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Storage of vitamin D in adipose tissue and associations between vitamin D related genetic variants and diabetes, myocardial infarction, cancer, death, and low bone mineral density

Results from a high-dose vitamin D study and the Tromsø Study

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Summary

Our original contribution to existing knowledge showed that long-term cholecalciferol (vitamin D) supplementation maintains high serum 25-hydroxyvitamin D (25(OH)D) levels at 1 year after cessation, likely due to the storage of vitamin D in adipose tissue. We also evaluated the changes of 25(OH)D and vitamin D levels in subcutaneous (sc.) adipose tissue during 1 year. In addition, we identified associations between the gene variations, related to vitamin D receptor (VDR), vitamin D metabolism and the estrogen receptor (ESR), and risk of type 2 diabetes (T2D), myocardial infarction (MI) and low bone mineral density (BMD) in a population from Tromsø, Northern Norway.

Vitamin D is a biologically active substance, which acts in both skeletal and non-skeletal metabolic pathways, and is stored most in adipose tissue. Its active metabolite 1,25-dihydroxyvitamin D (1,25(OH)2D), bound to the nuclear VDR, regulates gene expression and initiates rapid responses in the cells that regulate bone and adipose tissue homeostasis, and lead to anti-diabetic, anti-atherosclerotic, and anti-carcinogenic effects. Vitamin D is found in several body tissues, including the adipose tissue. Vitamin D levels correlate with serum levels in adipose tissue, and release into the circulation is known to be slow. Nevertheless, the clinical relevance of storage of vitamin D and its metabolites in adipose tissue has not yet been established.

Therefore, we followed-up the 1-year changes in serum and fat 25(OH)D, as well as vitamin D levels, in adipose tissue after ceasing weekly (for 3–5 years) vitamin D supplementation of 20,000 IE or placebo. A total of 76 individuals underwent serum 25(OH)D measurements. In addition, 29 and 12 of these individuals had undergone optional adipose tissue biopsies at inclusion and after 1 year, respectively. The levels of serum 25(OH)D in the vitamin D group decreased less than expected, based only on the half-life, and were higher than in the placebo group during 1 year after cessation. This effect could be explained by the storage of vitamin D in adipose tissue, as the levels of fat vitamin D decreased by 52% over the follow-up period. We concluded that 25(OH)D and vitamin D stored in adipose tissue after 3–5 years of vitamin D supplementation had a clinically relevant effect on serum 25(OH)D levels the following year.

It is not only storage in adipose tissue that may be relevant for a vitamin D effect. In observational studies, the association between low serum 25(OH)D and low BMD, risk for T2D, MI, cancer, and mortality is well known. Nevertheless, randomized controlled trials (RCTs) have not shown an indisputable effect of vitamin D in non-skeletal outcomes, to date.

One possible explanation for this may be that genetic differences lead to different responses and activity of vitamin D and VDR. Therefore genome-wide association studies (GWAS) are performed, where candidate gene variations in the form of single nucleotide polymorphisms (SNPs) are identified. Nevertheless, risk SNPs may vary among different geographical areas, and GWAS may not detect all of the SNPs, if they exert only minor effects; therefore mapping of risk alleles is warranted across the populations.

Thus, in the second article we explored whether the VDR-related SNPs were associated with T2D, MI, cancer, and mortality risk in a community-based north Norwegian population. Of 6 VDR SNPs tested, only rs7968585 was a significantly associated with T2D, and possibly with MI, even after the conservative correction for multiple testing, which increased probability for type II error. The functional mechanism of this SNP has not yet been clarified, but it likely affects VDR activity or affinity to 1,25(OH)2D.

Low serum 25(OH)D levels are associated with low BMD, as well as osteoporosis, and it is considered that 50–90% of BMD can be genetically determined. Genetic, autocrine, and endocrine mechanisms mean that bone remodeling processes may likely be affected by the genes regulating vitamin D and estrogen metabolism. Although it has been reported that estrogen receptor 1 (*ESR1*) gene variations are associated with BMD, previously conducted studies had no consensus on the effect of vitamin D SNPs on BMD.

Therefore in the third article, we evaluated VDR SNPs, the SNPs associated with vitamin D metabolism, and serum 25(OH)D levels, as well as *ESR1* SNP rs4870044 as determinants for lower BMD. With regard to vitamin D-associated SNPs, only SNP rs6013897 in the gene involved in the degradation of 25(OH)D and synthesis of 1,25(OH)2D was associated with total hip BMD in the Tromsø population. The association was weak and requires confirmation in further studies. Nevertheless, *ESR1* SNP rs4870044 had a strong association with both forearm and total hip BMD, indicating that the study design was probably sufficient to discover existing associations. We also evaluated predictors for BMD, and, surprisingly, serum 25(OH)D levels were not among them, possibly due to generally sufficient vitamin D levels in our study population.

List of papers

This thesis is based on the following papers:

Paper I:

Ieva Martinaityte, Elena Kamycheva, Allan Didriksen, Jette Jakobsen, Rolf Jorde

Vitamin D stored in fat tissue during a 5-year intervention affects serum 25-hydroxyvitamin D levels the following year

The Journal of Clinical Endocrinology and Metabolism. 2017 Jul 26; jc.2017-01187*. doi: 10.1210/jc.2017-01187.*

Paper II:

Ieva Martinaityte, Rolf Jorde, Henrik Schirmer, Ellisiv Bøgeberg Mathiesen, Inger Njølstad, Maja-Lisa Løchen, Tom Wilsgaard, Ragnar Martin Joakimsen, Elena Kamycheva

Genetic Variations in the Vitamin D Receptor Predict Type 2 Diabetes and Myocardial Infarction in a Community-Based Population: The Tromsø Study

PLoS One. 2016 Sep 19;11(9):e0163573. doi: 10.1371/journal.pone.0163573.

Paper III:

Ieva Martinaityte, Rolf Jorde, Nina Emaus, Anne Elise Eggen, Ragnar Martin Joakimsen, Elena Kamycheva

Bone mineral density is associated with vitamin D related rs6013897 and estrogen receptor polymorphism rs4870044: The Tromsø Study

PLoS One. 2017 Mar 2;12(3):e0173045. doi: 10.1371/journal.pone.0173045.

Abbreviations

1,25(OH)2D – total 1,25-dihydroxy vitamin D (calciferol)

25(OH)D – total 25-hydroxy vitamin D (calciferol)

25(OH)D_2 – 25-hydroxy vitamin D_2 (ergocalciferol)

25(OH)D_3 – 25-hydroxy vitamin D_3 (cholecalciferol)

BMD – bone mineral density

CV – coefficient of variation

DBP – vitamin D binding receptor

ESR(1) – estrogen receptor (1)

GWAS – genome-wide association study

HPLC-UV – high performance liquid chromatography coupled with ultraviolet detector;

