

Saranya Palaniswamy

VITAMIN D STATUS AND
ITS ASSOCIATION WITH
LEUKOCYTE TELOMERE
LENGTH, OBESITY AND
INFLAMMATION IN YOUNG
ADULTS

A NORTHERN FINLAND BIRTH COHORT 1966 STUDY

UNIVERSITY OF OULU GRADUATE SCHOOL;
UNIVERSITY OF OULU,
FACULTY OF MEDICINE;
CENTER FOR LIFE COURSE HEALTH RESEARCH;
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SARANYA PALANISWAMY

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A Northern Finland Birth Cohort 1966 study

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Abstract

Vitamin D deficiency, obesity and short telomere length are reported to be associated with increased risk of metabolic diseases and all-cause mortality, through modulation of inflammatory pathways. The season of blood sampling, obesity and physical activity have been identified as determinants of 25-hydroxyvitamin D [25(OH)D], but their association with 25(OH)D₂ (D2) and 25(OH)D₃ (D3) is still poorly understood. In addition, relationships between 25(OH)D, body mass index (BMI), inflammation, and leukocyte telomere length (LTL) has not been previously established. A better understanding of the determinants, risk factors of vitamin D deficiency, and their relationship with BMI, inflammation, and LTL is needed.

The study was based on the 31-year follow-up study of the Northern Finland Birth Cohort 1966 (N=4,758). Statistical analyses were used to 1) examine potential determinants of D2 and D3, and identify risk factors associated with hypovitaminosis D, 2) investigate the relationship of 25(OH)D and BMI with LTL and test whether it is independent of inflammatory pathways and, 3) assess how the association of BMI with inflammatory biomarkers might be mediated through 25(OH)D.

Our results showed that D2 contributed 5% and D3 95% of the total 25(OH)D concentrations. When examined, the determinants for each isoform, periods of low sunlight exposure associated with increased D2, but with decreased D3. Oral contraceptives associated with increased concentrations of both. We confirmed the known risk factors of low vitamin D: low sunlight periods, residing in northern latitudes, and physical inactivity. Serum 25(OH)D was not an important determinant of LTL, and inflammation may partly mediate the BMI-LTL association. Higher serum 25(OH)D was inversely associated with inflammatory biomarkers, and the association between BMI and biomarkers was modestly mediated through lowered 25(OH)D.

In conclusion, our results support the role of known risk factors in vitamin D deficiency and add information on specific determinants of D2 and D3. 25(OH)D did not associate with LTL in young adulthood. We have also provided new insights into a plausible role of vitamin D in BMI associated inflammation. An improved understanding of the role of vitamin D benefits public health in many ways (it can help prevent vitamin D deficiency by implementing lifestyle modification and supplementation).

Keywords: 25(OH)D, 25-hydroxyvitamin D, cytokines, determinants, inflammation, leukocyte telomere length, NFBC1966, obesity, risk factors, vitamin D

Palaniswamy, Saranya, D-vitamiinin yhteys lihavuuteen, tulehdukseen ja leukosyyttien telomeerien pituuteen nuorilla aikuisilla. Pohjois-Suomen syntymäkohortti 1966 -tutkimus

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Tiivistelmä

D-vitamiinin puutos, lihavuus ja lyhyt telomeerien pituus liittyvät mahdollisesti lisääntyneeseen riskiin sairastua metaboliisiin sairauksiin sekä yleisemmin kuolleisuuteen. Eräs selitys tälle voi löytyä tulehdustekijöistä. Lihavuuden, liikunnan puutteen ja verinäytteenoton ajankohdan tiedetään vaikuttavan 25-hydroksi-D-vitamiinin [25(OH)D]-pitoisuuteen, mutta niiden yhteys D-vitamiinin isomuotoihin (D2, D3) on vielä huonosti tunnettu. Aiemmin ei ole selvitetty 25(OH)D:n, painoindeksin (BMI), tulehduksen ja leukosyyttien telomeerien pituuden (LTL) välisiä yhteyksiä, ja siksi näistä tarvitaan lisätutkimusta.

Tutkimusaineistona oli Pohjois-Suomen 1966 syntymäkohortin, 31-vuoden seurantaan osallistuneet henkilöt (N=4,758). Tutkimuksessa keskityttiin 1) selvittämään D2- ja D3-vitamiinipitoisuuksien määrittäviä tekijöitä ja tunnistamaan D-vitamiinin puutteeseen liittyviä riskitekijöitä, 2) tutkimaan 25(OH)D-pitoisuuden ja BMI:n suhdetta LTL:n kanssa sekä testaamaan, onko suhde riippumaton tulehdussellista tekijöistä ja 3) arvioimaan ilmeneekö BMI:n ja tulehdussytokiinin välinen yhteys 25(OH)D-pitoisuuden kautta.

Tutkimus osoitti, että D2-isomuodon osuus oli 5 % ja D3:n osuus 95 % koko 25(OH)D-pitoisuudesta. Näitä isomuotoja määrittäviä tekijöitä tutkittaessa havaittiin, että vähäisellä auringonvalolle altistumisella on todennäköisesti yhteys lisääntyneeseen D2-pitoisuuteen, mutta alhaisempaan D3-pitoisuuteen. Suun kautta otettavien ehkäisytablettien käytöllä oli yhteys molempien muotojen lisääntyneisiin pitoisuuksiin. Tutkimus vahvisti alhaisten D-vitamiinipitoisuuksien tunnetut riskitekijät: lyhyt altistus auringon valolle sekä fyysinen passiivisuus. 25(OH)D-pitoisuus ei ollut yhteydessä LTL:ään mutta tulehdus näytti osittain vaikuttavan BMI-LTL-assosiaatioon. Korkeampi 25(OH)D-pitoisuus yhdistyi matalampiin tulehdussytokiinipitoisuuksiin, kun taas matala 25(OH)D-pitoisuus muokkasi BMI:n ja biomarkkereiden välisiä yhteyksiä, tosin heikosti.

Yhteenvedon voidaan todeta, että tulokset tukevat tunnettujen riskitekijöiden merkitystä D-vitamiinin puutoksessa ja tuovat lisää tietoa eri isomuotoihin vaikuttavista tekijöistä. Tutkimus antaa myös uusia näkemyksiä D-vitamiinin roolista lihavuuteen liittyvässä matala-asteisessa tulehduksessa. D-vitamiinin vaikutuksien tarkempi tunteminen on merkityksellistä myös kansanterveyden kannalta.

Asiasanat: 25(OH)D, D-vitamiini, leukosyyttien telomeerien pituus, liikalihavuus, määräävät tekijät, NFBC1966, riskitekijät, sytokiinit, tulehdus

Dedicated to my Family

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Oulu, April 2018

Saranya Palaniswamy

Abbreviations

1,25(OH) ₂ D ₃	Calcitriol
25(OH)D	25-hydroxyvitamin D
25(OH)D ₂	25-hydroxyvitamin D ₂
25(OH)D ₃	25-hydroxyvitamin D ₃
7-DHC	7-dehydrocholesterol
ActivePAI-1	Active plasminogen activator inhibitor-1
AGP	Alpha-1-acid glycoprotein
ALTM	All-Laboratory Trimmed Mean
AT	Adipose tissue
BMI	Body mass index
CI	Confidence interval
CV	Coefficient of variation
CVD	Cardiovascular disease
DBP	Vitamin D binding protein
DEQAS	Vitamin D External Quality Assessment Scheme
DNA	Deoxyribonucleic acid
GWAS	Genome-wide association study
hs-CRP	High sensitivity C-reactive protein
IL	Interleukin
IL-1RA	IL-1 receptor antagonist
IOM	Institute of Medicine
IP-10	Interferon gamma-induced protein 10
IUD	Intrauterine device
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LTL	Leukocyte telomere length
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MET	Metabolic equivalent of task
MR	Mendelian randomisation
NFBC1966	Northern Finland Birth Cohort 1966
NF-κβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NNC	National Nutrition Council
NNR	Nordic Nutrition Recommendation
OCP	Oral contraceptive pill

OR	Odds ratio
PR	Prevalence ratio
RR	Relative risk
sCD40L	Soluble CD40 ligand
SD	Standard deviation
SE	Standard error
SEP	Socioeconomic position
sE-selectin	Soluble E-selectin
sICAM-1	Soluble intercellular adhesion molecule-1
sVCAM-1	Soluble vascular adhesion molecule-1
TERC	Telomerase RNA component
TNF- α	Tumor necrosis factor alpha
TRF1	Telomere repeat binding factor 1
TRF2	Telomere repeat binding factor 2
T2D	Type II diabetes
VDSP	Vitamin D Standardization Program
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue
WC	Waist circumference
WHO	World Health Organisation

Relevant units and conversions

Dietary intake of vitamin D will be mentioned in micrograms (μg)
 nmol/L units will be used for serum concentration throughout the thesis
 $25(\text{OH})\text{D}$ concentration $[\text{nmol/L}] = 2.496 \times \text{Concentration} [\text{ng/ml}]$
 Supplementation units mentioned in experimental design are used without modification.

Institute of Medicine (IOM) cutoffs for vitamin D deficiency will be used in presenting vitamin D status from epidemiological studies.

Original publications

This academic dissertation is based on four publications, which are referred throughout the text by their Roman numerals (I–IV):

- I Palaniswamy S, Hyppönen E, Williams DM, Jokelainen J, Lowry E, Keinänen-Kiukaanniemi S, Herzig KH, Järvelin MR & Sebert S (2017) Potential determinants of vitamin D in Finnish adults: a cross-sectional study from the Northern Finland Birth Cohort 1966. *BMJ Open* 2017;7:e013161.
- II Williams DM*, Palaniswamy S*, Sebert S, Buxton JL, Blakemore AI, Hyppönen E & Järvelin MR (2016) 25-Hydroxyvitamin D concentration and leucocyte telomere length in young adults: findings from the Northern Finland Birth Cohort 1966. *Am J Epidemiol* 183(3):191–8. *Equal contribution.
- III Palaniswamy S, De Silva NM, Lowry E, Jokelainen J, Karhu T, Mutt SJ, Dehghan A, Chasman DI, Timonen M, Viinamäki H, Keinänen-Kiukaanniemi S, Hyppönen E, Herzig KH, Sebert S & Järvelin MR (2018) Does vitamin D mediate the association of body mass index with chronic inflammation? – Review of Randomised controlled trials, observational, and Mendelian Randomisation approach. Manuscript.
- IV Palaniswamy S, Williams D, Järvelin MR & Sebert S (2016) Vitamin D and the promotion of long-term metabolic health from a programming perspective. *Nutr Metab Insights* 8 (Suppl 1):11–21 (review article).

Contribution: In Study I and III, SP did all the statistical analyses and had a key role in designing the study, interpreting the results and writing the manuscript. In Study II, SP did the initial analyses that led to the final research focus, participated in the study design, interpretation, and writing the manuscript. In Study IV, SP did the literature search and wrote the article. Parts of the literature review (IV) on the topic “vitamin D and inflammation” that is linked to the objectives of Study III has been updated and used in the thesis.

Permission is not required for articles (I, II & IV) under the terms of the Creative Commons Attribution License.

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1 Introduction

Our knowledge of the physiological role of vitamin D has significantly advanced over the last few decades. The principal function of vitamin D is to control the calcium metabolism and its deficiency is associated with diseases of bone metabolism, such as rickets and osteomalacia. Besides its effects on musculoskeletal system, various other non-skeletal health outcomes have been linked with vitamin D deficiency [indicated by low serum 25(OH)D concentration]. Subsequently, the prevalence of vitamin D deficiency has piqued the attention of both the scientific community and the general public.

