study, found that lower levels of 25(OH)D were associated with thicker plaques and greater cIMT in subjects who had plaques in the carotid vessels, whereas no associations between 25(OH)D and cIMT or plaques were encountered in the whole cohort[145]. Another study of an elderly European population with symptomatic peripheral arterial disease demonstrated an inverse association between levels of serum 25(OH)D and cIMT, which was independent of cardiovascular risk factors[121]. In contrast, no 25(OH)D-cIMT association was found in a non-diabetic Amish population[144]. Finally, a study conducted in individuals with low socioeconomic status found no evidence of an association with cIMT or presence of plaques[146].

Vascular calcification is a risk factor for cardiovascular mortality[45]. The presence in vascular SMCs of 1α-hydroxylase, which converts 25(OH)D to $1,25(OH)_2D$, suggests that vitamin D may have direct effects on the vascular wall, potentially including vascular calcification[90]. A study measuring $1,25(OH)_2D$ in 153 individuals with high risk of CHD and 13 patients with hypercholesterolemia reported an inverse association between $1,25(OH)_2D$ and coronary calcium levels determined by EBCT (electron beam computed tomography)[147]. Similarly, $1,25(OH)_2D$ independently and inversely predicted CAC quantity in a sample of 283 subjects with risk factors for CHD[148]. Low 25(OH)D levels were also associated with incident CAC in the Multi-Ethnic Study of Atherosclerosis[126]. Taken together, these results suggest that $1,25(OH)_2D$ may regulate the deposition of calcium in the vascular wall, thus inhibiting vascular calcification. The effect may be mediated through VDR-binding to vitamin D response elements in the promoters for various calcification-related genes. In addition, macrophages in the atherosclerotic lesions associated with vascular calcification express 1α-hydroxlylase activity, producing $1,25(OH)_2D$[205]. In contrast, no associations between serum 25(OH)D or $1,25(OH)_2D$ were detected in two other cross-sectional studies[144, 149]. In addition, associations have been demonstrated between vitamin D metabolites and the vitamin D metabolism gene *CYP24A1*[206], the gene product of which converts the

stored form of vitamin D to inactive 24,25-dihydroxyvitamin D. Accordingly, $1,25(OH)_2D$ enhances the metabolism of its own precursor[88].

Reasons for contradictory findings across studies may be differences in selection criteria and demographic factors, outcome measures, presence of cardiovascular risk factors and adjustment for confounders. Of note, our study represents the first with a longitudinal design.

Finally, few studies have been performed with vitamin D supplementation in prevention of atherosclerosis. However, intervention studies on vitamin D supplementation in vitamin D-deficient individuals significantly reduced plasma levels of CRP, matrix metalloproteinases 2 and 9, and tissue inhibitor of metalloproteinases 2[207], and had beneficial effects on the elastic properties of the common carotid artery in postmenopausal women[208].

Study IV

A potential causal role of vitamin D in atherosclerosis

We examined 25(OH)D-associated genetic variants, serum 25(OH)D concentration and measures of cIMT in subjects with and without T2D. Our results demonstrate a significant association between the 25(OH)D-decreasing allele of rs3829251 in the *DHCR7* locus and faster progression of cIMT, which depended on interaction with T2D status and was independent of serum 25(OH)D levels and established cardiovascular risk factors. These findings suggest that a pathway associated with the metabolism of vitamin D is causally implicated in subclinical atherosclerosis in subjects with T2D. However, the serum 25(OH)D concentration might be a poor reflection of vitamin D actions in target cells and tissues.

The three genes that contribute to the variation of 25(OH)D were chosen[166] [167]. The genes encode three key enzymes in the metabolism of 25(OH)D: *DHCR7* codes for 7-dehydrocholesterol (responsible for the availability of 7-HDC in the skin), *CYP2R1* for the liver 25-hydroxylase (involved in the conversion of vitamin D into 25(OH)D), and *GC* is the gene encoding vitamin D binding-protein.

Few studies have examined vitamin D-associated genetic variants in relation to CVD. A population-based study using SNPs associated with serum 25(OH)D concentration to explore the hypothesis that vitamin D is causally related to risk of MI, diabetes, cancer and mortality only found an association with breast cancer[209]. Similarly, a prospective cohort study including women and men scheduled for coronary angiography found no association between vitamin D-related SNPs and mortality[210].