HR – hazard ratio

HbA_{1c} – glycated hemoglobin A_{1c}

LC-MS – liquid chromatography mass spectrometry

LC-MS/MS –liquid chromatography with tandem mass spectrometry

LOQ – limit of quantification

MI – myocardial infarction

OGTT – oral glucose tolerance test

PTH – parathyroid hormone

RCT – randomized control trial

Sc. – subcutaneous

SNP– single nucleotide polymorphism

T1D – type 1 diabetes

T2D – type 2 diabetes

VDR – vitamin D receptor

VDSP – The Vitamin D Standardization Program

Vitamin D – cholecalciferol

W. – week

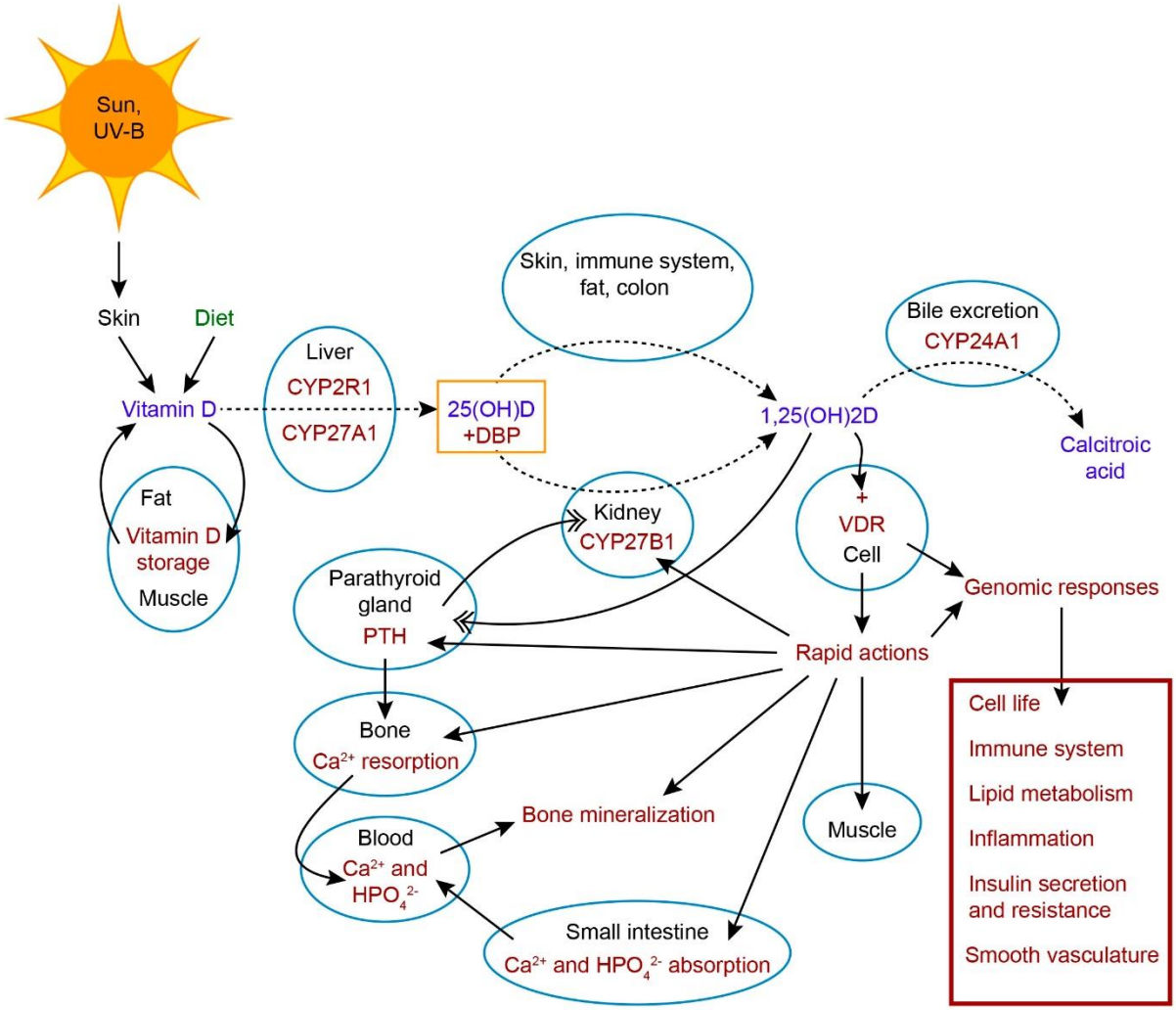
Introduction

Vitamin D: background

Vitamin D is known to be a pro-hormone with established functions in calcium and bone metabolism, and is also considered to have a positive effect on non-skeletal outcomes. The action of vitamin D is complex, and the effects of separate factors in metabolism or disease pathogenesis appear to be weak and additive, and remain under investigation. Vitamin D is still a hot topic in research, currently generating over 27,000 research articles with "vitamin D" in the title, out of which over 400 were meta-analyses and 1,700 were clinical trials. Only last year (2016), 2,406 new articles were published, and the publication rate also appears to be steady in 2017.

Vitamin D, obtained via skin production or with food (mainly fatty fish such as salmon and Atlantic mackerel, cod liver oil, or food supplementations), is either stored, mainly in the adipose tissue, or undergoes 2 hydroxylations: first to 25(OH)D in the liver, and then to physiologically active 1,25(OH)2D in the kidneys (Figure 1) (1). In addition, smaller amounts of 1,25(OH)D may be hydroxylated in the multiple other tissues, such as adipose tissue (2), placenta, bone, macrophages, keratinocytes as well as pathological tissues (3). Vitamin D and its metabolites are transported bound to vitamin D binding protein (DBP) (4). Both transported from the kidneys and locally in the tissues synthetized active 1,25(OH)2D then binds to the VDR and initiates rapid and genomic actions of vitamin D, as described below in the section '**VDR action**' (1, 5).

Figure 1. A simplified chart of vitamin D acquisition and the main metabolic pathways in selected tissues.



Vitamin D storage in adipose tissue

Although it is well-established that vitamin D is primarily stored in adipose tissue, it is also stored in smaller amounts in muscles and other tissues (6, 7). Vitamin D is gradually released when the levels of circulating vitamin D and serum 25(OH)D decline (2, 8-10). There is no consensus on whether the storage is of clinical importance, and some have argued that the amount of vitamin D stored in adipose tissue is too little to maintain adequate serum 25(OH)D levels (7), although these calculations are rough. However, in so-called vitamin D winter, when there is no UVB radiation effective for vitamin D synthesis, serum 25(OH)D levels fell by only 20–40% (when vitamin D intake was known) (11), and it is logical to assume that gradual release from the accumulated storage in adipose tissue moderates serum 25(OH)D levels (2, 10). The gradual release (in addition to the intake) also appears to explain

the prolonged calculated half-life of vitamin D metabolites, for example, serum 25(OH)D has a traced half-life of approximately 15–25 days (12), while the calculated half-life of vitamin D is around 82 days (13).

Although it is supposed that vitamin D is a stored substance, and is detected in higher levels in adipose tissue, the smaller amounts of fat 25(OH)D are likely to be important for local functions in adipose tissue (14-16). Both 25-hydroxylase, required to convert vitamin D to 25(OH)D, and 1α-hydroxylase, needed for the next conversion to 1,25(OH)2D, are expressed in adipose tissue, allowing local conversion of vitamin D to 1,25(OH)2D (2). Furthermore, degradation enzyme 24-hydroxylase is also expressed in various adipocytes (2). Activated VDR in adipose tissue regulates gene expression and is involved in adipogenesis regulation, adipocyte apoptosis, energy homeostasis incorporating leptin, and has an anti-inflammatory effect (2).

As yet, there are few data on the kinetics of vitamin D and its metabolites in human adipose tissue, and no longitudinal observations have exceeded 12 weeks. To date, two studies have been published in which vitamin D levels were followed in repeatedly taken fat biopsies (7, 15). In the first of these, Heaney et al. found that high-dose supplementation (50,000 IU/week) resulted in an increase of both serum 25(OH)D levels and vitamin D levels in sc. adipose tissue (7). In the second, a weight loss study conducted by Piccolo et al., in which there was approximately 200 IU vitamin D intake/day, no significant changes in 25(OH)D serum or fat levels were observed (15).

The other six studies describing vitamin D and its metabolites in adipose tissue present cross-sectional data for fat biopsies (14, 17-20) (Supplemental Table in Paper I). In general, the variation of fat vitamin D and fat 25(OH)D levels was large, due to small group sizes. The correlations between fat vitamin D and serum 25(OH)D, as well as serum vitamin D, were inconsistent. Therefore, a study with longer follow-up and a larger population was warranted.

Issues in evaluating vitamin D status: serum 25(OH)D

In addition to the methodological considerations, questions around what should be measured and what is a sufficient vitamin D status are the issues involved in evaluating sufficient vitamin D level. Although 25(OH)D is an inactive metabolite, its 15–25 days half-life in serum (12, 21), and higher concentration than circulating vitamin D (a half-life of 15–24 hours (22)), and 1,25(OH)2D (a half-life of 7 hours (12)), implies greater stability and convenience in measuring vitamin D status in clinical practice. Moreover, it is known that serum 25(OH)D levels are less affected by hormones and immobility than 1,25(OH)2D (12).

Nevertheless, in some cases, such as renal function impairment, 1,25(OH)2D measurements may be preferred, as normal 25(OH)D levels do not exclude vitamin D deficiency. Furthermore, extra-renal synthesis of 1,25(OH)2D in, for example, lymphoproliferative conditions, may lead to hypercalcemia, due to uncontrolled macrophage activation (23); however, these conditions are usually excluded when clinical vitamin D studies are planned.

It is difficult to identify the universal cut-off for serum 25(OH)D levels expressing sufficient vitamin D status. In general, the levels of serum 25(OH)D representing vitamin D insufficiency are defined by simultaneously higher serum parathyroid hormone (PTH) levels (12). Although there is no consensus on that issue, Tromsø Endocrine Research Group has generally considered vitamin D deficiency by serum 25(OH)D level < 50 nmol/L, and optimal serum 25(OH)D levels > 70–75 nmol/L. This choice is also supported by the review carried out by Bischoff-Ferrari, in which low serum 25(OH)D levels (< 50 nmol/L) were not associated with any beneficial effects for diverse outcomes, and high serum 25(OH)D levels (≥ 75 nmol/L) appeared to be advantageous (24).

As different techniques and assays are used to measure serum 25(OH)D, the Vitamin D Standardization Program (VSDP), internationally initiated in 2010, intends to alleviate determination of sufficient vitamin D levels, develop universal guidelines, and increase vitamin D research to higher level by having the standardized and comparable serum 25(OH)D measurements (25). The serum 25(OH)D values used in the analyses of Paper II were readjusted, based on the VSDP protocol (25).

To date, epidemiological studies have suggested that low vitamin D is associated with various conditions: obesity, diabetes, cancer, cardiovascular diseases, low BMD, autoimmune diseases, and mortality (26-30). Despite these observed associations, causality is not yet established (31, 32). Therefore, it is speculated that factors other than serum 25(OH)D levels explain the effect and action of vitamin D.

VDR action

One of the further interesting aspects in vitamin D metabolism is the VDR and its related genetics. The VDR is a nuclear receptor found in most human tissues, and is activated after binding to 1,25(OH)2D. The genomic action of activated VDR is explained by creation of a complex with the retinoid X receptor (RXR) (1). The VDR-RXR complex further interacts with the VDR responsive elements in the DNA material, and induces or represses gene expression in this manner (1, 5). The VDR is considered to modulate up to one third of

human genes (33), including those involved in the regulation of bone metabolism, cell-life processes, the immune system, and lipid metabolism (1).

The non-genomic action of activated VDR is explained by rapid responses caused by 1,25(OH)2D binding to the alternative pocket (5). The VDR for rapid response, generated within 1–45 minutes, is found in plasma membrane cavaeolae, and it has been observed that it affects calcium and chloride transport in the intestine, osteoblasts, and Sertoli cells, as well as insulin secretion (5). Both a genomic and a direct action of VDR has been observed in non-skeletal body tissues, and it is thus considered to delay multifactorial aging diseases, such as T2D, MI, cancer, and possibly mortality (5, 26-30).

The function of VDR itself is regulated by environmental, epigenetic (transient functional changes in genes without changes in the nucleotide sequence), and genetic factors, as well as a complex interaction between these factors (34). Environmental factors include sources of exogenous vitamin D and bodily functions that regulate levels of vitamin D and its metabolites; in this manner, the environment induces autoregulation of VDR (34). In addition, epigenetic and genetic factors, that is, functional and structural changes in genes, modify the environmental regulation of VDR. VDR gene-related SNPs are an example of a genetic factors, where the associations with diverse outcomes are revealed by GWAS or smaller observational studies (e.g., *VDR* SNPs associated with serum 25(OH)D levels (35)).