The role of vitamin D in non-classical functions has been hypothesised from observational, genetic and experimental studies. Evidence from epidemiological observations has implicated vitamin D status in the risk of many metabolic diseases, including: cardiovascular diseases, diabetes, and infections. Furthermore, a genome-wide map of the vitamin D receptor (VDR) by chip sequencing has reported the presence of 2776 genomic positions occupied by VDR and 229 genes activated in response to vitamin D. The VDR and enzymes involved in vitamin D metabolism have been identified from cardiac muscle, brain tissue, immune cells, and many other tissues. Observational and randomised controlled trials (RCTs) have estimated that increasing vitamin D concentration would help to reduce direct and indirect healthcare costs associated with these diseases in Europe. Currently, the various factors associated with vitamin D status and the risk factors for vitamin D deficiency are not clearly identified. In addition, the exact role of vitamin D in various disease mechanisms and the strategies to reduce this risk are still unknown.

Telomeres located at the end of the chromosomes, undergo gradual attrition in the length with every successive cell division. An inverse relationship between BMI and leukocyte telomere length (LTL) has been reported in various studies. Yet, very limited information is available on the association of vitamin D with telomere length. If a sufficient vitamin D status acts as a rate-limiting step in telomere attrition, then vitamin D status could be targeted as a modifiable factor for improving and maintaining telomere length.

Obesity and its association with low vitamin D status has been reported in multiple studies, and several possible mechanisms for this have been suggested. Recent estimates from the NCD Risk Factor collaboration (NCD-RisC) examining adult body mass index (BMI) trends in 200 countries with 19.2 million participants (1975–2014) have reported an increase in obesity from 3.2% to 10.8% and 6.4% to 14.9% in men and women, respectively. In Finland, the adult (20–64 years) obesity

rates has increased (1978–2016) from 7% to 22% and 8% to 16% in men and women, respectively. Mounting evidence links obesity to various metabolic diseases, including musculoskeletal disorders, diabetes and cardiovascular diseases. This emerging obesity pandemic is expected to cost the European Union €70 billion annually through healthcare costs and decreased productivity.

Vitamin D is proposed to have immunomodulatory properties. Previous experimental investigations have reported the inhibition of inflammatory biomarker secretion through direct inhibition of molecular pathways (e.g. NF- κ B, MAPK). It is hypothesised that reduced vitamin D status with obesity may mediate its association with obesity-induced inflammation. However, the role of vitamin D and its association with BMI, inflammation and leukocyte telomere length in the early stages of disease progression is still lacking. There exists an important gap in our understanding about the extent of these associations in a young general population. This lack of evidence in the literature is problematic; an understanding of the modifiable lifestyle and dietary factors that contribute to inflammation or obesity could provide potential insights for the prevention and targeting of early intervention.

Therefore, the primary focus of this dissertation is to identify the various factors determining vitamin D isoforms, risk factors for low vitamin D status, and examine the association between vitamin D and leukocyte telomere length. I also investigate the association of vitamin D with inflammation including its role in BMI-associated inflammation.

2 Review of the literature

The first chapter of the thesis starts briefly with a general introduction of vitamin D, its history, and vitamin D research in Finland. It is preceded by an overview of the vitamin D isoforms, metabolism and their mechanism of action by binding to vitamin D receptor. In addition, the cutoffs proposed for vitamin D, vitamin D standardization program, the impact of fortification and the prevalence of vitamin D deficiency in Finland, are outlined.

Terminology used in the thesis: In the literature, the term ‘vitamin D’ can be used alternatively to define the molecule, the supplement, the active or inactive form in the blood. To allow consistency and avoid the reader’s misinterpretation, I am using the following terminology throughout the thesis. The terminology refers to the International System (IS). Vitamin D will be used as a global term. 25(OH)D₂ or D₂ and 25(OH)D₃ or D₃ terms are used for the vitamin D isoforms. 25-hydroxyvitamin D [25(OH)D] is the term used to define the circulating concentration measured in serum or plasma. 1,25-dihydroxyvitamin D₃ or calcitriol is the term used to refer the active form of vitamin D. The dietary intake of vitamin D and fortification units will be mentioned as µg (micrograms). The serum concentration of vitamin D will be mentioned as nmol/L throughout the thesis.

2.1 History, structure, metabolism and vitamin D status

Vitamin D is a fat soluble (hydrophobic), secosteroid hormone (1). It is well known for its traditional role of calcium homeostasis in serum, kidney, bone and intestinal tissues, which are vital for supporting cellular processes, neuromuscular functions, and bone osteogenesis (2). The discovery of vitamin D receptor in the 1980s and its presence in over 30 tissues ignited the research on extra skeletal functions of vitamin D (3, 4). In addition, the emerging role of vitamin D in eliciting potent pro- and anti-inflammatory processes in modulating the immune system makes an area of further exploration (5, 6).

2.1.1 History of vitamin D

Until the beginning of the twentieth century, only scarce information was available about the causes of paediatric diseases, such as rickets and scurvy (7). The extreme incidence of rickets in the United Kingdom (particularly in Scotland), which was also known as “the English Disease” led to initial research on vitamin D (7). In

1913, McCollum and Davis discovered that vitamin A came from cod liver oil. Mellanby hypothesised that rickets may be caused by a dietary deficiency disease, deficient in something similar to vitamin A, and investigated the diet of Scottish people who had greater prevalence of rickets. He fed dogs with the diet consumed by the Scottish people (primarily oatmeal porridge) and kept them indoors away from sunlight (8). The dogs developed rickets in a similar phenomenon to humans. Mellanby then used cod liver oil with oatmeal in the diet and assumed vitamin A was responsible for curing rickets (8). Later, McCollum examined the aforementioned hypothesis and eliminated vitamin A from the treatment (cod liver oil). This was found to cause vitamin A deficiency, but was still able to cure rickets (8). McCollum *et al.* (1920) named the new vitamin that cured rickets vitamin D (9). Towards the end of the nineteenth century, Huldshinsky (Austria) and Chick *et al.* (England) examined the children living in sun-deprived cities developing skeletal deformities in contrast to those dwelling in sunny climates (7). Following this, scientists started treating children with rickets either using cod liver oil, sunlight exposure or artificially produced ultraviolet B (UV-B) light (10). The chemist Windaus clarified the chemical structure of vitamin D (D3) (11). He later received the Nobel Prize in chemistry (1928) for his contribution “on the constitution of the sterols and their connection with vitamins”.

2.1.2 Summary of vitamin D research and recommendation in Finland

In the late 1940s, Schultzer and Christensen studied vitamin D in Finland and reported treatment of parathyroid tetany with vitamin D2 (12). In the 1950s, a few articles were published about the “Principles of vitamin D administration for children” and on “Vitamin D requirements in infant feeding” (13, 14). The seasonal variation of vitamin D in the Finnish population was reported from a few studies in the 1980s (15–17). Later, studies examining vitamin D status during pregnancy (18), in children (19), and in old adults (20) were reported.

In 2001, Hypponen *et al.* examined the association between the frequency of vitamin D supplementation during the first year of life and the risk of type 1 diabetes (T1D) in the Northern Finland Birth Cohort 1966 (NFBC1966) (21). The results of the study reported a reduced risk of type 1 diabetes with vitamin D supplementation (21). Similarly, using the same data from NFBC1966, the reduced risk of schizophrenia at a later age (31 years) with vitamin D supplementation during first year of life was reported by McGrath *et al.* (2004) (22).

In Finland, vitamin D supplementation for children in the form of cod liver oil was common in 1930s. From the 1940s to 1960s, the recommended daily dose was 125–100 µg (23). In 1964, the recommended dose was decreased to 50 µg due to reports on vitamin D intoxication (23). Later, in 1972, because of the decline in rickets from 7% to 0.6% in Finland, the recommendation was further lowered to 25 µg (24). The daily dose of supplements of 10 µg was implemented in 1992 (Sosiaali-ja terveystallitus 1992).

In 1980, the first Nordic Nutrition Recommendation (NNR) was published with the latest update in 2013 (<http://www.norden.org/nnr>). Finland has implemented dietary and nutrient intake recommendations based on the common guidelines set in collaboration with Nordic countries by the NNR. The National Nutrition Council (NNC) has monitored the dietary consumption of Finnish population since 1954 and made its first recommendation regarding vitamin D in 1987, with the most recent update in 2014 (25).

2.1.3 Vitamin D isoforms

Vitamin D is obtained from two isoforms, 25-hydroxyvitamin D₂ (ergocalciferol; D₂) and 25-hydroxyvitamin D₃ (cholecalciferol; D₃) (26). D₂ is obtained naturally from plant-based sources such as shiitake mushrooms, portobello mushrooms, irradiated yeast and mushrooms, and fortified dairy products (27). D₃ is obtained by the photochemical reaction of UV-B radiation on the epidermis of the skin (28). About 90–95% of the vitamin D is synthesised naturally by the action of sunlight on the skin (29). D₃ can also be obtained from animal-based sources such as egg yolk, fatty fish such as salmon, tuna, mackerel and small amounts from meat from animal organs (2). Various food and milk products, such as margarine, cheese and orange juice can be fortified with analogues of both isoforms (30).

With limited availability of vitamin D from dietary sources and reduced exposure to sunlight due to apprehension for skin cancer, individuals are turning to supplements to fulfill their vitamin D needs. Both forms (D₂ and D₃) could be obtained as supplements (2, 31). The natural, dietary, fortified and pharmaceutical sources of vitamin D₂ and D₃ isoforms are shown in Table 1.

Table 1. Natural, dietary, fortified and pharmaceutical sources of vitamin D isoforms (Global).

Sources	Composition of vitamin D isoforms
Natural source	
Sunlight exposure (UV-B radiation)	D3
Dietary sources	
Salmon	D3
Sardines	D3
Mackerel	D3
Tuna	D3
Cod liver oil	D3
Shiitake mushrooms	D2
Portobello mushrooms	D2
Egg yolk	D3
Fortified products*	
Milk	D3
Dairy products (butter, cheese, margarine, yoghurt)	D3
Breakfast cereals	D3
Infant formulas	D3
Bread	D3
Alternative milk (soya milk, almond milk, etc.)	D2 or D3
Pharmaceutical (supplements)	
Vitamin D drops or tablets	D2 or D3
Multivitamin tablets	D2 or D3

*The fortified food products and supplements predominantly contain only D3 in Finland. In other countries, the food products are fortified with either D2 or D3.

There is some evidence that vitamin D3 is more biopotent than vitamin D2 isoform (32–34). In Finland, the fortification of margarine with vitamin D began in 1941 (35). Since 2003, all dairy products and dietary fats are fortified with vitamin D3 (<http://www.norden.org/nnr>) (35). In 2010, the amount of fortification with vitamin D was increased in milk and dietary fats (for detailed information, see section 2.2.3, paragraph “Food fortification in Finland”) (35).