The use of genetic markers associated with variation in vitamin D metabolism allow conclusions regarding long-term effects of vitamin D on atherosclerosis and risk of CVD. This approach circumvents the limitation of observational studies where most often serum 25(OH)D levels are measured on one occasion. In addition, the use of genetic variants as instruments for studying modifiable exposures has the potential to avoid

confounding (as the genotype is assigned at random) and is free from reverse causality since the genotype is not modified by disease[211]. However, a genetic variant may result in multiple biological alterations (pleiotropy)[211]. Our selection of SNPs was based on previous GWA studies. However, the GWA approach has limitations since risk variants identified in general have small effects (ORs between 1.05 and 1.30)[56]. Therefore, SNPs alone explain only a very small proportion of the population-attributable variance in a phenotype. Moreover, not every SNP in the genome is detected by genotyping, which means that the variants reported from GWA studies may not be the true causal variants, but through linkage disequilibrium (LD) act as proxies. In addition, genotyping in GWA studies only includes SNPs that occur commonly in the population, with minor allele frequency (MAF) >5%. Thus, low-frequency variants (MAF< 5%) that may have larger effects are not detected[56]. To identify which genes might be influenced by genotypes of certain SNPs, we examined the allele-specific expression of genes at each 25(OH)D-associated locus in relevant tissues. The gene expression analyses demonstrated that lower levels of *DHCR7* mRNA were associated with the 25(OH)D-lowering alleles of both rs12800438 (proxy for rs12785878) and rs3829251.

Participants in the top quartile of the genotype scores had a 2-fold increased risk of vitamin D deficiency[166, 167]. These results indicate that some genetic variant(s) might protect against, or increase risk for vitamin D deficiency. The *GC* polymorphism follows a strong latitude gradient, and the GC2 haplotype (combination of alleles/DNA sequences at adjacent locations, a loci) is more frequent in populations living at northern latitudes and associated with lower levels of 25(OH)D and vitamin D-binding protein[168]. Moreover, the genetic variation in *DHCR7* that is associated with higher vitamin D levels has been shown to confer a survival advantage, which may have allowed early humans to avoid severe vitamin D deficiency when migrating to northern latitude[212]. However, out of these three genes, *CYP2R1* has the highest affinity and specificity to vitamin D. A genetic polymorphism in *CYP2R1*is known to be associated with vitamin D deficiency[168].

Estimates of season and sunlight exposure have been estimated to explain up to 15% of the inter-individual variation of serum 25(OH)D, and additional characteristics such as age and adiposity account for together 21-32% of the variation of 25(OH)D[213]. Vitamin D status has shown a heritability of 28-57% in a study of twins[214]. The Framingham Offspring study found a significant heritability of 28.8% whereas 24% of the between-individual variation in 25(OH)D was explained by environmental factors such as season, vitamin D intake, waist circumference, HDL cholesterol and current use of hormone replacement therapy[215]. In our cohort, waist-hip ratio, LDL cholesterol, HDL cholesterol, triglycerides, CRP, smoking and lipid-lowering therapy explained 9% and 12%, respectively, of the variation of 25(OH)D in non-diabetic and diabetic subjects. Addition of the 25(OH)D-associated SNPs (rs2282679, rs7041, rs12785878 and rs3829251) to the variables above explained an additional 2% and 3% of the variation of 25(OH)D levels in non-diabetic and diabetic subjects, respectively.
Our study is the first to use the combination of genetic markers, serum 25(OH)D levels, progression of cIMT and change in plaque score measures in an adequately powered population to assess the role of vitamin D in atherosclerosis and to specifically address

whether T2D status influences this process. SNPs found to be robustly associated with serum 25(OH)D levels or 25(OH)D insufficiency in previous GWA studies were chosen, and the genetic architecture of each locus was taken into account. Access to relevant human target tissues also enabled us to link SNPs to allele-specific expression of neighbouring individual genes. In all, results show that a 25(OH)D concentration-associated genetic variant in *DHCR7* interacts with T2D status to influence subclinical atherosclerosis. This is the first demonstration of a causal protective role of a vitamin D-associated mechanism against atherosclerosis. The longitudinal design of the study and availability of genetic data, 25(OH)D levels and uniquely detailed cIMT phenotypes in one and the same study constitute considerable strengths in relation to previous studies of the role of vitamin D in atherosclerosis.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