Genetic variations: SNPs

Nevertheless, the genetic variations associated with vitamin D may play a role in untangling the vitamin D puzzle. The complex phenotypical traits are denoted by a large number of genetic variations with small effects (36). A SNP located in the DNA is a most common genetic variation (as per the definition, it must occur in at least 1% of the population), whereby the single nucleotides differ (e.g., the sequence may include C:C, C:G, or G:G nucleotides). The small effects mean that the SNPs do not frequently reach statistical significance in the GWAS and are neglected, although, evaluated together, they account for a significant part of the complex trait and can explain the “missing-heritability problem” (which means that the genetic variations reported by GWAS explain only small fractions of the expected genetic predisposition) (36, 37). Moreover, an analysis of SNP associations may be used instead of the risk factors of interest; the so-called Mendelian randomization approach allows control for confounding (38). Finally, it has been shown that the association of particular SNPs varies in different geographical/ethnic areas (39, 40). Therefore, an

exploration of the associations between SNPs and complex phenotypical traits, such as T2D or cancer, is warranted across populations.

Vitamin D and non-skeletal effects

Considerations regarding VDR might explain causality of observed associations between low serum 25(OH)D levels and risk of T2D, MI, cancer, and mortality (26-30). VDR is expressed in the pancreatic islets (41), cardiac myocytes, and fibroblasts (42). It has been observed *in vitro* that VDR regulates insulin secretion, reduces insulin resistance by decreasing inflammation in the pancreatic islet cells, and maintains normal mitochondrial activity, epigenome, and glucose, as well as lipid homeostasis (43, 44). Of relevance to cardiovascular diseases, vitamin D appears to suppress the renin system, have an antiatherosclerotic effect, and protect against myocardium hypertrophy and heart failure, and coronary artery disease (CAD) (26, 45, 46). The anticarcinogenic effect of vitamin D is explained by modulation of cell proliferation, cell differentiation, apoptosis, inflammation, angiogenesis, and invasive/metastatic potential, although this effect appears to be suppressed by epigenetic factors in advanced disease (47).

To date, clinical intervention studies have not shown that vitamin D supplementation has an indisputable protective or positive modifying effect on tumorigenesis, MI, T2D, and mortality (26, 47, 48). In addition to the probability that vitamin D has no effect, the other possible explanation of failure to show an effect may be the multifactorial pathogenesis of the outcomes, and consequently study samples that were too small, as well as choice of study design (vitamin D doses, dosage, study population). For example, in some studies, the participants receiving vitamin D supplementation have high vitamin D baseline levels, and then relatively normal functions that are potentially related to the vitamin D. Others have argued that daily vitamin D supplementation is necessary to maintain steady vitamin D and 1,25(OH)2D levels, and that studies based on weekly vitamin D supplementation should be redone.

The associations between the outcomes of interest and SNPs modulating VDR, or other vitamin D-related metabolic processes, have been identified via candidate studies and GWAS (35, 49). Thus, SNPs associated with low serum 25(OH)D levels and T2D may indicate the risk of disease when vitamin D levels are insufficient (50). Associations between *VDR* SNPs and our clinical outcomes of interest have been reported, but the data are inconsistent. For example, many studies have reported associations between risk of cancer, as well as T1D and *VDR* SNPs *FokI*, *BsmI*, *ApaI*, and *TaqI* (35), while the risk between *VDR*

SNPs and T2D is less prominent (39, 51). Although Levin et al. reported an association between the *VDR* SNP rs7968585 and a composite outcome, including hip fracture, MI, cancer, and mortality (50), the associations between *VDR* SNPs and MI, as well as mortality, have rarely been reported, and it appears that they have not been replicated (52-54).

Vitamin D and the skeleton

Although the role of vitamin D is more established in bone metabolism (55), some unknowns remain, including the effect of SNPs. Activated VDR modulates gene expression and activation of the second-messenger systems in the parathyroid gland, small intestine, kidney, and bone, which are all involved in calcium and bone homeostasis (1). Therefore, defects in VDR (1, 56), as well as low serum 25(OH)D levels, lead to osteomalacia and osteoporosis over time (26). With its consequent fractures, and increased morbidity and mortality, osteoporosis is a world-wide epidemic, and has a great social and economic burden (57). Scandinavia has high osteoporosis and fracture rates, despite lower prevalence rates of vitamin D deficiency (58). Although some of these higher rates may be explained by more thorough health registries and a higher survival age, with consequently increased comorbidities, the question of other risk factors, such as genetics, is of interest. Several genes with VDR responsive elements, regulating bone anabolism, catabolism, and calcium homeostasis, have been identified (1). Moreover, associations between osteoporosis or fracture and some *VDR* SNPs have been reported, but unfortunately the data are inconsistent (59-61). Associations between estrogen receptor gene variation and BMD have also been reported (62, 63). The long-term clinical rationale of identifying the risk-gene variations is to evaluate the risk, then optimize and personalize the vitamin D supplementation, according to the expected vitamin D effect.

Aims of the thesis

The overall aim of the thesis was to evaluate the effect of vitamin D supplementation on serum 25(OH)D levels and storage in adipose tissue, and to explore genetic risk factors related to vitamin D and estrogen for T2D, MI, cancer, death, and low BMD in a Norwegian population.

The specific aims of the subprojects were:

- To determine the effect of high-dose vitamin D supplementation on serum 25(OH)D levels, as well as levels of vitamin D and 25(OH)D in adipose tissue at 1 year after cessation.
- To evaluate whether *VDR*-associated SNPs predict clinically important outcomes, such as T2D, MI, cancer, and mortality in a community-based Norwegian population.
- To explore whether SNPs associated with vitamin D and ESR1 predict forearm and total hip BMD.

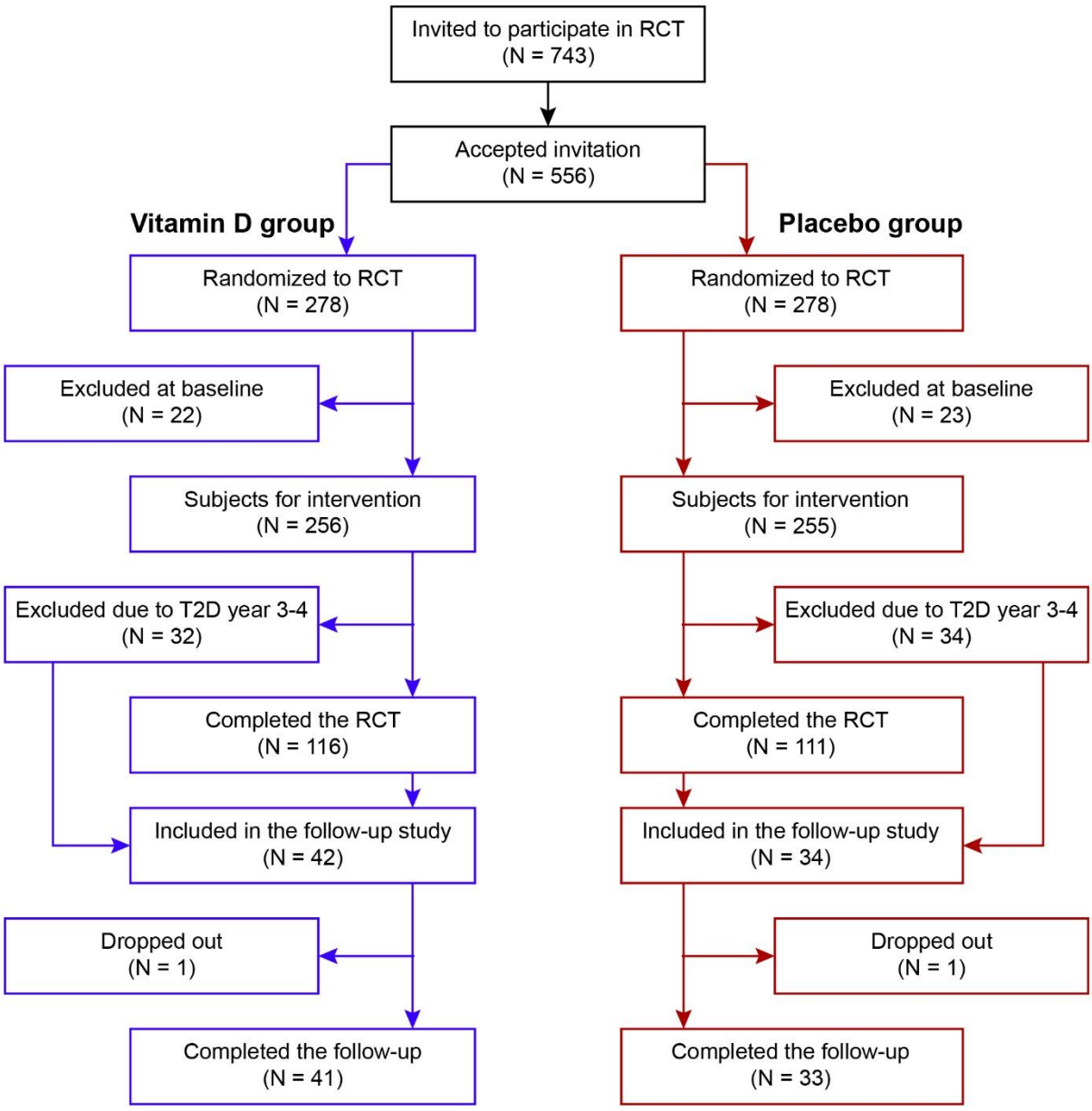
Materials and methods

Study design and study population

Paper I (Vitamin D storage in adipose tissue)

We performed a 1-year, prospective, double-blind cohort, follow-up study at the Clinical Research Unit at The University Hospital of North Norway from November 2012 until January 2015. A total of 76 individuals were recruited after finishing 5 years' participation in the "Prevention of T2D with vitamin D supplementation in subjects with reduced glucose tolerance" RCT, or after exclusion due to development of T2D based on an annually performed oral glucose tolerance test (OGTT) (32, 64) (Flowchart 1).