2.1.4 Metabolism of Vitamin D

Endogenous synthesis: Vitamin D is an inert biological compound which undergoes various steps of conversion to form an active hormone (2). The *de novo* synthesis of vitamin D occurs in the skin, by the photochemical reaction of UV-B radiation on 7-dehydrocholesterol (7DHC) in the range of 290–320 nm to form the

previtamin D₃ (2, 29). This is followed by thermal isomerisation of previtamin D₃ to vitamin D₃ (2, 29). In circulation, previtamin D₃ concentration increases 10-fold within a few hours of sun exposure (2, 29). However, it may take between 24 hours and a few days to eventually increase the serum concentrations of 25-hydroxyvitamin D [25(OH)D] (29, 36). Furthermore, prolonged sun exposure converts previtamin D₃ into biologically inactive byproducts namely lumisterol, tachysterol and these are stored in the skin as a reservoir (37). These compounds could be converted back to active compounds in the circulation when the serum concentration of previtamin D₃ decreases (37). The steps involved in vitamin D synthesis and metabolism are depicted in Figure 1.

Endogenous vitamin D₃ in the epidermal basal layers, or the dietary vitamin D₂/D₃ absorbed by the intestine, is then bound to a carrier protein, vitamin D binding protein (DBP), and transported to the liver (2, 29). Vitamin D (D₂, D₃) is then hydroxylated at the C25 position by vitamin D-25-hydroxylase enzyme to form 25(OH)D, which is present in an inactive form (2). The hydroxylation of vitamin D to 25(OH)D is not tightly regulated and the circulating concentration of 25(OH)D is proportional to vitamin D intake. 25(OH)D is the main circulating form of vitamin D and has a half-life of approximately 2–3 weeks (28). Serum 25(OH)D concentration is considered to be the best biomarker to measure the vitamin D status of an individual (2). This is because serum 25(OH)D concentration reflects the combined intakes of D₂ and D₃ and the epidermal synthesis during the preceding 3–4 weeks (38, 39).

In the kidney, 25(OH)D is absorbed by the epithelial cells of the proximal tubules and is 1 α -hydroxylated to active metabolite 1,25(OH)₂D (calcitriol) by the mitochondrial enzyme, 25-hydroxyvitamin D-1-alpha-hydroxylase (2, 40). Calcitriol concentrations are tightly regulated and its half-life is approximately 8–18 hours depending on various physiological functions (41). 1-alpha hydroxylase is predominantly expressed in renal tissues, however, it is also present in non-renal tissues, including immune cells (dendritic cells, T-lymphocytes, macrophages) (40).

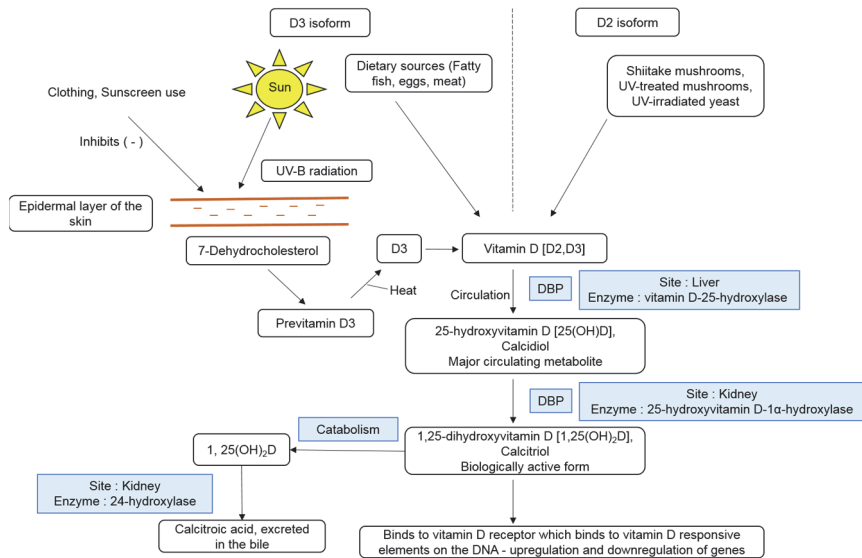


Fig. 1. Synthesis and metabolism of vitamin D. The “vitamin D” represents the combined sources of D2 and D3.

The role of DBP-binding protein: In the circulation, both 25(OH)D and calcitriol are bound to DBP (site of synthesis: liver), a 52–59 kDA protein, for solubilisation and transportation to target tissues (2). Vitamin D toxicity is prevented by the binding of active vitamin D metabolites to DBP (2). The rapid catabolism of vitamin D metabolites is also prevented by DBP, thus increasing its half-life (2). The availability of DBP in serum is 100-fold higher than that of 25(OH)D (2). DBP has the highest binding affinity for 25(OH)D when compared with other vitamin D metabolites (2). DBP binds 88% of 25(OH)D in the circulation (42). Due to structural similarity, albumin, which has lower affinity to 25(OH)D, binds the remaining 12% and a much lower proportion is present in an unbound form in the circulation (42).

Catabolism: When 1,25(OH)₂D is no longer needed for physiological processes the compound is broken down into numerous byproducts (2). These byproducts are then excreted from the body through the bile into the feces (43), and a small proportion excreted through urine (44). Both calcitriol and 25(OH)D are inactivated by the same enzyme, 25-hydroxyvitamin D-24-hydroxylase (2). Although 25(OH)D does not elicit any response in the body, it is inactivated to prevent conversion to calcitriol (2). 1,25(OH)₂D is hydroxylated to produce 1,24,25(OH)₃D, and it is cleaved to produce 1,23(OH)₂D and calcitroic acid, which

are water soluble and excreted in the bile (43). Although this is the primary pathway of vitamin D catabolism, there are other secondary pathways that are not clearly elucidated (45).

Strict transcriptional feedback mechanisms involving parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23) control the renal production of calcitriol, and all target cells express 25-hydroxyvitamin D-24-hydroxylase that converts calcitriol into an inactive form [1,24,25(OH)₃D]. Various extrarenal tissues, including many cell types (macrophages, dendritic cells etc.), also express 25-hydroxyvitamin D-1-alpha-hydroxylase and are therefore able to produce calcitriol. This local production and breakdown is not subject to the same feed back controls as renal production. The functions of calcitriol in locally produced cells are explained in detail in section 2.4.11 (vitamin D and inflammation).

2.1.5 Vitamin D receptor

Calcitriol then binds to the vitamin D receptor (VDR), which undergoes heterodimerisation with the retinoid X receptor (RXR) (46, 47). This further interacts with specific vitamin-D response elements (VDREs) near the promoter region of target genes (27, 46, 47), resulting in initiation of genomic responses. The 1,25(OH)₂D-VDR complex regulates about 3% of the human genome (46, 47). VDR may be located in the cell nucleus (nVDR) or in the plasma membrane (mVDR) (46, 47). One or both types of the VDR are present throughout the body (3, 47). The presence of nVDR in tissues, including adipose tissue and immune cells (T- and B-lymphocytes), has been reported (48–51).

Genomic response: An nVDR genomic response takes place within hours to days leading to transcription of genes and translation of proteins (46, 47). A study that provided a high-resolution map of VDR binding on the human genome using lymphoblastoid cell lines has reported 2,776 VDR binding sites and 229 genes, which could be up- or down-regulated by calcitriol stimulation (46, 47). Genome-wide association studies (GWAS) show high VDR binding activity around genes associated, for example, with T1D, and multiple sclerosis, among others (46, 52). The expression of several genes responsible for bone health and calcium homeostasis, including PTH, and FGF23, are also regulated by VDR activation (46, 47).

Non-genomic response: The mVDR leads to non-genomic functions by rapidly activating the signal transduction pathways (46, 47). mVDR mediates the non-genomic responses within a few seconds to maximum of 10–60 minutes (46, 47).

The second messenger system may include phospholipase (phospholipase C, phospholipase A2), kinases (protein kinase C, mitogen-activated protein kinase (MAPK), phosphatases, G-protein coupled receptors or the opening of ion channels (46, 47). These signalling pathways may alter or regulate the gene expression (46, 47).

Biological functions of vitamin D

The functions of vitamin D are classified as classical and non-classical (2). The classical function of calcitriol is the regulation of mineral homeostasis and bone metabolism (2). Calcitriol binds to the nuclear vitamin D receptor and increases absorption of calcium and phosphates in the intestine and reabsorption in the kidneys. It is also involved in the regulation of serum PTH and FGF23, thereby controlling bone mineralisation, remodeling and bone growth (2).

The non-classical functions of vitamin D are the inhibition of cell proliferation, the role in innate and adaptive immunity by regulating the activation, expression, and differentiation of macrophages, dendritic cells and B and T-cells (2, 47). Based on these biological functions, vitamin D insufficiency/deficiency is hypothesised to have an influence on the immune, endocrine and musculoskeletal health as reported from the literature (53). The non-classical functions of calcitriol in immune cells (locally synthesis) are explained in detail later in sections 2.4.11–13.

Deficiency: Vitamin D deficiency primarily causes rickets in children (2). Vitamin D deficiency in adults leads to an increase in PTH concentration that further leads to increased osteoclastic activity, resulting in the removal of the matrix and mineral from the skeleton (2, 47). The bone mineral content is low, resulting in osteopenia and osteoporosis (2).

2.1.6 Vitamin D: levels and cutoffs

Serum 25(OH)D concentration is considered as the best biomarker of vitamin D status, which reflects both the endogenous production as well as dietary intake of vitamin D (D2+D3) over the preceding 3–4 weeks (38, 39). The cutoffs of 25(OH)D sufficiency and deficiency as proposed by multiple governing bodies are presented in Table 2. The cutoffs of vitamin D sufficiency proposed in Table 2 were based primarily on its association with bone health (IOM, Endocrine society) and very little information is available on the non-skeletal health benefits of vitamin D.

Table 2. Cutoffs used for defining the vitamin D [25(OH)D] status.

Vitamin D status	Institute of Medicine (IOM) (54)	Endocrine Society (55)
Deficiency	≤30 nmol/L	≤50 nmol/L
Insufficiency	30–50 nmol/L	50–75 nmol/L
Sufficiency	≥ 50 nmol/L	>75 nmol/L
High Normal	-	-

Important note: The IOM cutoff for vitamin D will be used throughout this thesis in defining the vitamin D deficiency. The different cutoffs used for reporting vitamin D deficiency will be converted to IOM criteria for consistency and will be mentioned in the following sections.

Apart from these cutoff values for vitamin D sufficiency shown in Table 2, there are also other vitamin D cutoffs used. A study, which examined the vitamin D status in Central Europe, has proposed an increased cutoff range for 25(OH)D concentration to 75–125 nmol/L (vitamin D sufficiency) that could contribute to the reduction of non-skeletal disease outcomes (56). Current recommendations (2016) from the Scientific Advisory Committee on Nutrition, United Kingdom, has stated serum 25(OH)D concentrations to be maintained >25 nmol/L throughout the year (57).

Controversies regarding the optimal concentrations

The determination of “optimal” serum 25(OH)D concentration, as proposed by IOM, were computed using repeated sample analyses and plotting the distribution with the sample mean of the populace (58). The argument exists that normal levels should not be based on the average vitamin D status of the population because these levels may be influenced by people’s lifestyle, behaviour and other socioeconomic factors (59). In addition, optimal cutoffs may be influenced by cultural practices and body weight (60). Endocrine society guidelines acknowledged the differences of vitamin D with body weight and recommended additional vitamin D intake for people who are obese, however, they reported there is no substantial evidence to justify this recommendation (55).