Conclusions

I. A low level of circulating 25(OH)D was an independent risk factor for developing T2D in subjects with prediabetes, but not in subjects with NGT. This indicates that low levels of 25(OH)D may primarily exert an adverse effect in subjects with other predisposing risk factors, in whom the disease process is already underway. Accordingly, vitamin D supplementation should preferentially be evaluated for prevention of T2D in subjects who have attained a pre-diabetic state. Also, in line with other studies, patients with prediabetes had lower levels of serum 25(OH)D, and low levels of 25(OH)D are common in people with T2D, which indicates that vitamin D insufficiency might be an underestimated, "novel" risk factor for T2D. In addition, the association between vitamin D deficiency and T2D suggests that vitamin D exerts a direct effect on insulin action and insulin secretion. Furthermore, since diabetes and obesity are accompanied by increased levels of cytokines in the circulation, including adipokines, TNFα, IL-6 and PAI-1, which all reflect an inflammatory response, vitamin D may suppress inflammation in subjects with prediabetes.

II. In our case-control study of premature MI, the serum 25(OH)D concentration was found to be inversely associated with MI in univariable analyses. However, this association did not persist in multivariable analyses. Of note, the variables mainly responsible for attenuating the inverse association of 25(OH)D with MI were plasma glucose and HDL cholesterol. Secondly, proinulsin and PAI-1 were inversely and independently associated with 25(OH)D concentrations. High plasma proinsulin levels indicate β-cell stress due to insulin resistance, impaired β-cell function, and/or abnormal secretion. PAI-1 is an inhibitor of plasminogen activation and constitutes an important regulatory protein in fibrinolysis, and hyperinsulinemia is suggested to increase PAI-1 in patients with and without diabetes. Thus, low levels of circulating 25(OH)D may predispose to atherothrombosis by mechanisms involving β-cells and/or effects on the vessel wall.

III. There were no consistent independent relationships between the serum 25(OH)D concentration and measures of segment-specific and composite cIMT or plaque area. In addition, there was a positive relationship between 25(OH)D levels and latitude.

However, subjects with T2D had thicker cIMT and a faster rate of cIMT progression compared with subjects without diabetes, which was independent of established risk factors. The corollary is that low levels of vitamin D may accelerate the development atherosclerosis in subjects with manifest T2D.

IV. We discovered an association between the 25(OH)D-decreasing allele of the rs3829251 SNP in the *DHCR7* locus and faster progression of cIMT, which depended on the T2D status. This finding suggests that a pathway, which is associated with the metabolism of vitamin D, is causally implicated in subclinical atherosclerosis in subjects with T2D. Since the relationship between rs3829251 and cIMT progression was independent of 25(OH)D, an unknown pathway is implicated that is regulated by the same enzymatic processes. The question arises of whether low levels of 25(OH)D may be associated with vascular disease in the presence of particular variants of vitamin D-related genes and whether individuals with particular vitamin D-related phenotypes may require different recommendations in order to optimize their vitamin D status. Since 25(OH)D is the best marker of vitamin D stores and $1,25(OH)_2D$ is the active metabolite, it can be speculated that local $1,25(OH)_2D$ production in the vasculature and pancreas is primarily involved in progression of atherosclerosis and in predisposing to clinical atherothrombotic events rather than circulating levels of 25(OH)D.

Future perspectives

The incidence of T2D is rapidly increasing, and CVD remains the leading global cause of death. Simultaneously, vitamin D insufficiency is frequent in many populations, and established risk factors cannot completely explain the pathogenesis and occurrence of T2D and CVD. Therefore, additional interventional studies are necessary to clarify whether vitamin D supplementation would be protective against T2D and CVD and to determine the mechanisms behind such protection. Besides interventional and mechanistic studies, genetic association studies are warranted to assess the connection between vitamin D deficiency, T2D and CVD. Genetic studies are important to identify individuals who are more susceptible to vitamin D deficiency and to developing T2D and CVD in the population.

Interventional studies using long-term vitamin D supplementation would have to address the following issues:

- Choice of primary end-point, such as T2D, MI, stroke, hypertension, heart failure, and fatal cardiovascular event
- Consider study design to control for UVB exposure
- Decision on predefined target levels for the serum 25(OH)D concentration
- Measurement of both 25(OH)D and $1,25(OH)_2D$ concentrations
- Monitoring of serum levels of calcium, phosphate, magnesium and PTH
- Consider whether the vitamin D dose should vary across populations (dark skinned may need larger doses)
- Other factors to control are latitude, seasonal variation, body weight and physical activity

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