Flowchart 1. Study population of the fat biopsy study (Paper I).



Inclusion in the diabetes prevention study has previously been described in detail (64). In short, the participants had primarily been recruited via the 6th survey of Tromsø Study conducted in 2007–2008 (65), where those with glycated hemoglobin A_{1c} (HbA_{1c}) measured at 5.8–6.9% and without known diabetes (HbA_{1c} was not included as a criterion of diabetes at that time), as well as randomly selected participants with lower HbA_{1c} values were invited for an OGTT to diagnose those with impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) (66). In addition, some participants with diagnosed IFG/IGT were recruited from the former RCT of vitamin D supplementation in overweight and obese subjects (67) study, as well as from the Renol Ionexol-clearance Survey (68). A total of 511 included individuals were randomized to a 1:1 ratio of 20,000 IU vitamin D or placebo weekly by the Randomization Unit at our Clinical Research Unit (64). The randomization was double-blinded and not stratified, and the copy of the randomization list and codes was stored at the hospital pharmacy.

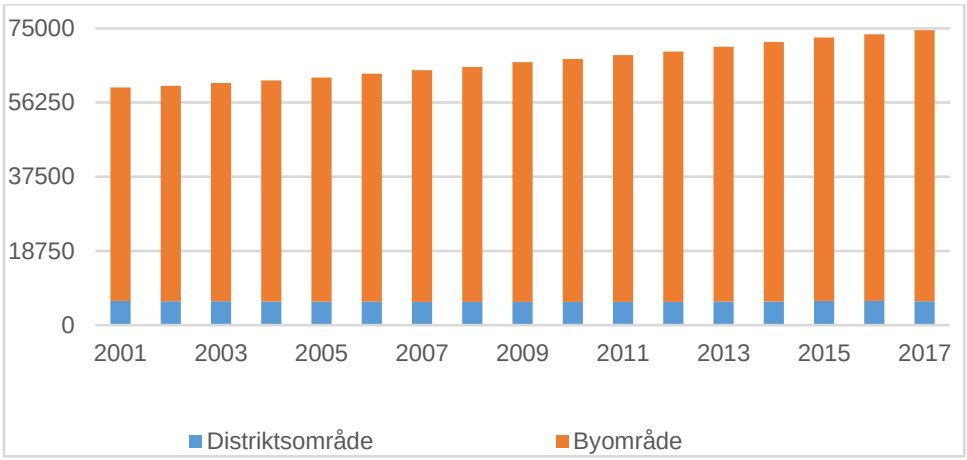
Exclusion criteria to the diabetes prevention study were primary hyperparathyroidism, granulomatous disorders, urolithiasis, cancer in the past 5 years, unstable angina pectoris, acute MI or stroke in the past year, reduced kidney function (serum creatinine >125 µmol/L in men and >105 µmol/L in women), pregnancy, lactation, and fertile age in women and no use of contraception.

During our follow-up study, the participants were not permitted to take vitamin D supplementation exceeding 400 IU/day, use a solarium more than twice a month, or plan to stay in the tropics for more than 2 months.

Paper II (SNPs as risk factors for T2D, MI, cancer, and mortality), and Paper III (SNPs as risk factors for low BMD):

The data for papers II and III were acquired from the population-based Tromsø Study. This is a unique, large-scale, longitudinal, community-based epidemiological survey that has been conducted every 6–7 years since 1974 (69). Tromsø city is located 69°N and 344 km north of the polar circle, and is a special case, as the vitamin D-winter (no skin-produced vitamin D) lasts from September until April (70). The population of Tromsø city is growing, and now stands at 74, 040 inhabitants (as of the end of the first quarter of 2017) (Figure 2).

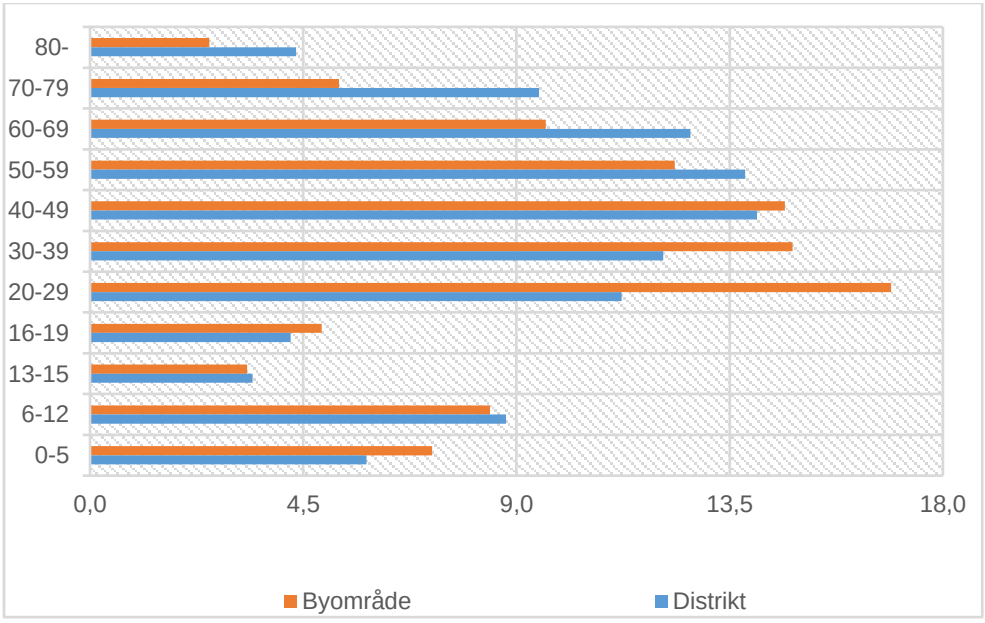
Figure 2. Population of Tromsø in the city and district areas.



Source: Statistics Norway.

Tromsø as the biggest city in Northern Norway, and it is also known as "Northern Paris", due to its beauty and cultural heritage. The local population is multiethnic, and consists of Norwegians, Samis and Kvens (exact numbers are unknown, as ethnicity is not registered for those living in Norway). Moreover, the parents of every seventh Tromsø inhabitant are immigrants, and one seventh of the population works in the health sector. A great number of young adults inhabit the city area, rather than the district area, as the Arctic University of Norway and other work and study places are centrally located (Figure 3).

Figure 3. Age profile in the city and district areas of Tromsø out of the of total population of the respective areas, as of 01.01.2017.

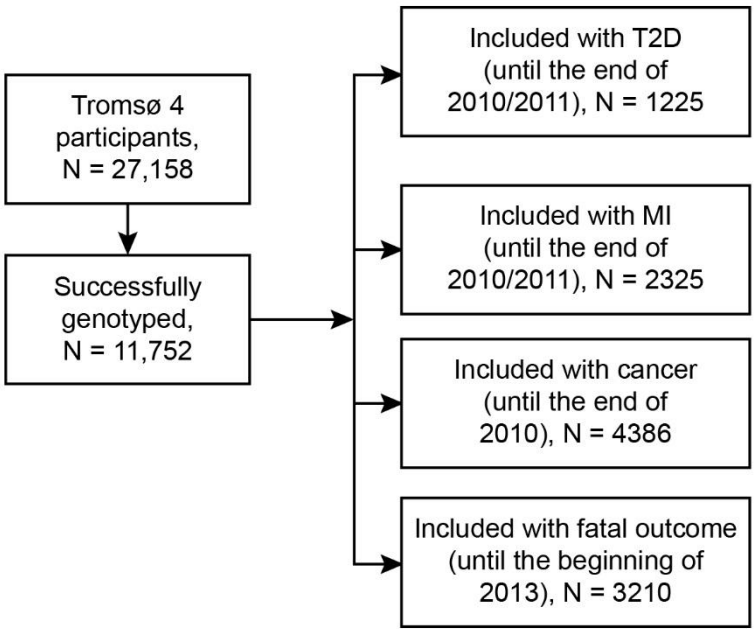


Source: Statistics Norway.

Individuals living in Tromsø and aged over 25 years were invited to participate in the 4th Tromsø survey, conducted in 1994/1995, and 77% (27,158) of those invited took part in the first visit (71).

For Paper II, 11,752 individuals from the 4th Tromsø survey were genotyped for SNPs, based on nationally and locally registered endpoints of interest: T2D, MI, cancer, or death (for T2D and MI, endpoints included until the end of 2011, fully updated until the end of 2010; for cancer, data were updated until the end of 2010, and for the death register, updated until the beginning of 2013), as well as randomly selected individuals (72). Limited financial capacity meant that the case-cohort or nested cohort study design was used, where a randomly assigned control group was taken from the same cohort as a specific endpoint group (73). The number of endpoints and at least one successfully analyzed *VDR* SNP are shown in Flowchart 2.