Methods for assessing serum 25(OH)D concentration

Serum 25(OH)D concentration is considered to be the best biomarker of vitamin D and defines the vitamin D status in an individual (2). The measurement of serum 25(OH)D is, however, challenging, because the circulating 25(OH)D is highly

lipophilic, is strongly bound to protein, and exists in two vitamin D isoforms, 25(OH)D₂ and 25(OH)D₃ (61). The detection of both vitamin D isoforms is important for accurate assessment of vitamin D status and monitoring of vitamin D deficiency. There are several established methods for determining 25(OH)D concentration, including high performance liquid chromatography, radioimmunoassay, competitive protein-binding assays, Enzyme-Linked Immunosorbent Assay (ELISA) kits, liquid chromatography tandem mass spectrometry (LC-MS/MS) and some recently developed automated immunoassays (62). Among these methods, LC-MS/MS is considered to be the gold standard reference method for the measurement of serum 25(OH)D (62, 63). The advantages of using the LC-MS/MS method is that it is able to distinguish 25(OH)D₂ and 25(OH)D₃ isoforms, has a low batch-to-batch variation and lower limit of detection (measure even the smallest concentration) (64). However, the instrument is very expensive, requires specialised expertise to operate the system and is not available in most clinical laboratories (64).

As various methodologies are employed to assess serum 25(OH)D concentration, it hinders the comparison and interpretation of results between individual studies for determining the optimal cutoffs, for setting dietary reference values and food-based strategies for prevention of vitamin D deficiency (65, 66). The estimated difference in serum 25(OH)D concentration measured from various analytical procedures may exceed 20 nmol/L (67). In order to overcome this challenge, the International Vitamin D External Quality Assessment Scheme (DEQAS) was established in 1989 with the aim to constantly monitor the different 25(OH)D assays performed in individual laboratories (61). In addition, the Vitamin D Standardization Program (VDSP, 2011) established and organised by the National Institute of Health (NIH) strives to promote the need for accurate and comparable 25(OH)D measurements (68). A standardised laboratory measurement of 25(OH)D is one that is accurate and comparable to other studies over time, location and laboratory procedure (68). VDSP recognises DEQAS as one of the accredited external quality assessment schemes. Until recently, DEQAS assessed the performance of various assays for the determination of serum 25(OH)D by using consensus mean based on all-laboratory trimmed mean (ALTM) of N=1000 participants in 56 countries (69). As more laboratories continue to participate in the DEQAS assessment, the inter-laboratory precision has increased (69). Furthermore, a lack of reference standard for 25(OH)D has been another issue resulting in poor between-method comparability (68, 70). Since October 2012, the National Institute of Standards and Technology (NIST) in collaboration with the US Office of Dietary

Supplements (ODS) of the NIH, has developed and certificated Standard Reference Materials (SRM 972) for determination of 25(OH)D in human serum (68, 70). The NIST, as part of the VDSP has participated in DEQAS by analysing the quarterly serum sample sets to assign an accuracy-based target value for serum total 25(OH)D (69). NIST analysed 90 DEQAS samples between October 2012 and January 2017 to assign target values (69). The NIST-assigned values are compared with the ALTM and the biases assessed for various assays used by the participants. The absolute mean bias between the NIST-assigned values and the DEQAS ALTM was 5.6%, with 10% of the samples having biases >10% (69). Now the serum 25(OH)D measurements are compared against the NIST, thus NIST was incorporated in the DEQAS since 2012.

2.1.7 Vitamin D status in Finland and Europe

Finland lies approximately between latitudes 60°N to 70°N. The population used in this thesis comprises of people born in the two northernmost provinces, Oulu and Lapland (latitude >65°N) and are at risk of vitamin D deficiency throughout the year (71). There is only a few hours of daylight in long winter months, which makes people incapable of synthesising vitamin D naturally (72). In addition, weather conditions may be cold and consequently the hours spent outside are fewer (72).

Impact of first degree of fortification (2003): A cross-sectional data from ‘The Cardiovascular Risk in Young Finns Study’ (2007) has reported prevalence of vitamin D deficiency (<50 nmol/L) in 38% and 34% of males and females respectively (73). The mean concentration reported in the above-mentioned study (N=2,204; age: 30–45 years) was 57 and 61 nmol/L in men and women, respectively (73). In addition, the Finnish National Diabetes Prevention program (FIN-D2D, 2007), including 45–74 year old participants (N=2,822), has reported a lower prevalence of vitamin D deficiency (<50 nmol/L) among the younger age category when compared to older group (74). The mean 25(OH)D concentration reported in that study was 58.2 (men) and 57.1 nmol/L (women), respectively (74). Both the study samples were collected during 2007 and reported the 25(OH)D status after the first degree of vitamin D fortification in Finland (2003). The main message from these studies was that although 25(OH)D concentration increased when compared to earlier reports in Finland, a significant percentage of the subjects remained with inadequate vitamin D status in all the age groups (75).

Impact of second degree of fortification (2010): A recent national survey (FINDIET), which examined the levels of fortification using data collected at three time points, before (2003) and after fortification (2007, 2012) has reported an efficacy of fortification (D3) in increasing the vitamin D status in Finland (76). The study (age: 25–64 years) has reported an average serum 25(OH)D concentrations of 63 nmol/L and 67 nmol/L in men and women, respectively (76). Serum 25(OH)D in this study was measured using high through-put automated chemiluminescent microparticle immunoassay and the laboratory has participated in the DEQAS (76). Another 11-year follow-up study (age: 30–75 years) has reported an increase in mean serum 25(OH)D concentrations from 48 nmol/L to 65 nmol/L (77). This study was in adherence with the VDSP protocol (77). When comparing the two studies, there are still differences in the analytical procedures and use of internal standard reference materials (76, 77). The current studies did not provide any additional information on the factors influencing vitamin D status, such as sun exposure, BMI, lifestyle and others (76, 77).

VDSP standardised vitamin D deficiency – pandemic: A recent review from Europe, which included 55,844 individuals from 14 studies, including Finland, has reported the presence of an average serum 25(OH)D concentration of <30 nmol/L (VDSP protocol standardized) in 13% of the studied population (78). When a cutoff of <50 nmol/L was used, the prevalence of vitamin D deficiency was 40.4% in the population (78). Furthermore, there was a 17.7% prevalence of vitamin D deficiency in extended winter (October–March) while only 8.3% in summer (April–November) (78). The dark-skinned ethnic subgroup of the studied population had a 3–71-fold higher prevalence of vitamin D deficiency (<30 nmol/L) when compared to Caucasians (78).

2.1.8 Summary

In summary, this section has outlined the history, main sources of vitamin D (D2, D3) and the metabolism and function of vitamin D in the body. 25(OH)D concentration, which is obtained as the sum of D2 and D3, are considered to be the best biomarker of vitamin D status in an individual. However, controversies exist regarding the definitions and optimal cutoffs proposed for vitamin D sufficiency.

As vitamin D exists as two isoforms, it is important to consider how the known determinants contribute to the concentration of each, as well as the overall 25(OH)D concentration. The literature lacks knowledge on the association between multiple factors and the individual vitamin D isoforms (D2, D3).

Study I of this thesis proposes to better understand the factors (environmental, anthropometry, lifestyle, socioeconomic, sex-specific) and their association with two isoforms of vitamin D (D2, D3). Along with the investigation of the determinants associated with vitamin D isoforms, Study I will also examine the factors influencing the 25(OH)D concentration, which will be discussed in the following section 2.2.

A proper knowledge on the factors associated with D2 and D3 concentrations could help in achieving vitamin D sufficiency through either natural sources (sunlight and diet) based on season or by supplementation.

2.2 Factors influencing the vitamin D status (study I)

The aim of this section is to provide an introduction to the known environmental, anthropometric, lifestyle, socioeconomic and sex-specific determinants associated with vitamin D, and the risk factors of vitamin D deficiency. In addition, the proposed mechanisms behind all of these factors and their association with vitamin D deficiency has been elaborated in this chapter.

2.2.1 Environmental determinants

Seasonal variation, atmospheric conditions (pollution, cloud cover) and latitude are the environmental determinants, that influence the cutaneous synthesis of vitamin D.

Seasonal variation, pollution, cloud cover: The first major source of vitamin D is obtained through sun exposure. Although the conversion of 7-DHC to previtamin D3 occurs at the wavelengths between 290 and 320 nm, these wavelengths are less likely to reach earth's surface in considerable amounts due to residing in higher latitudes (heavy ozone cover) and thick air pollution (79). Besides air pollution, heavy cloud cover could inhibit the cutaneous synthesis of 25(OH)D by interference with UV-B radiation (79). In addition, the season of the year largely influences the amount of solar radiation reaching the atmosphere (79). Figure 2 shows maximal vitamin D synthesis by UV-B radiation in summer (example from the NFBC1966 data).

Latitude: The UV-B radiation from the sun is absorbed efficiently by the ozone layer before it reaches the earth's surface. Only 1% of the UV-B radiation reaches the earth surface in summer (midday, 12.00 noon) (80). This is proposed to be the reason for the insufficient vitamin D synthesis in individuals residing in latitudes

greater than or lesser than 33°. In addition, people residing in the far north or south (72) are unable to synthesise vitamin D for nearly half (6 months) of the year (80). Subsequently, the mean serum 25(OH)D concentrations are lower in northern hemisphere when compared to the southern hemisphere (81).

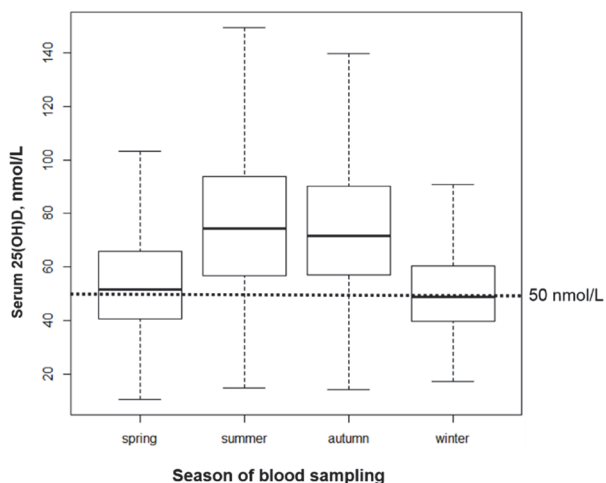


Fig. 2. Box plot of serum 25(OH)D by the season of blood sampling in NFBC1966 [age: 31 years; N=4,758 (males and females)]. According to Finnish Meteorological Institute (82), the season of blood sampling was classified as spring (April–May), summer (June–August), autumn (September–October), and winter (November–March). The dark central line in the boxes represents the mean 25(OH)D concentration (nmol/L). The dotted line represents IOM criteria for vitamin D sufficiency (50 nmol/L). Higher mean 25(OH)D concentration was observed in the summer.

Other factors influencing the cutaneous synthesis of vitamin D

Age, skin pigmentation and sun exposure habits (use of sunscreens and sun avoidance) are the other determinants, that influence cutaneous synthesis of vitamin D.

Age: Age is a factor that affects the D3 synthesis. A study that examined skin biopsies obtained from individuals aged 8–92 years has observed an age-dependent decrease in the quantity of 7-DHC (83). Comparison of skin samples obtained from older (77–82 years) and younger persons (8–18 years) reported a two-fold decrease in the capacity of skin to synthesise D3 in older subjects (83). In addition, damage to the skin from burns or scars affects vitamin D cutaneous synthesis (83–85).