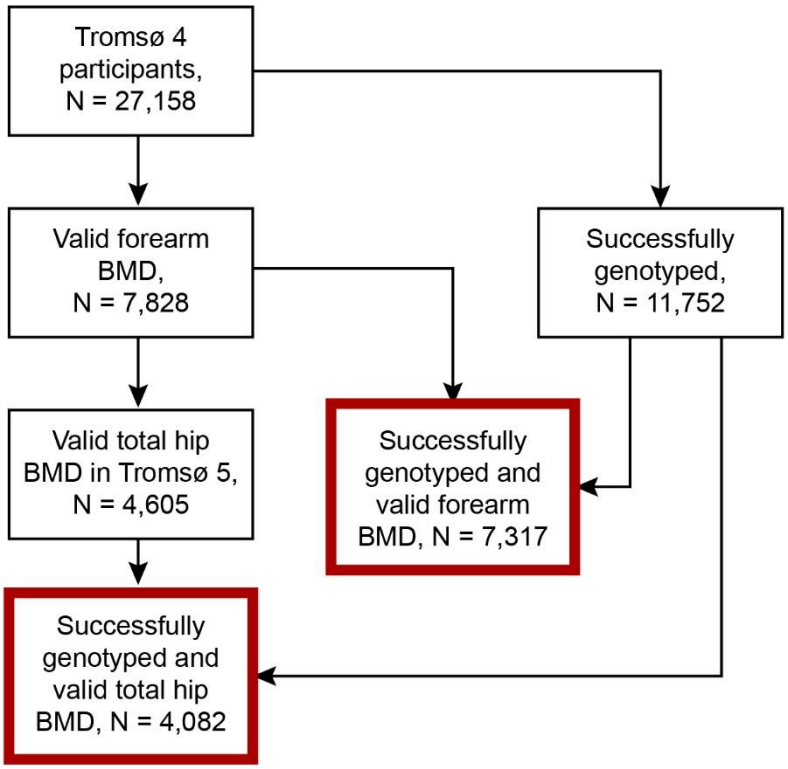
Flowchart 2. The population of the *VDR* SNPs and T2D, MI, cancer, and mortality study (Paper II).



For Paper III, 7,965 individuals (78% of those invited) participated in the more extensive examination at the second visit, where distal forearm BMD was successfully measured in 7,828 participants (74). Furthermore, in the 5th Tromsø survey, conducted in 2001/2002 (participants of the 4th Tromsø survey and randomly selected inhabitants aged 30–75 years were invited to take part; 8,130 attended with a participant rate of 79%), 4,605 participants were successfully measured for total hip BMD (75). Participants with at least one successfully analyzed SNP of

interest from the 4th Tromsø survey, and successful BMD measurements of the distal forearm, total hip or both, were included (Flowchart 3).

Flowchart 3. The study population of the SNPs and BMD study (Paper III).



Safety (Papers I-III)

All participants had signed informed consent forms prior to participation in the studies. The studies were approved by the Regional Committee for Medical and Health Research Ethics (REK Nord), and the fat biopsy trial was registered at ClinicalTrials.gov. Participants in the fat biopsy study were informed of risks of infection, bruising, and pain, and instructed to contact the study investigators or the Clinical Research Unit in case of infection or increasing pain. The participants were informed of coverage by Norwegian patient insurance, and that they would be informed of their results and receive further recommendations for supplementation and follow-up after study completion.

Questionnaires

Paper I

At every visit, the participants were interviewed by a trained nurse in order to ascertain intake of vitamin D (as tablets or cod liver oil), intake of calcium supplementation, use of solarium, vacations in tropical/sunny areas, and medical events (Appendices 1-3).

Papers II and III

In both the 4th and 5th Tromsø surveys, self-administered questionnaires were used to collect information on modulating risk factors, such as smoking status, physical activity, drugs and supplements use, and medical conditions. For Paper III, the use of supplements and drugs was also registered, according to the written list of drugs used on a regular basis, and checked by research personnel at the study site.

Body measurements

Height and weight were measured wearing light clothing without shoes. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared.

Adipose tissue biopsies - Paper I

Participants could optionally consent to sc. abdominal fat biopsy, performed at inclusion and then after 1 year. The biopsies were performed using the needle-aspiration technique, as previously described (76), and aspirated adipose tissue was immediately washed with saline water, placed in liquid nitrogen, and then stored at –80˚C until analyses. Vitamin D and 25(OH)D were analyzed by a liquid chromatography-tandem mass spectrometry LC-MS/MS method, as previously described (14, 77). In short, the saponified sc. adipose tissue samples were liquid–liquid extracted, and then cleaned-up by a normal-phase solid-phase extraction method. The mass spectrometer was operated in a positive multiple reaction monitoring mode, and the limit of quantitation (LOQ) was <0.1 ng/g. Using house-reference pork fat materials (N=6), the analyses of variation were assessed as 8.2% for vitamin D_3 (5.8 ng/g) and 8.5% for 25(OH)D_3 (2.4 ng/g). The accuracy was 84–96% for vitamin D_3 and 113–114% for 25(OH)D_3, assessed on spiking (N=9).

Blood pressure - Paper I

Systolic blood pressure (BP) was measured by trained personnel, using an automatic device (Dinamap Vital Signs Monitor 1846, Critikon Inc., Tampa, FL, USA). The standardized procedure was followed, with 2 minutes resting in a sitting position before the measurement; BP was taken three times via the right upper arm, with 2-minute breaks, and the average of the last two readings was recorded.

BMD measurements - Paper III

All BMD measurements were performed by specially trained technicians according to the standardized protocols. BMD of the distal forearm was measured once in the 4th Tromsø survey, as described previously (78). In short, BMD was measured in the non-dominant arm if possible, in the radius and ulna at the point where these two bones are separated by 8 mm, and

by 24 mm proximally. A single x-ray absorptiometric device (DTX-100; Osteometer MediTech, Inc., Hawthorne, CA, USA) was used. To ensure the quality of the data, the forearm BMD measurements were adjusted for artifacts and long-term drift throughout the measuring time, using the European forearm phantom (79).

The BMD of the total hip was measured once in the 5th Tromsø survey, using a dual-energy x-ray absorptiometric device (GE Lunar Prodigy, LUNAR Corporation, Madison, WI, USA) (58). If available, the means of BMD scans of both sides of the body were used.

Blood samples

Paper I

At the end of the study, serum 25(OH)D was analyzed in a batch using a sensitive LC-MS/MS method, as previously described (64). In short, serum 25(OH)D_2 and 25(OH)D_3 were simultaneously measured, with a LOQ of <4 nmol/L; a between-day coefficient of variation (CV) of <9% and a within-day CV of <2%. Accuracy of 25(OH)D_3 was 104.6% over 3 days. In most of the samples, 25(OH)D_2 was not measurable; therefore we presented the total 25(OH)D. Serum calcium and phosphate were analyzed using the Hitachi 917 (Roche Diagnostics, Basel, Switzerland), with reagents from Boehringer-Mannheim, Mannheim, Germany. Serum PTH was measured by an Immulite 2000 Intact PTH analyzer (Siemens Healthcare Diagnostics, Los Angeles, USA, and HbA_{1c} was analyzed using an immunoturbidimetric method with Unimate 5 HbA_{1c} (Hoffmann-La Roche, Basel, Switzerland).

Papers II and III

Non-fasting blood samples for serum 25(OH)D, serum PTH, serum total cholesterol, and HbA_{1c} were collected in the 4th Tromsø survey. For serum 25(OH)D, the samples stored at -70°C, were thawed in 2008 and analyzed via electro-chemiluminescence immunoassay with an automated clinical chemistry analyzer (Modular E170, Roche Diagnostics, Basel, Switzerland). For Paper III, serum 25(OH)D values were adjusted after the VDSP protocol according to the LC-MS/MS method (25). Serum PTH was measured by two-site chemiluminescent immunometric assay using an automated Immulite 2000 analyzer (Siemens Healthcare Diagnostics, Los Angeles, CA, USA). Total serum cholesterol was analyzed via the enzymatic colorimetric method, using a CHOD-PAP kit (Boehringer-Mannheim, Mannheim, Germany), and HbA_{1c} in EDTA whole blood was analyzed via an immunoturbidimetric assay with Unimate 5 HbA_{1c} (Hoffmann-La Roche, Basel, Switzerland).

Non-fasting blood samples for serum TSH, creatinine and calcium were collected in both the 4th and 5th Tromsø surveys. Serum TSH was analyzed using the same method, with Immulite analyzer used for serum PTH. Serum creatinine and serum calcium were analyzed via a spectrophotometric method with an automated Hitachi 917 analyzer (Hoffmann-La Roche, Basel, Switzerland) and reagents from Boehringer-Mannheim, Mannheim, Germany.

SNPs selection and genotyping - Papers II and III

For Paper II, we selected several *VDR* gene-associated SNPs: *FokI* (rs2228570/rs10735810), reported to be associated with T2D and cancer (39, 40, 80, 81); *ApaI* (rs7975232), *BsmI* (rs1544410), and *TaqI* (rs731236), associated with CAD, cancer, and cancer-related mortality (54, 82, 83); *Cdx2* (rs11568820), as it has been reported that some *Cdx2* haplotypes are associated with colorectal cancer (84); and rs7968585, reported as being associated with a composite outcome (MI, hip fracture, cancer, and mortality) in people with low serum 25(OH)D in a North American population (50).

For Paper III, we considered the abovementioned *VDR*-associated SNPs. First, we selected *BsmI*, as it has been most consistently associated with BMD (60, 85). The other SNPs in high or moderate linkage disequilibrium (LD $\geq$0.4) with *BsmI* were then excluded (*ApaI*, *TaqI*, and rs7968585) (86). *FokI* and *Cdx2* were selected, due to low LD, but the associations with low BMD and fracture risk have been reported as inconsistent to date (59, 61, 85, 87, 88). We also chose to test the *VDR* SNP haplotype *BsmI-ApaI-TaqI* as an association with osteoporotic fracture has been reported (89). From the available SNPs associated with the biggest differences in serum 25(OH)D levels across the alleles in our cohort, we included rs2298850 in the *DBP* gene, rs10741657 in the *CYP2R1* gene, rs3794060 in the *NADSYN* gene, and rs6013897 in the *CYP24A1* gene (72).

For Paper III, we also selected an SNP, rs4870044, close to the *ESR1* gene, both as a quality control for our study, as a strong association with hip and lumbar BMD has previously been reported (62, 90), and to evaluate the association with distal forearm BMD, which has not previously been reported.

DNA was prepared from whole blood samples collected during the 4th Tromsø survey, first visit, via the manual isolation method. Genotyping was performed via the KBioScience Allele-Specific Polymorphism (KASP) system, as previously described (72).

Statistical analyses

Distribution of the continuous variables was evaluated for skewness, kurtosis, and visual inspection of histograms and Q-Q plots. The genotype frequencies were evaluated via the chi-square test for Hardy-Weinberg equilibrium (91). LD was calculated via an SNP Annotation and Proxy Search, which is based on International HapMap Project data (86). Trends across the SNP genotypes were evaluated with linear regression for the continuous variables, and with the chi-square test with linear-by-linear association for the categorical variables. Major homozygote was used as reference in the SNP analyses.

The tests were performed two-sided, and a P-value <0.05 was considered statistically significant. The data were analyzed with Statistical Package for the Social Sciences IBM software, version 22 (SPSS Inc. Chicago, IL, USA).

Paper I

The values missing from one participant at the last visit were replaced by the series mean method for the measurements, and "the last observation carried forward" for the answers.

An independent samples t-test, Mann-Whitney U test, and Fisher's exact test were used to compare the intervention groups at baseline. One-way analysis of variance or the related samples Wilcoxon signed rank test were used to evaluate the variation of continuous variables over time. Correlations were assessed via Spearman's test. The independent samples median test was used to compare concentrations in fat biopsies between the intervention groups.

The terminal half-life of 25(OH)D was defined as the time required for the serum concentration of 25(OH)D to fall by 50% during the terminal phase (92). The terminal phase was visually determined when the logarithmically transformed levels of serum 25(OH)D appeared to be linear over time. The serum terminal half-life of 25(OH)D, as well as the half-life in the pre-terminal phase, was then calculated using the following formula:

$$\text{Halflife} = \frac{\text{Elapsed time in days x ln2}}{\ln\left[\frac{\text{Delta of baseline serum levels between vitamin D and placebo groups}}{\text{Delta of end serum levels between vitamin D and placebo groups}}\right]}$$

for exponential decay on the web calculator http://www.calculator.net/half-life-calculator.html, where ln2 is the natural logarithm of 2, a constant of 0.693.

Paper II

It emerged that the method of measuring serum 25(OH)D with the Modular E170 biased the serum 25(OH)D values if the participants smoked (93), therefore smokers and non-smokers were separately analyzed. The hazard ratio (HR) of the SNP genotypes was evaluated via Cox

regression. We tested three statistical models: adjusting only for age and gender; additional adjusting for known risk factors; and additional adjusting for T2D while evaluating MI risk. The interactions between serum 25(OH)D levels and SNP genotypes, as well as associations with endpoints of interest, were also tested via Cox regression.

We used a conservative Bonferroni approach for correction for multiple testing, which could eventually result in false negative results (overcorrection).

Paper III

Missing categorical data in the questionnaires were interpreted as negative answers (for example, if questions about diseases were unanswered, it was assumed that the disease(s) was/were not present). Linear regression was used to evaluate the risk factors for low BMD in the study population, to evaluate risk genotypes, and to examine the interactions between *BsmI*, *ApaI*, and *TaqI*. Serum 25(OH)D values were adjusted for the season using months as dummy variables.

Summary of results

Paper I

Of 92 individuals invited, 76 consented and were included. One participant of 42 in the vitamin D group withdrew after 1 month, and was excluded from all but baseline analyses. One participant of 34 in the placebo group did not attend the last visit. A total of 29 participants (18 in the vitamin D group and 11 in the placebo group) consented for sc. adipose tissue biopsy at inclusion, and 12 (eight in the vitamin D group, and four in the placebo group) did so after 1 year. No complications other than local bruising were registered following the biopsy procedure.

Serum 25(OH)D levels were significantly higher in the vitamin D group compared to the placebo group during the entire 1-year follow-up period. At inclusion, mean serum 25(OH)D in the vitamin D and placebo groups was 122 nmol/L and 71 nmol/L, respectively. After 1 year, serum 25(OH)D remained significantly higher in the vitamin D group, at 85 nmol/L vs 73 nmol/L in placebo group. The half-life in the pre-terminal phase (0–3 months) was 83.4 days, and – 255 days in the terminal phase.

Significantly higher levels of vitamin D and 25(OH)D were observed in the adipose tissue of those given vitamin D vs placebo at baseline (a median of 209 ng/g vs 32 ng/g for vitamin D, and median of 3.8 ng/g vs 2.5 ng/g for 25(OH)D). Adipose tissue vitamin D levels decreased by 52% over 1 year in the vitamin D group, and became similar to those in the placebo group.

Paper II

A total of 1,054 individuals with T2D, 2,287 with MI, 3,166 with cancer, and 4,336 with death were successfully genotyped for rs7968585. The mean follow-up time for T2D and MI from birth was 60.8 years, for cancer 61.2 years, and for death from the examination time 16.5 years. The 20th percentile of serum 25(OH)D for non-smokers was 33.9–46.6 nmol/L, and was 50.4–64.2 nmol/L for smokers. The levels of mean serum 25(OH)D did not differ across the *VDR* SNP genotypes. A significant difference in HRs across the *VDR* genotypes was found only for rs7968585 for the risk of T2D (HR 1.25, 95% CI 1.05–1.49; minor homozygotes (C:C) vs major homozygotes (T:T)), and for risk of MI (HR 1.14, 95% CI 1.02–1.28) adjusted for age and gender. After Bonferroni correction for multiple testing, there remained a 25% increased risk of T2D for minor homozygotes, but the risk of MI was insignificant. No interactions between serum 25(OH)D status and rs7968585 genotypes were observed for any of the endpoints.

Paper III

We obtained 7,317 valid BMD measurements of the forearm, and 4,082 of total hip. Women and men were separately analyzed for the risk factors in our study population. BMI, height, serum creatinine, and calcium, and use of vitamin D and estrogen, had a positive association with forearm BMD in women. Age, serum PTH, cancer history, early menopause, and use of calcium were associated with lower forearm BMD in women. BMI and physical activity were positively associated with total hip BMD in both genders, while age and smoking were negative predictors. For hip BMD in women, serum PTH and use of bisphosphonates were also negative predictors, while for men, self-reported ulcer surgery was associated with lower BMD.

Of the evaluated vitamin D SNPs, only SNP rs6013897 (located near the *CYP24A1* gene that encodes 24-hydroxylase) had a negative trend across the genotypes (standardized β coefficient of -0.031, P-value of 0.024) for the BMD of total hip adjusted for age and gender. The difference between major and minor homozygote was 0.02 g/cm^2. The trend became insignificant after adjusting for the other total hip BMD predictors.

ESR1-associated SNP rs4870044 had a β coefficient of -0.016 for forearm BMD and -0.034 for total hip BMD across the genotypes (P-values of 0.036 and 0.045, respectively), adjusted for age and gender. The difference between major and minor homozygote was 0.004 g/cm^2 for forearm BMD and 0.004 g/cm^2 for total hip BMD. The trend for forearm BMD remained significant for men, but not for women, after adjusting for the other forearm BMD

predictors, and the trend for total hip BMD remained significant after similar adjustment for both genders. Serum 25(OH)D was not associated with BMD in our population.

Discussion

Methodological considerations

Study design, validity, errors, sample size, and power calculations

Significant associations in observational studies suggest, but cannot confirm, causality. Nevertheless, observational studies are the source of new causality theories, which may then be confirmed or rejected by intervention studies. Studies of vitamin D are complicated by the intake and skin synthesis of exogenous vitamin D, which is difficult to quantify and control for. Therefore, Tromsø, with ineffective skin production of vitamin D in 8 months of the year, is clearly an advantageous study site. We must always consider study validity (ability to distinguish who does and who does not have a condition), due to systematic errors, so that we are able to critically evaluate the results. Furthermore, study reliability, or the ability to replicate its results in other populations, is also an important aspect of the results evaluation.

Paper I

Cohort studies investigate whether exposures are associated with disease incidence. The usual manner of selecting the exposure groups in a cohort study is according to the known exposure. One must then always consider the selection- and information-biases. In our prospective, longitudinal, vitamin D storage cohort study, the participants were selected by inviting the volunteers to participate in an RCT, where they were subsequently randomized to the vitamin D or control groups. Selection bias might be an issue in clinical studies conducted in a more motivated, and usually healthier, population. Nevertheless, in our follow-up study, where we aimed to investigate the dynamics of vitamin D and its metabolites in serum and adipose tissue, this bias would have had a minor effect. Furthermore, the double blinding was kept until the end of the observation, which minimized observer-bias. Although random differences between intervention groups may occur, the problem of the known and unknown confounding factors in our cohort study was minimal, as randomization to the original RCT was used.

The cohort study design, when the exposed and unexposed groups are selected before the outcome occurs, leads to a reduced probability of differential selection. We lost only two participants during follow-up (one from each intervention group), and their characteristics were similar to those continuing in the study; therefore, the differential losses to follow-up were minimal. Recall bias was also avoided, as our study was prospective. Nevertheless,

some questions in the questionnaires used at every visit were retrospective, which could have led to misclassification of answers (due to respondent bias). However, such risk was small and it should not have affected the results.

With expected mean serum differences in 25(OH)D levels of 20 nmol/L after 3 months, and an SD of 17 nmol/L, according to the earlier observation, the minimum number of participants to be included was 38. However, uncertainty of the assumptions for the actual half-life and serum levels meant that we included twice that number. Retrospectively, the power in our study was 0.74, which meant that the probability of correctly rejecting the null hypothesis was 3 out of 4.