Melanin (skin pigmentation): Melanin that gives pigmentation to the skin is produced by melanocytes. Melanin pigmentation acts as a natural sunscreen against UV-B radiation (86). Individuals with darker pigmentation form less D3 when compared with individuals having lower concentration of melanin pigmentation (36). This is also supported by the presence of varying concentration of serum 25(OH)D in different ethnic groups. Individuals from ethnicities with darker melanin pigmentation have lower 25(OH)D when compared with lighter skin individuals (87).

Use of sunscreen: Application of sunscreen creams interfere with the cutaneous synthesis of vitamin D by absorbing UV-B radiation before it enters the skin. Sunscreens, which have a sun protection factor (SPF) of 15, reduce the synthesis of D3 by 98% (88). The synthesis of vitamin D through UV-B radiation without the use of sunscreens is based on both melanin pigmentation as well as phototype of the skin (89). An individual's skin phototype (I–VI) influences the extent of tanning and sun burning behaviour with the exposure to UV-B radiation. The skin phototypes and sun exposure characteristics are explained in the Table 3.

Table 3. Skin phototypes (I–VI) and sun exposure characteristics (89).

Skin phototype	Melanin pigmentation	Time for vitamin D synthesis (June–August, 11AM–2 PM) needed per day with no use of sunscreen ^{3,4} .	Sun exposure characteristics ¹
I	Light, pale white	5 minutes	Never tan, always burn
II	White, fair	10 minutes	Tan slightly, burn slightly
III	Cream white	15 minutes	Tan moderately, rarely burn
IV	Moderate brown	20 minutes	Tan darkly, never burn
V	Brown, Dark brown	25 minutes	Tan easily, very rarely burn ²
VI	Deeply pigmented dark brown to black	30 minutes	Tan easily, may never burn ²

¹Sun exposure characteristics time: reaction to 30 minutes of direct sun exposure after winter (e.g. first warm day of spring).

²The classification of skin phototypes was based on race. The individuals are identified to have more protection from UV-B radiation when compared to Caucasians.

³Approximate calculation of vitamin D synthesis from UV-B radiation. Add 5 minutes more of sun exposure if residing in Northern Europe/USA and 10 minutes far from equator (Finland/Alaska). Add 3 times for older adults, cloudy day, being in shade, 2 times if the weather is indefinite. Decrease by half if residing near equator.

⁴Calculated based on the percentage of the body exposed to skin in young individual.

2.2.2 Anthropometric factors: BMI and waist circumference

The association of BMI and fat mass with vitamin D status is described in section 2.4 (see section 2.4.9). This section only describes the association of waist circumference with vitamin D.

Waist circumference: Measurement of waist circumference (WC) is a convenient way to assess central (abdominal) obesity. It partially reflects the presence of visceral adipose tissue (VAT) in an individual. The adipose tissue compartments will be discussed later in section 2.4 (see section 2.4.4).

Waist circumference is measured at the level midway between the lowest rib and the iliac crest. Table 4 shows the current recommendation for abdominal obesity based on the guidelines by different governing bodies.

Table 4. Waist circumference thresholds for abdominal obesity (90).

Ethnicity/Country	Organisation	Men (cm)	Women (cm)
European Origin	IDF (90)	≥ 94	≥ 80
USA	ATP-III (91)	≥ 102	≥ 88
Asian (Chinese, Malaysian and Asian-Indian, Japanese)	IDF (90)	≥ 90	≥ 80
Japanese	Japanese obesity society (92)	≥ 85	≥ 90
China	Cooperative Task Force (93)	≥ 85	≥ 80
Ethnic south and Central Americans	IDF (90)	South Asian recommendations are used; no specific data available	
Eastern Mediterranean and Middle East (Arab) populations	IDF (90)	European recommendations; no specific data available	
Sub-Saharan Africans	IDF (90)	European recommendations; no specific data available	

* International Diabetes Federation (IDF).

* National Cholesterol Education Program Adult Treatment Panel III (ATP III).

A prospective study from Norway (N=2,460; age: 19–55 years) has observed 25 nmol/L reduction in 25(OH)D with an increase of 0.5 cm in WC during follow-up, who had normal WC at baseline (94). Another study (country: New Zealand; N=250; age: >18 years) on overweight and obese subjects has reported a decrease in 0.29 nmol/L vitamin D per 1 cm increase in WC. From these studies, it is inferred that abdominal obesity may influence serum 25(OH)D concentration (95).

2.2.3 Lifestyle and behavioural factors

Together with factors influencing cutaneous synthesis, an individual's lifestyle and behavioural factors may play a role in determining the vitamin D status. Although people residing in lower latitudes (around 40°N) (96) receive abundant sun exposure throughout the year, they are still vitamin D insufficient probably because of their lifestyle and behavioural practices (85). Cultural factors (covered clothing) (97), working patterns (indoor or outdoor) (98) and behavioural factors (time spent outdoors, use of sunscreen, sun avoidance) largely influence vitamin D synthesis (99).

Cigarette smoking, alcohol consumption, physical activity

Smoking: Some studies have reported an inverse association between cigarette smoking and vitamin D deficiency (100–103) while others have reported no relationship (104, 105). The mechanism underlying smoking status and vitamin D metabolism is unclear.

Proposed mechanism: In a recent investigation, smoking has been reported as an effect modifier between vitamin D and C-reactive protein (CRP) concentrations (Figure 3) (106). A study that compared vitamin D markers between smokers and non-smokers has reported lowered mean concentrations of 25(OH)D and calcitriol in smokers (107). Prolonged cigarette smoking may result in increased inflammation in smokers when compared to non-smokers.

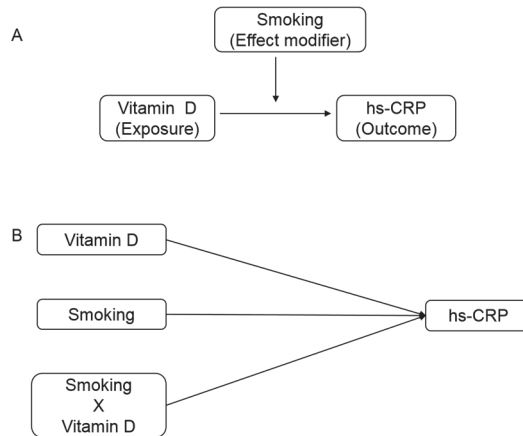


Fig. 3. (A) A conceptual diagram of effect modification. (B) Smoking was reported as an effect modifier that changed the association between vitamin D and CRP concentration. The association between 25(OH)D and CRP was reported to be stronger in smokers vs. nonsmokers (106).

Alcohol: A recent systematic review that assessed the association between alcohol consumption and vitamin D from studies published between 1976 and December 2015 has reported 15 studies with positive association [increase 25(OH)D], 18 studies with an inverse association, and 16 studies with no association described (108). Alcohol consumption is suggested to decrease PTH secretion, thereby preventing the conversion of 25(OH)D to calcitriol, resulting in an increase in serum 25(OH)D concentration in the circulation (108). However, the plausible biological mechanisms between alcohol consumption and increase in 25(OH)D still remains unknown.

Proposed mechanism: Due to ethical reasons, no study has examined the association between 25(OH)D and alcohol administration in humans. A study conducted in mice fed with 30% ethanol diet and cosupplementation with vitamin D has reported maintenance of normal serum calcium concentration in the alcohol-vitamin D group (vs. alcohol group alone) (109). This mechanism suggests that vitamin D may act as an important factor in balancing the calcium homeostasis and bone resorption (a process that results in loss of calcium from bone into blood) in chronic alcohol condition (109). Whether these mechanisms exist in humans is unknown.

Physical activity: Physical activity has been reported to be positively associated with serum 25(OH)D concentration (110–113). A study which examined

both objectively measured and self-reported moderate to vigorous physical activity (N=6,370; mean age: 47.7 years; country: USA) has reported an increase of 0.80 nmol/L and 0.45 nmol/L of 25(OH)D with 10 min increase per day of physical activity (112).

Proposed mechanism: Sun exposure during outdoor physical activity is proposed to increase 25(OH)D concentration. Sun exposure may act as proxy to increase the cutaneous synthesis of D3 during outdoor leisure and vigorous physical activity, thereby increasing serum 25(OH)D. However, Scragg *et al.* reported higher 25(OH)D status in individuals, irrespective of indoor and outdoor activity (110). Another study conducted in postmenopausal women (country: USA; N=1,343; age: 50–79 years) has reported an association between physical activity and vitamin D, even after adjustment for sun exposure (114). In addition, vigorous physical activity was positively associated with increased serum 25(OH)D in people residing at higher latitudes (64°N; Norway) (115). These results may suggest physical activity as an independent determinant of vitamin D status independent of sunlight exposure. In addition, physical activity has been reported to be inversely associated with decreasing inflammatory biomarkers (116, 117). By these mechanisms, physical activity may influence both vitamin D status and inflammatory biomarkers, simultaneously.

The plausible mechanisms in all of these behavioural lifestyle factors, and their associations with vitamin D status, has yet to be clarified in future investigations.

Vitamin D and dietary consumption

The second major source of vitamin D is obtained through the consumption of vitamin D-rich foods. The dietary sources of vitamin D is shown in Table 1 (see section 2.1.3). During vitamin D synthesis, the D3 isoform undergoes complex conversions (Figure 1; see section 2.1.4) to form serum 25(OH)D concentration. However, the dietary sources of D2 and D3 isoforms are able to bypass these complex reactions and could enter directly into the same metabolic pathway (2). D3 cutaneous synthesis requires exposure to sun throughout the year, which is not possible in higher latitudes (e.g. Northern Finland). Therefore, the best source for maintaining serum 25(OH)D concentration throughout the year is through dietary intake of vitamin D-rich foods.

The Nordic Nutrition Council has reported the average dietary intake of vitamin D in the Nordic countries (Table 5) (25, 96). This average consumption was calculated based on the published literature from the Nordic countries (Finland,

Sweden, Norway, Denmark and Iceland). Finland was reported to have highest dietary intake of vitamin D compared with other Nordic countries (Table 5). The reported dietary intake of vitamin D from Finland was based on National FINDIET survey in 2012 (118). The higher dietary intake of vitamin D in Finland when compared with other Nordic countries is attributable to fortification of food products with vitamin D (75).

Table 5. The average dietary intake of vitamin D in Nordic countries (supplement use not included) (2014).

Country	Age group (years)	Dietary intake of vitamin D (µg/day)
Denmark	4–75	3.5
Finland	25–74	12.8
Sweden	18–80	8.8
Norway	18–70	6.2
Iceland	15–80	9.8

Report from the Nordic Council of Ministers (2014) (25, 96).

The presence of vitamin D in the average diet in the Nordic Countries.

A longitudinal study (N=4,185; Denmark) examining 25(OH)D concentration at baseline and 5-year follow-up has reported a positive association between healthier diet consumption [vs. unhealthy diet] and the vitamin D status (119). The above-mentioned study utilised a food frequency questionnaire and the dietary habits (healthy, average, or unhealthy) was computed based on intake of vegetables, fish, saturated fat and fruit (119). In addition, subsample (N=10,674) longitudinal analyses at two time points has reported 4.54 nmol/L difference in serum 25(OH)D concentration between healthy vs. unhealthy diet consumers (119). The multivariable analyses included adjustment for time point, season, sex, age, education, alcohol, smoking, diet, physical activity and BMI (119). This study may suggest a direct influence of diet on vitamin D status, through mediation by BMI or socioeconomic factors or other pathways (119). The influence of SEP and BMI on dietary intake is discussed in the section 2.2.5.

Studies investigating the trend of vitamin D consumed through “traditional” diet (fish) and “modern day” diet (imported meat, sweets, cakes, chips, soft drinks) in Alaska (1960–2010) (120) and in Greenland (1987–2010) (121) have reported a greater decline in vitamin D in those consuming a “modern day” diet. The study from Alaska was performed based on the higher incidence of rickets and vitamin D deficiency during 2001–2010 in Alaskan native children (122). The proposed mechanism behind obesity and low vitamin D status through diet is explained in

detail in section 2.4.9. These reports suggest the importance of diet in influencing 25(OH)D concentration, which may act as a modifiable factor to prevent vitamin D deficiency. Along with natural dietary sources of vitamin D, the following section will discuss on the importance of fortification and supplements to maintain optimal vitamin D status.

Food fortification in Finland

Vitamin D fortification of dairy products was adopted in Finland in 2003 (75). The initial fortification levels were 0.5 µg per 100 g for all fluids and 10 µg per 100 g of D3 for spreadable fats (75). Following this national policy, many studies in the 2000s examined the efficacy of vitamin D fortification in increasing the serum 25(OH)D status of individuals (see section 2.1.7). The studies reported the presence of vitamin D insufficiency/deficiency in all age groups and inefficacy of food fortification in increasing vitamin D status (75). In April 2010 the National Nutrition Council in Finland revised the old recommendation by increasing the fortification levels to 1.0 µg per 100 g and 20 µg per 100 g of D3 for all fluids and spreadable fats respectively (75). Table 6 shows the recommended daily intake of vitamin D and supplement in Finland (2016).

A total dietary intake of 10 µg per day of vitamin D is recommended for individuals with little or no sun exposure. In addition, 10 µg per day of vitamin D supplement is recommended throughout the year, if vitamin D-rich foods are not consumed regularly through diet (96). In recent years, multiple food products, including bread, cereals, pasta and flours, were fortified with vitamin D (123, 124).

Stability: A study which investigated the stability of D3 and D2 in food products has reported that the retention of vitamin D is based on the household cooking procedures used (125). The retention of D3 from eggs and margarine cooked in oven (40 min; heat: 170–200°C) was reported at 39–45% and frying at 82–84%. The retention of D3 in boiled eggs was reported at 86–88%, wheat bread at 85% and rye bread with lower retention at 69%. D2 was reported to have a slightly higher retention of 73% (rye bread) and 89% (wheat bread). Other studies that examined the stability and shelf life of dairy products fortified with D3 reported insignificant loss of vitamin D from these products (125–127).

Table 6. The Nutrition Council recommendations for dietary intake and supplementation of vitamin D in Finland (2016). Data obtained from evira.fi (128).

Age group/years	The recommended total dietary intake, µg/day ^a	Additional consumption (along with dietary intake of vitamin D) ^b
2 weeks to 2 years	10	10 µg/day throughout the year
2–17 years	10	7.5 µg/day throughout the year
18–60 years	10	10 µg/day if necessary ^b
61–74 years	10	10 µg/day if necessary ^b
≥ 75 years	20	20 µg/day throughout the year ^c
Pregnant and breast feeding women	10	10 µg/day throughout the year

^{*}No recommendation for micronutrients given for children under 6 months. Breastfeeding is the preferred source of nutrition for infants.

^a Recommended total dietary intake of vitamin D and through fortified food products.

^b If vitamin D fortified food products and fish are not consumed during the dark time of the year (October–March).

^c A smaller dose, 25nmol/L (10 µg/day) of vitamin D supplement is sufficient, if used on a regular basis along with consumption of a lot of vitamin D fortified food products.

Therefore, the availability of vitamin D consumed through dietary products is based on the household cooking procedure. This may result in considerable loss of vitamin D obtained through diet. The impact of fortification on serum 25(OH)D concentrations in Finland and their limitations are mentioned earlier in section 2.1.7.

Biopotency

An introduction of vitamin D isoforms (D2, D3) is mentioned earlier in section 2.1.3. There is some controversy regarding the biopotency of D2 and D3 in raising and maintaining vitamin D status. D2 is considered less biologically active when compared with D3 (96, 129, 130). RCT examining D2 (N=20) and D3 (N=20) supplementation (dose: 25 µg per day; age: 18–84 years) for 11 weeks have reported D2 as effective as D3 in raising 25(OH)D concentration (131). However, systematic review and meta-analysis on 7 studies that examined D2 and D3 supplementation on serum 25(OH)D concentration, reported D3 to be more potent in raising 25(OH)D when compared to D2 (mean difference=15.23 nmol/L) (130). The study reported greater heterogeneity among the studies included in the meta-analysis (130). There are some reports hypothesising on the lower affinity of D2 in binding to the vitamin D receptor, which may lead to rapid clearance from the

circulation (32–34). However, controversies still exist on the efficiency of the vitamin D isoforms in increasing individuals serum 25(OH)D concentration (131).

2.2.4 Sex-specific factors

Serum 25(OH)D concentration in relation to sex has been debated. Studies have reported higher serum 25(OH)D concentration in men (81), in women (73, 87, 132, 133), or no association (134). There are few factors that influence the serum 25(OH)D concentration in relation to sex and is discussed below.

Proposed mechanisms: The sex differences in relation to vitamin D status could be influenced by the dietary intake of vitamin D, differences in body composition and oral contraceptive practices.

Women: oral contraceptives

A study conducted in 2,204 adults from Finland (age: 30–45 years) has reported a low serum 25(OH)D concentration in men versus women (mean=57 vs. 61 nmol/L) (73). Women using oral contraceptive pills (OCPs) versus nonusers had higher serum 25(OH)D concentration (mean=69 vs. 59 nmol/L) (73). The use of oral contraceptive in women may reflect an increase in serum 25(OH)D concentration in the circulation. This may explain the difference in average serum concentration in the general population being higher in women when compared to men.

Harris and Dawson-Hughes (1998) were the first to report an association between plasma 25(OH)D concentration and use of oral contraceptive pills in women (135). Following this, some cross-sectional studies and one randomised controlled trial have reported a similar association between OCPs and the vitamin D status (136–140).

Proposed mechanisms: The estrogen content in oral contraceptive is suggested to increase the VDBP concentrations. Cross-sectional examination of women (N=75; age: 25–35 years) using hormonal contraceptives (N=52) when compared with nonusers (N=23) has reported an increase in the vitamin D binding proteins (median: 358 vs. 271 µg/mL) (139). Binding of 25(OH)D to VDBP can increase its half-life, that could reflect a higher pool of serum 25(OH)D concentration in women using contraceptives. Contraceptive users had 13–25% higher concentrations of 25(OH)D, calcitriol and VDBP. A study conducted in postmenopausal women (N=187; age: 45–58 years), which examined over a period of 5-years the variability in 25(OH)D concentration with VDBP, has reported an 8%

increase in VDBP in response to estrogen therapy (141). Another study which examined African American women (N=1,662; age: 23–34 years) has also reported similar observations (138).

Oral contraceptive, vitamin D and inflammation: Oral contraceptive use in women has been reported to be positively associated with increased CRP concentration when compared with nonusers (142). This study has utilised the same population as examined in this thesis (142). Oral contraceptive increases 25(OH)D (136–139) as well as inflammatory biomarkers (142). This increase in 25(OH)D concentration in the circulation may be because of its immunomodulatory role to decrease the inflammation arising in women using oral contraceptive, as shown in Figure 4. However, this mechanism is not clearly understood and requires future studies to investigate the above-mentioned association.

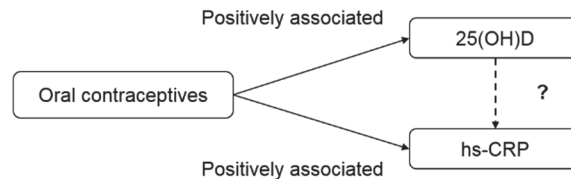


Fig. 4. Oral contraceptive and their association with vitamin D and inflammation. Oral contraceptive increase both 25(OH)D and hs-CRP in the circulation. This increase in 25(OH)D may occur as a result of anti-inflammatory function to decrease the inflammation arising with the contraceptives.

2.2.5 Socioeconomic factors

Socioeconomic position (SEP) is considered to be another factor affecting the vitamin D status, and this may explain many of the above-mentioned associations. A systematic review examining the socioeconomic determinants of micronutrient intake in studies published from Europe has reported a positive association between SEP and vitamin D (143). The socioeconomic factors examined in relation to vitamin D include occupation, education status and income.

Occupation, education, income: A recent systematic review that examined different occupations in relation to vitamin D has reported indoor workers, shift workers and healthcare workers to be at high risk for vitamin D deficiency (144). A study from Finland (N=5,714; age: 30–79 years), which examined socio-demographic factors in relation to vitamin D, has reported positive association

between education and higher vitamin D status. Participants with higher education (>12 years) had 7.4 nmol/L higher 25(OH)D concentration when compared with the lowest education category (<7 years) ($p<0.001$; adjusted for age, sex and season) (145). Similar associations have been reported from other studies (146, 147). Lower education levels may suggest low food diversity choices and poor quality of dietary intake (148). Another study from Belgium (N=915; age: 20–69 years) has reported participants with low household income (<2250€ per month) to have 1.73 times greater odds of being vitamin D deficient than with high household income (3000–10000€ per month) (149). Few other epidemiological studies have reported similar observation between income and vitamin D status (150, 151).

Proposed mechanisms: BMI, diet, physical activity, smoking and alcohol consumption are proposed to explain the influence of socioeconomic factors on vitamin D status. Systematic review and meta-analysis, which examined socioeconomic position over the life course, has reported an inverse relationship between SES and obesity in women from developed nations (152). BMI was reported to be higher in lower SEP groups versus high SEP over the life course (152). Individuals of low socioeconomic gradient may adopt the consumption of an unhealthy dietary pattern when compared with other socioeconomic groups (153). Vitamin D dietary sources, such as fish (seafoods), dairy products and supplements, may be of higher cost to buy and consume regularly in low SEP groups. A study that examined the relationship between SEP and quality of diet, has reported the cost of diet in mediating the pathway between income and quality of dietary intake (154). Another study from France, that examined [N=564; mean age: 43y] class III obese individuals with socioeconomic predictor score, has reported low SEP as an independent risk factor for vitamin D deficiency (155). However, studies examining the association between SEP and vitamin D have not included a correction for BMI or other lifestyle factors. Thus, socioeconomic factors may contribute to vitamin D deficiency directly or indirectly through diet, BMI, and other lifestyle factors.

2.2.6 Genetic factors

The serum 25(OH)D concentration may be influenced by multiple genetic variations. Identification of those variants could be useful to prevent vitamin D deficiency in at-risk individuals. The genetic variations have been examined by single nucleotide polymorphisms (SNPs) in the genes that are present in the vitamin D pathway. The genes that are commonly assessed are the two cytochrome enzymes

CYP2R1 (vitamin-D-25-hydroxylase), CYP24A1 (25-hydroxyvitamin D-1 α -hydroxylase), 7-dehydrocholesterol reductase (DHCR7; enzyme that converts 7-DHC to cholesterol), and vitamin D binding protein (DBP; GC). A twin study performed in Sweden (N=204, same-sex monozygotic and dizygotic; age: 39–85 years, 60°N) has reported the influence of genetic factors on serum 25(OH)D concentration (156). A genome wide association study (GWAS) using 33,996 individuals (European origin; including NFBC1966), has reported the presence of variants in three loci of vitamin D pathway genes (157). The genes reported from GWAS were DHCR7 (enzyme lowers the substrate availability of 7-DHC for 25(OH)D formation), CYP2R1 [mutation in this gene, inability of hydroxylation to form 25(OH)D from vitamin D3], and DBP (GC variant) (157). However, a recent study reported nil observation (158), but was conducted on smaller samples. These variants may influence 25(OH)D concentration and result in variability in vitamin D status.