Papers II and III

Our case-cohort study exploring SNPs as risk factors for T2D, MI, cancer, and mortality, as well as the cross-sectional BMD study, were both community-based, and could have some of the same biases described above. Participation was voluntary, which could have led to selection bias, as volunteers could have had a higher socioeconomic status, be healthier, etc.

The case-cohort study had a robust control group, but the study was retrospective and could have led to some more extensive information biases, particularly with regard to not documenting outcome risk factors, other than gene variations, as the study data were based on medical records.

Both the clinical outcomes in Paper II, and BMD measurements in Paper III were registered in a standardized and validated manner, thus minimizing measurement bias. Temporal bias was not an issue, as genetic influence is life-long.

In the observational studies, in addition to random differences, the factors associated with exposure and outcome (but outside their causal pathway), may confound (distort) the explored association. As confounders are based on *a priori* knowledge, their effects may be assessed, for example, with multivariable methods. One approach was to examine whether the exposure-outcome association remained in the same direction and of the same magnitude after adjusting for confounding factors. This approach is questionable in gene variation studies, as Mendelian randomization may already be interpreted as a valid randomization (94). Nevertheless, we chose to adjust for possible confounders in Papers II and III.

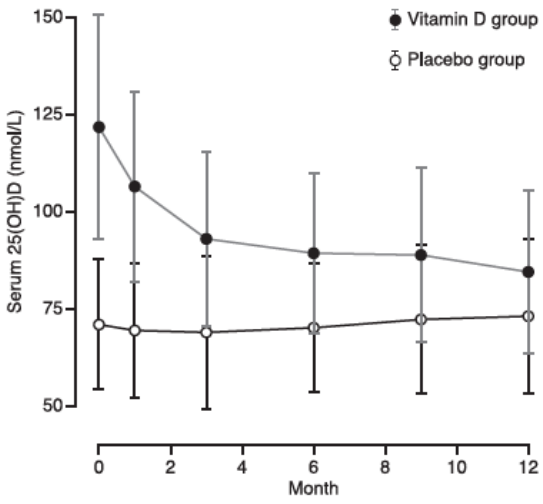
Discussion of main results

Paper I (Vitamin D storage in adipose tissue)

Our results show that long-term vitamin D supplementation leads to maintenance of higher serum 25(OH)D levels 1 year after cessation; the baseline difference of 51 nmol/L

between the vitamin D and placebo groups decreased to 11 nmol/L at this time (Figure 4), and would probably have become insignificant a short time later. The half-life of approximately 82 days calculated in the follow-up study was similar to that reported in previous studies (13, 95). This indicates that there are mechanisms extending serum 25(OH)D circulation, as the half-life of traced serum 25(OH)D is known to be much shorter, at 15–25 days (12, 21). Such a prolonged effect may be explained by storage of vitamin D, mainly in adipose tissue, and gradual release into the circulation (2, 10).

Figure 4. Serum 25OH)D levels during 1 year of follow-up.



Nevertheless, there has been no consensus on whether this storage is of clinical importance. Heaney et al. argued that to maintain sufficient vitamin D levels (approximately 80 nmol/L), around 4,000 IU vitamin D is required daily (96). Vitamin D accumulated in adipose tissue, assuming that the concentrations in the sc. tissue represent the average, was calculated to last no longer than 20–50 days (7). In our study, the level of fat vitamin D was 209 ng/g in the vitamin D group, and decreased to 100 ng/g after 1 year. Assuming the same storage concentrations in the adipose tissue and a BMI of 29.5 kg/m^2, a mean weight of 96 kg and an approximate body fat percentage of a minimum of 30% and a maximum of 40% (7, 97), our stored and released vitamin D of 3,130–4,185 mcg, or 125,000–170,000 IU, would only cover the vitamin D demands of 31–43 days. Nevertheless, these calculations, based on generalization, are rough as there are few data and little correlation between levels of vitamin D and its metabolites in different types of adipose tissue (18-20). Furthermore, this vitamin D released from adipose tissue may work as an additive supplementation to vitamin D intake or

skin production, thus prolonging sufficient serum 25(OH)D levels, as observed after a single high-dose intake of vitamin D (95).

Although the diabetes prevention study was conducted over 3–5 years, which was far longer than the length of vitamin D supplementation in other published studies related to fat biopsies, the mean concentration of vitamin D in the adipose tissue of the vitamin D supplementation group was only 209 ng/g. Heaney et al. found that the average concentration reached after 3 months of 50,000 IU/week vitamin D supplementation was 180 ng/g (7). The relatively small difference suggests that there may be a limited storing capacity, or "saturation limit" of vitamin D in adipose tissue. Assuming that the accumulation of vitamin D in adipose tissue is linear, the 209 ng/g level found by Heaney et al. (7) could be reached in approximately 25 extra days. If this was confirmed in further studies, it would have a clinical and economic benefit: an optimal high-dose vitamin D supplementation duration of 4 months could be followed by a 1-year vitamin D-free period to maintain serum 25(OH)D levels in the physiologic range. However, although high-dose vitamin D supplementation seldom has side effects, intermittent vitamin D use is questionable, as serum vitamin D has a short half-life of 19–25 hours (22), which leads to inconsistent vitamin D serum levels.

We tracked 25(OH)D in sc. adipose tissue, following the study by Didriksen et al. (14), and also detected it in smaller amounts after 1 year. The fat 25(OH)D levels were similar to those measured by Piccolo et al. (15). Both hydroxylases required to convert vitamin D to 1,25(OH)2D are found in adipose tissue, therefore this 25(OH)D measured is assumed to be important for vitamin D functions in adipose tissue: gene expression regulation, adipogenesis modulation, adipocyte apoptosis, energy homeostasis involving leptin, and an anti-inflammatory effect (2).

Paper II (*VDR* SNPs as risk factors for T2D, MI, cancer, and mortality)

The minor *VDR* SNP rs7968585 (C:C) homozygotes were associated with a 25% increased risk for T2D in our study population, even after conservative adjusting for multiple testing. To our knowledge, no other studies reporting the association between rs7968585 variations and T2D have been published. The British Birth cohort study involving *VDR* SNP rs739837, which is in high LD with rs7968585, did not find an association with T2D, although this study included 5,160 participants (51). Knowing that vitamin D maintains many cellular processes through the VDR, and may increase insulin secretion, reduce insulin resistance in peripheral tissues, regulate the adipokines important for normal glucose and lipid metabolism, and have an anti-inflammatory action (43, 44), we may assume that the observed

association between *VDR*-related rs7968585 and T2D might be genuine, although the action of this SNP remains unknown.

Neither in our study, nor in the studies by Levin et al. and Barry et al., did rs7968585 variations predict serum 25(OH)D levels (50, 98). However, the rs7968585 genotypes modified the effect of vitamin D supplementation on serum 25(OH)D levels by up to 10% (per variant allele compared to the wild-type) (98). If the absence of association between the risk SNP and low serum 25(OH)D was genuine, according to Mendelian randomization, the effect of rs7968585 on T2D should be other than dependent on serum 25(OH)D levels. However, there is also a possibility that an existing association between vitamin D levels and *VDR* SNPs was not found as a result of it being a generally vitamin D-sufficient population.

We also observed a 14% increased risk for MI for minor homozygotes of rs7968585, but the association was insignificant after adjusting for multiple testing. This SNP has been associated with a risk of composite clinical outcome, including MI, cancer, hip fracture, and mortality, when the mean serum 25(OH)D was 36 nmol/L, but not in individuals with sufficient vitamin D levels (50). There was also observed that the effect of vitamin D supplementation on advanced adenomas was modified according to the rs7968585 genotype (99). To date, no other publications on this SNP and significant associations have been published, possibly indicating few effects in limited populations.

Rs7968585 is in the non-coding region of the *VDR* gene, but in moderate-to-high LD with other common *VDR* SNPs, *ApaI*, *TaqI*, and *BsmI* (86). Nevertheless, the latter *VDR* SNPs, as well as *FokI* and *Cdx2*, (which are in low LD with rs7968585 (r^2<0.4)) showed no significant associations either with serum 25(OH)D levels or with the investigated clinical outcomes in the study population. It has previously been inconsistently reported that the aforementioned *VDR* SNPs are associated with T2D, MI, hypertension (only *FokI*), cancer, cancer-related mortality (35, 39, 40, 50-54), as well as with vitamin D supplementation modification to improve metabolic profiles (100). In particular, rs7968585 has rarely been reported (Table 1), therefore our data contribute to the effect of *VDR* SNP mapping, although we found no significant effect on the outcomes investigated.

Table 1. Overview of the studies reporting *VDR* SNP rs7968585 associations with clinical outcomes: T2D, MI, cancer, mortality, and modifying the vitamin D supplementation effect.