2.2.7 Summary

In summary, this section has outlined the factors influencing vitamin D status and the proposed mechanisms underlying the association were discussed.

The determinants and risk factors associated with vitamin D vary within different populations. It is not clear what factors influence vitamin D status in the Finnish young adult population at 31 years. In addition, the risk factors for vitamin D deficiency in this population is unclear. Identification of modifiable determinants influencing vitamin D status may help prevent vitamin D deficiency during the long winter months. The NFBC1966 data at 31 years was collected before a general degree of fortification was introduced to dairy products in Finland.

Study I of this thesis proposes to understand the factors (environmental, anthropometry, lifestyle, socioeconomic and sex-specific) and their association with 25(OH)D₂ and 25(OH)D₃ concentrations, and the factors associated with being in low 25(OH)D status.

A better understanding of the modifiable determinants and factors of low vitamin D status, will be essential to maintain serum 25(OH)D concentration as well as direct appropriate intervention strategies to prevent deficiency/insufficiency.

2.3 Vitamin D, BMI and Leukocyte telomere length (Study II)

The aim of this section is to provide a general introduction on telomeres, telomere shortening and their association with metabolic health outcomes. In addition, the association of BMI and vitamin D with leukocyte telomere length (LTL) will be discussed. The key biological terms used in this section and their descriptions are provided in section 8, Appendix 8.1.

2.3.1 Telomeres and telomere maintenance

Telomeres are the DNA-protein complexes located at the terminus of the eukaryotic linear chromosomes (159, 160). Telomeres are noncoding nucleotide sequences consisting of repeated hexamer 5'-TTAGGG-3'/5'CCCTAA-3' at the end of eukaryotic chromosomes (159, 160). In humans, the length of the telomere sequence can extend from 5 to 15000 base pairs (161). These nucleoprotein complexes serve to cap the ends of the chromosome and protect against chromosomal deterioration and fusion with neighbouring chromosomes during cellular mitosis (159, 160). In addition, they protect the chromosomal ends from being mistakenly recognised by the DNA-damage repair system as double-stranded breaks (162, 163). It is estimated that the length of the telomere inexorably shortens with each successive rounds of cell division (164, 165). This results in the loss of 30 to 200 base pairs of telomeres during each replication (161, 166). The mechanism, which results in loss of base pairs, is known as the end replication problem that leads to a gradual attrition in length of the telomere. Cell division in humans usually occurs 50–70 times before the telomere reaches cell senescence. During each cell division, the telomeres present at the end of the chromosome becomes shorter, and eventually, no longer telomere is present on the chromosome leading to a replicative senescence (Table 6) (162, 167, 168).

Although telomere length shortening with each cell division is inexorable, telomere length (repeats) can be elongated and maintained by a ribonucleoprotein enzyme, the telomerase (169, 170). Telomerase enzyme is comprised of a catalytic protein subunit (telomerase reverse transcriptase, TERT) and an RNA component (telomerase RNA component, TERC) (171, 172). The reverse transcriptase enzyme (TERT) functions by adding the TTAGGG repeats at the 3' end of the chromosome along with the TERC component, which acts as a template. This results in the addition of new telomeric sequences from the TERC component, and hence, involved in the elongation of telomeres (169, 170). Telomerase enzyme prevents

the telomere shortening, thereby maintaining the length of the telomere (173). In addition to the telomerase enzyme, two other proteins play an important role in the maintenance of telomere length (161, 174). They are telomere repeat binding factors 1 (TRF1) and 2 (TRF2). The approach of telomerase to the telomere complex is regulated by the TRF1 and hence plays an important role in telomere length regulation (174). TRF2 protects the ends of the telomeres thereby suppressing the DNA damage repair system (174). The single-stranded 3' end of the guanine-rich overhanging of the telomere folds back on itself, ultimately forming a protective structure termed as telomere-loop (t-loop) (174). The t-loop is maintained by TRF2 that halts the recognition of single- and double-stranded DNA breaks and suppressing the senescence and apoptotic pathways (161, 174).

2.3.2 Telomere length measured from leukocytes

Leukocytes, or white blood cells (WBCs), are constituted of cells originating from both myeloid progenitor, which gives rise to granulocytes (neutrophils, eosinophils, and basophils), monocytes/macrophages, and lymphoid progenitor, which produces T-cells, B-cells, and Natural Killer (NK) cells (175, 176). All of the WBC cells are present with a nucleus, which differentiates these cell types from red blood cells and thrombocytes that are anucleated. In the peripheral leukocytes, neutrophils are comprised of 50–70%. In the blood leukocyte count, lymphocytes constitute 20–40% (predominantly T-cells) (175, 176). Telomere lengths present greater inter-individual variability with age (175, 176). However, within an individual exists a significant correlation in mean leukocyte telomere length measured from peripheral leukocytes and the telomere length, which are found in different organ/tissue compartments (177–179). Therefore, LTL measured from blood leukocytes act as a proxy for the telomere length obtained from different tissues within an individual.

In epidemiological studies, telomere length has been measured primarily from peripheral blood leukocytes (comprises of all the WBC subsets arising from both the myeloid and lymphoid progenitors) (175, 176). The length of the telomeres vary among different immune subsets that are present in the blood (175, 176). Therefore, telomere length measured from peripheral leukocytes denote the average length comprising the heterogeneous group of immune cells in the circulation.

2.3.3 Factors influencing LTL and its association with metabolic health

Telomere loss primarily depends on cell division frequency (normal cell division process), genetic, environmental factors and telomerase activity. It is, therefore, plausible that LTL may be modified by environmental and lifestyle factors (180–183). The association between multiple lifestyle and behavioural factors have been examined in relation to LTL. Smoking (182), oxidative stress (166, 184), and obesity ($\text{BMI} \geq 30 \text{ kg/m}^2$) (182) have been reported to be associated with shortened LTL. Telomere length shortening leads to increased risk of cell death, abnormal cell proliferation, and senescence in humans (185, 186). Shorter telomeres trigger cell senescence and were reported to be positively associated with secretion of proinflammatory cytokines, which may negatively influence the tissue microenvironment ultimately leading to pathological conditions (187, 188).

Longer LTL is considered to be a biomarker of cellular longevity (189). Factors such as physical activity (190–192), dietary patterns (193), consumption of omega-3 and 6-fatty acids (194), and multivitamin use (195) were reported to be associated with longer LTL. In addition, recent RCTs have suggested that incorporating lifestyle and behavioural changes, including physical activity, weight loss and management, and stress management may modify telomerase enzyme and prevent telomere shortening (190, 191). However, recent findings suggest that abnormally long telomeres are associated with various cancers including colorectal, breast and lung (196, 197).

In recent years, epidemiological studies have provided evidence on the association between LTL and the risk of chronic conditions, including various facets of cardiovascular diseases and T2D (187, 188, 198). The association between shorter LTL and mortality has also been reported by two twin studies (199, 200). However, a few other studies, which examined the above-mentioned association, have reported null results (201–203). A recent genome-wide meta-analysis study ($N=37,684$) has reported seven loci that were associated with mean leukocyte telomere length and supported a causal role of TL variation with metabolic health outcomes (204). Figure 5 shows the factors associated with both 25(OH)D and leukocyte telomere length.

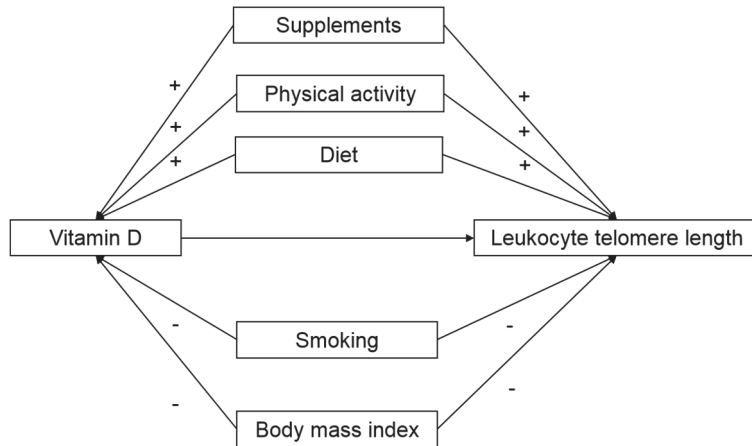


Fig. 5. Factors associated with both vitamin D and leukocyte telomere length.

2.3.4 BMI and leukocyte telomere length

Body mass index has been reported to be inversely associated with telomere shortening in epidemiological studies (182, 185, 205). In addition, two systematic review and meta-analyses have reported a negative association between BMI and LTL (206, 207). However, a greater heterogeneity among the studies included in both the systematic reviews were reported (206, 207). A study (N=309; age range: 8–80 years), which examined different parameters of body composition, has reported an inverse association between BMI, total body fat, abdominal adiposity and shortened LTL (208).

A recent study (N=7,008; age range: 20–85 years) which examined the association between life-course overweight trajectories and LTL, has reported a 0.2% decrease in telomere length with 1 kg/m² increase in BMI (207). A follow-up study of young and middle age adults over 10 years has reported a similar negative association between BMI and telomere restriction fragment (TRF) (209). TRF is another analytical procedure used to measure leukocyte telomere length (209) (Figure 6).

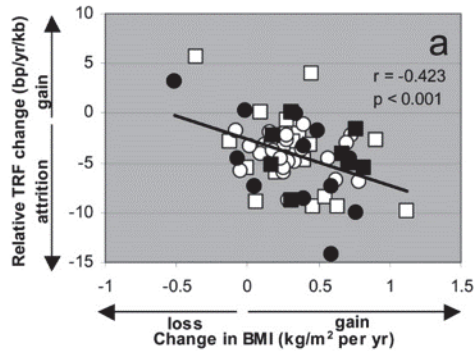


Fig. 6. Illustration of BMI vs. relative telomere restriction fragment. Change in BMI (N=70) per year and its association with relative change in TRF length. Light symbols (Caucasians) and dark symbols (African) represent ethnicity. Squares represent men; circles represent women; r -value represents the model adjustment for follow-up period, age, sex and race. Gardner J P. *et al.* (2005), Published by permission of Wolters Kluwer Health, Inc., *Circulation* (209).

A study conducted on women (N=647) using retrospective self-reported weight during the 30–39 years and comparison with their current BMI (≥ 40 years) has reported being overweight and obese at two time points were inversely associated with LTL (*vs.* normal weight). In addition, high BMI in the 30s age range, weight gain and weight cycling were inversely associated with LTL (210).

Proposed mechanisms between BMI and telomere attrition

Inflammation and oxidative stress are proposed to be the underlying mechanisms between the associations of BMI with telomere length shortening (Figure 7).