***VDR* SNP**	**Study design, population**	**N**	**Association with**	**Authors, year**	**Comments**
Rs7968585	Candidate gene; I) White US II) Italians III) Swedish men	I) 2,312 II) 2,998 III) 1,453	Composite outcome: hip fracture, MI, cancer and mortality	Levin et al., 2012 (50)	Only in those with lowest quintile of serum 25(OH)D
Rs7968585	Candidate gene; Non-Hispanic whites	1,787	Vitamin D effect on serum 25(OH)D	Barry et al., 2014 (98)	
Rs739837 (LD of 0.87 with rs7968585)	Candidate gene; British Birth cohort	5,160	Metabolic and cardiovascular outcomes	Vimaleswaran et al., 2014 (51)	Non-significant association
Rs7968585	Candidate gene; Non-Hispanic whites	1,702	Vitamin D effect on advanced colorectal adenomas	Barry, 2017 (99)	No vitamin D effect on overall adenoma risk

Paper III (Vitamin D and estrogen related SNPs as risk factors for low BMD)

In our population, only the rs6013897 SNP of the tested vitamin D SNPs showed a significant, although minor, effect, whereby minor homozygotes had lower total hip BMD. SNP rs6013897 is located near the *CYP24A1* gene that encodes 24-hydroxylase, which degrades 25(OH)D and stimulates the synthesis of 1,25(OH)2D. In addition, in earlier studies in Norway and elsewhere, a negative association, although sometimes weak, between serum 25(OH)D levels and minor alleles was observed (38, 49, 72). Although it has also been observed that rs6013897 may modify the effect of vitamin D supplementation on serum

25(OH)D levels (98), only one study has been published regarding BMD, in which no association was found in a Chinese population (101).

The *ESR1* SNP rs4870044 was highly associated with both forearm and total hip BMD in our study, where the minor allele was disadvantageous. The action of ESR1 is mainly to mediate the estrogen effect on bone (102, 103). As expected, our results match the previously reported rs4870044 associations with hip and lumbar BMD, as well as BMD associations with other *ESR1* SNPs (62, 63, 104). To date, no associations between rs4870044 and forearm BMD have been reported, although this is also clinically important.

The significant predictors of BMD were not unexpected, and could be explained by a causal relationship with BMD, or as an artifact (for example, when bisphosphonates were negatively associated with BMD, one may assume that the women had already been diagnosed with osteoporosis and were receiving treatment). Surprisingly, serum 25(OH)D levels were not associated with BMD, possibly due to generally sufficient vitamin D levels in our study population.

Limitations and advantages

Paper I

The absence of a standardized diet was a disadvantage, although oral intake of vitamin D and sun exposure were registered and used as covariates. Nevertheless, the prevalence of vitamin D supplementation was high, and although its half-life was calculated as an effect of vitamin D supplementation (25(OH)D in the vitamin D group, subtracted from the levels in the placebo group, the confounding factor might not have been completely avoided. Active 1,25 (OH)2D in fat, as well as vitamin D in serum, were not measured. We did not perform simultaneous fat biopsies from other sites of adipose tissue or muscles, and the number of biopsies after 1 year was small.

The main advantage of the study was its design, with double-blinding, prospective, and simultaneous follow-up of the participants, and fat biopsies analyzing both 25(OH)D and vitamin D levels, taken twice over the year. External factors and other variables were comparable in both groups, and did not vary extensively over the follow-up period. The study power for serum measurements was good.

Paper II

Our population was generally vitamin D-sufficient, which could have led to false negative associations between serum 25(OH)D levels and *VDR* SNPs. Furthermore, not all of

the genotyped participants had available serum 25(OH)D, and the latter was measured only once – this reduced the power to find an association and obtain more accurate serum 25(OH)D levels. Moreover, the chosen correction for multiple testing by the Bonferroni method could have led to overadjustment and false negative results.

However, the Bonferroni correction showed that the association observed between rs7698585 and T2D could be assumed genuine, with higher probability. Furthermore, our endpoint registers were of high quality, and the length of follow-up time was long.

Paper III

Significant associations were still observed after adjustments, which, in addition to possible residual confounding, could have shown genuine associations between the SNPs and the outcomes. The vitamin D levels were generally sufficient, which could have masked existing associations.

The robust association between the *ESR1* SNP and BMD indicates that the study design was probably sufficient to discover existing associations. BMD measurements were standardized and validated (58, 79).

Conclusions, implications, and future perspectives

25(OH)D and vitamin D stored in adipose tissue after 3–5 years' vitamin D supplementation may have a clinically relevant effect on serum 25(OH)D levels the following year. This finding is novel, and should be confirmed by study replication. Furthermore, the optimal vitamin D supplementation time should be determined, as it appears that adipose tissue has a maximal capacity (saturation) for vitamin D storage. For the optimal supplementation period, a confirmatory study, such as a RCT with several parallel intervention groups, is warranted. Intermittent vitamin D supplementation practice could improve compliance and optimize the costs. Finally, the molecular mechanisms of vitamin D storage and release in adipose tissue remain to be clarified and further basic research is required.

Regarding the genetics of vitamin D, we found a significant association between the *VDR* SNP rs7968585 and risk of T2D, and possibly MI, in a general population, whereas interactions between rs7968585 and serum 25(OH)D levels regarding clinical endpoints were not found. We consider that confirmatory candidate gene (rs7968585) studies, but not GWAS from other populations, are required to identify whether this SNP is associated with T2D and MI risk. Knowledge of rs7968585 function could lead to improved understanding of this SNP, which is located in a non-coding region.

Finally, it was found that one *CYP24A1* SNP rs6013897 and *ESR1* SNP rs4870044, but not *VDR* or other serum 25(OH)D-related SNPs, were associated with forearm and total hip BMD in our study population. None of the *VDR* SNPs of interest have previously been consistently reported to be associated with low BMD, although VDR is *known* to act both by rapid and genomic action on BMD. Therefore the functions of separate *VDR* SNPs should be clarified, and candidate gene studies in large, but homogeneous, populations should be carried out, as the effect of a single SNP might be very little, and the inter-individual variability broad, and might not be easily detected in the GWAS.

T2D, MI cancer, osteoporosis, and probably mortality, are the outcomes of a complex multifactorial process, in which genetic and epigenetic risk factors have an additive effect. We believe that mapping of risk gene variations, other than those related to vitamin D and estrogen metabolism, is important, and, ideally, an examination of the effect of vitamin D supplementation in an RCT arranged according to risk SNPs is warranted, but it is technically very complicated. Future treatment strategies should focus on personalized evaluation of the risk of multifactorial undesired outcomes to tailor personalized treatment.

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Appendices

Appendix 1

Medikamentskjema ”Varighet av vitamin D lagre etter langvarig vitamin D tilskudd”

Navn…… Pas kode……(beholder gammelt kode nr fra diabstudien som nettopp har deltatt i)

Medikamentnavn	Dose	0	1 mnd	3mnd	6 mnd	9 mnd	12 mnd

Utfylling: dersom starter nytt medik i løpet av studien skriv ”start”
dersom kontinuerer medikament, skriv ” → ”
dersom slutter, skriv ”sep”
dersom endrer skriv den nye dosen (f.eks 50 mg x 1)

Overfør informasjon fra gammelt medikamentskjema i diabstudien ved tid 0. Signer alt med initialer 261012

Appendix 2

” Varighet av vitamin D lagre etter langvarig vitamin D tilskudd”

Syden og solarium skjema. Fylles ut ved 0, 1, 3, 6, 9, 12 mnd
Høyde og vekt måles ved 0, 6, 12 mnd
Navn……. Kode……

0 mnd (v/ inklusjon) dato: høyde: vekt :
Dato når tok siste studiemedisin kapsel ……..
Har du siste 3 mnd vært i ”Syden”? □ Ja □ nei
Hvis ”ja”, hvor og i hvilket tidsrom?

Har du siste 3 mnd vært i solarium? □ Ja □ nei
Hvis ”ja”, hvor mange ggr siste 3 mnd?

1 mnd . dato:
Har du siste 3 mnd vært i ”Syden”? □ Ja □ nei
Hvis ”ja”, hvor og i hvilket tidsrom?

Har du siste 3 mnd vært i solarium? □ Ja □ nei
Hvis ”ja”, hvor mange ggr siste 3 mnd?

3 mnd . dato:
Har du siste 3 mnd vært i ”Syden”? □ Ja □ nei
Hvis ”ja”, hvor og i hvilket tidsrom?

Har du siste 3 mnd vært i solarium? □ Ja □ nei
Hvis ”ja”, hvor mange ggr siste 3 mnd?

6 mnd dato: høyde: vekt :
Har du siste 3 mnd vært i ”Syden”? □ Ja □ nei
Hvis ”ja”, hvor og i hvilket tidsrom?

Har du siste 3 mnd vært i solarium? □ Ja □ nei
Hvis ”ja”, hvor mange ggr siste 3 mnd?

9 mnd dato:
Har du siste 3 mnd vært i ”Syden”? □ Ja □ nei
Hvis ”ja”, hvor og i hvilket tidsrom?

Har du siste 3 mnd vært i solarium? □ Ja □ nei
Hvis ”ja”, hvor mange ggr siste 3 mnd?

12 mnd dato: høyde: vekt :
Har du siste 3 mnd vært i ”Syden”? □ Ja □ nei
Hvis ”ja”, hvor og i hvilket tidsrom?

Har du siste 3 mnd vært i solarium? □ Ja □ nei
Hvis ”ja”, hvor mange ggr siste 3 mnd? 261012

Appendix 3

Kalk og vitamin D skjema ” Varighet av vitamin D lagre etter langvarig vitamin D tilskudd”

Navn…… Pas kode…… (beholder gammelt kode nr fra diabstudien som nettopp har deltatt i)

Navn på kalktablett	dose	0	1 mnd	3 mnd	6 mnd	9 mnd	12 mnd

Tran (flytende/kapsel)	dose	0	1 mnd	3 mnd	6 mnd	9 mnd	12 mnd

Navn på vitamin D tilskudd	dose	0	1 mnd	3 mnd	6 mnd	9 mnd	12 mnd

Utfylling: dersom starter nytt medikament i løpet av studien skriv ”start”
dersom kontinuerer medikament, skriv ” → ”
dersom slutter, skriv ”sep”
dersom endrer skriv den nye dosen (f.eks 1 tabl x 2) Signer med initialer

Overfør informasjon fra gammelt kalk/vit D skjema i diabstudien ved tid 0. OBS at tar med seg alle vitamin bokser uansett innhold til kontrollene

261012