Oxidative stress: Obesity is suggested to be associated with generation of reactive oxygen species (ROS) in the adipose tissue (211, 212) (see section 8, Appendix 8.7). The increase in ROS in adipose tissue may lead to disruption of the oxidative-antioxidative homeostasis leading to oxidative stress which may lead to shortening of telomeres, in addition to the cell division process (normal cell division) (166, 184). Indeed, telomeric ends contain guanine rich nucleotide sequences that are highly susceptible to single stranded/double stranded DNA damage as a response of increased oxidative stress (213). A study conducted in 62 women (age: 20–50 years), which compared mean BMI (23.9 *vs.* 27.4 kg/m²) and waist circumference (19.2 *vs.* 22.1 cm) in high and low telomerase groups, has

reported a low telomerase enzyme activity in relation to anthropometric measures (214). The lower telomerase enzyme may lead to disruption in the telomere maintenance function and loss of telomeres from the ends results in shortened LTL. The differences in oxidative stress markers and antioxidant enzymes in obese and nonobese subjects are explained under the section 2.4.7.

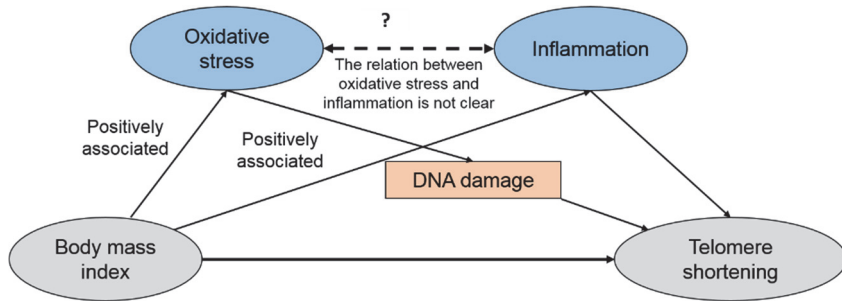


Fig. 7. Diagram illustrating the proposed mechanisms underlying the association between BMI and shortened telomere length.

Inflammation: Inflammation is considered to be another important driver of shortened telomere length in relation to BMI (182, 215). BMI and its suggested association with inflammation is explained later in section 2.4.7. An experimental study comparing the inflammatory profile of genetically and high-fat diet induced rats has reported a deteriorated immune function, premature immunosenescence, decreased natural killer cell activity, and proliferation of cells (216). A study conducted in old persons (N=1,962; age range: 70–79 years), which investigated the association between CRP and LTL, has reported a null association (217). However, another study in adolescents (N=1,080; age: 13–16 years) has reported an inverse association between CRP and LTL (218). A study based on a sample of 45,069 individuals has reported a decrease in seven base pairs of LTL per one unit increase in BMI (unadjusted model) (219). The association remained persistent when adjusted for covariates, but was attenuated when CRP was added into the model (219). Other studies investigating the association between inflammation and LTL were performed on diseased conditions. More studies are warranted in future to clarify the above-mentioned association.

2.3.5 Summary

In summary, an outline of the telomeres, telomere maintenance and the factors influencing telomere length was discussed. There is only limited knowledge available in the literature on the factors influencing telomere length.

BMI and its inverse association with telomere length has been reported from many epidemiological investigations including a previous investigation from NFBC1966. The proposed pathway (oxidative stress, inflammation) linking BMI with shortened LTL was also discussed. BMI and its positive association with increased CRP concentrations have been reported previously. However, it has not been tested whether BMI and its association with shortening of telomeres may be mediated through inflammatory pathways.

Vitamin D is proposed to have immunomodulatory and regulatory properties through VDR. Although VDR is not present in all WBC cells, monocytes, T and B lymphocytes (subsets of WBC cells) contain VDR. Telomere length is measured from peripheral blood leukocytes, which is comprised of all cell types from WBC. Incorporating lifestyle and behavioural changes is proposed to modify the telomerase enzyme and prevent telomere shortening. If sufficient serum 25(OH)D concentration would play a rate-limiting step in telomere attrition, then vitamin D status could be targeted as a modifiable factor for improving and maintaining telomere length.

Study II proposes to examine the association between 25(OH)D and leukocyte telomere length and test whether the previously observed inverse association between BMI and LTL is independent of vitamin D. The study will also test whether the association between BMI-LTL and 25(OH)D-LTL is mediated through inflammatory pathways (hs-CRP).

2.4 Body mass index, inflammation and vitamin D (Study III)

This section provides an overview of obesity, vitamin D and their association with inflammation.

2.4.1 Obesity

Obesity is defined by the World Health Organisation (2000) as a state of “abnormal or excessive accumulation of fat that may impair health” (220). Body mass index (BMI) is a simple-index used to classify normal weight, overweight and obesity in

adults. BMI is calculated as body weight in kilograms divided by height in meters squared (kg/m^2). Table 7 shows the classification of BMI according to WHO as underweight, normal weight, overweight and obesity (221).

Table 7. Classification of body mass index in adults (WHO, 2016) (221).

Classification	Body mass index (kg/m^2)
Underweight	< 18.5
Normal weight	18.5–24.9
Overweight	25.0–29.9
Obesity class I	30.0–34.9
Obesity class II	35.0–39.9
Obesity class III	≥ 40.0

2.4.2 Trends and worldwide prevalence of obesity

The prevalence of obesity has increased tremendously during the last decades in both developed and developing countries. According to a WHO 2017 report, 39% of adults (≥ 18 years) were overweight and 13% were obese (221). A recent article, which analysed the trend of adult BMI from 1975 to 2014, including more than 19.2 million individuals from 200 countries, has reported an increase in mean BMI from 21.7 to 24.2 kg/m^2 and 22.1 to 24.4 kg/m^2 in men and women, respectively (222). If this trend in BMI progresses, then 1 in 5 individuals are predicted to be obese by 2025 (222). The study has also reported the increase in obesity rates from 3.2% (1975) to 10.8% (2014) and 6.4% to 14.9% in males and females, respectively (222). Figure 8 shows the increase in obesity at two time points (1997 and 2012) in Finland (self-reported, age: 20–64 years). The obesity prevalence has increased from 12% to 17% in men and 10% to 20% in women, respectively (Statistics in Finland) (223). Only two time points are displayed in the Figure 8 to facilitate comparison of similar time points of data collection from NFBC1966.

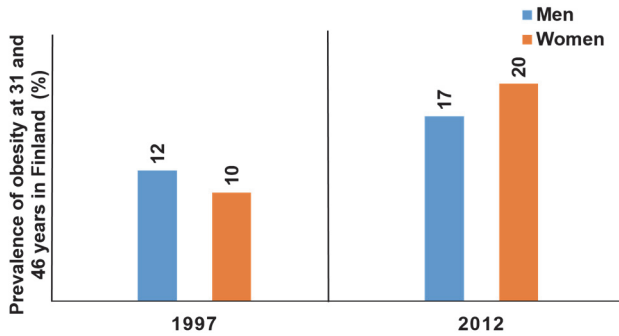


Fig. 8. Obesity prevalence in Finland at two time points, 1997 and 2012. BMI was calculated based on self-reported weight and height (age: 20–64 years). The obesity rates have increased from 12% to 17% (men; 1997) and 10% to 20% (women; 2012). The data from National Institute for Health and Welfare is presented only at two-time points (1997 and 2012) to facilitate comparison with NFBC1966 data collection (Source: National Institute for Health and Welfare (THL), 1978–2016, Finland) (223).

Obesity is suggested to be associated with various cardio-metabolic complications including type II diabetes mellitus (T2D), cardiovascular diseases (CVD), and many other metabolic health outcomes (216).

2.4.3 Adipose tissue

There are three types of adipose tissue present in humans. They are brown, white and brite/beige adipose tissue (224). White adipose tissue is comprised of adipocytes and stromal vascular fraction (SVF). The SVF includes the preadipocytes, vascular cells, endothelial cells, fibroblasts, macrophages and other immune cells (macrophages) (225). The SVF function in providing structural integrity and regulation of tissue development in AT (225). WAT is the main energy store (87%) of the body, storing the excess energy as triacylglycerols (lipid droplets). Triacylglycerols are broken down into free fatty acids (FFA) during the energy deficient periods, which eventually enter into the circulation for maintaining homeostasis (225).

WAT acts as a biochemically active endocrine organ and regulates numerous physiological processes through secretion of various inflammatory biomarkers into the blood stream. The cytokines secreted by the WAT function in an autocrine, paracrine or endocrine manner (226). The signals from these cytokines play an important role in the regulation of energy homeostasis, food intake, immunity,

inflammation and other metabolic functions (insulin sensitivity, lipogenesis and growth hormone signalling) (227).

This thesis primarily focuses on the release of inflammatory biomarkers from the white adipose tissue and only WAT will be described and discussed further.

2.4.4 Adipose tissue compartments: subcutaneous and visceral adipose tissue

Total body fat mass is distributed throughout the body as regional adipose tissue storage depots. Subcutaneous and visceral adipose tissue are the primary depots which store the excess energy consumed through dietary intake. Subcutaneous adipose tissue area accounts for about 80% of the total body fat in an individual (228). Visceral (abdominal) adipose tissue accounts for 10–20% and 5–10% of the total body fat in males and females, respectively (228). The abdominal fat percentage increases with age in both sexes (228). In addition, thin layers of adipose tissue were also reported to be present in the heart, kidney and muscles (227).

Subcutaneous adipose tissue comprises all the fat layers that are present below the skin. They are abdominal, gluteal or femoral fat deposits. In the abdomen, subcutaneous depot tissue is divided by a fascial plane (connective tissue) termed as fascia superficial, into superficial and deep subcutaneous adipose tissue (229). Visceral adipose tissue which covers the internal organs, can be further classified into: i) omental ii) mesenteric iii) retroperitoneal iv) gonadal, and v) pericardial (230). The omental depot starts near the stomach and spleen and expands over the viscera inside the abdomen. The deeper mesenteric fat depot is attached in a web-form, mainly surrounding the jejunum and ileum sections of the intestine (230).

2.4.5 Adipogenesis and the development of obesity

Over nutrition or positive energy deposition causes an increase in the white adipose tissue volume. Adipose tissue expansion causes adipocytes to undergo: i) hyperplasia (increase in cell numbers) and ii) hypertrophy (enlargement or increase in the size of the adipocytes) (231). Adipocytes are formed from the precursor cells, called as pre-adipocytes. During availability of excess nutrition, the energy needs to be stored into adipocytes, which results in the formation of new adipocytes from preadipocytes that are termed as adipogenesis (232). The newly formed adipocytes store the excess energy and this result in expansion of the adipocyte size with an accumulation of more and more triglycerides (Figure 9).

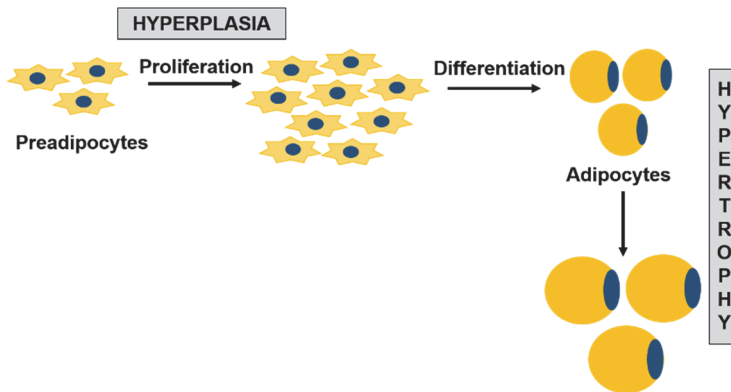


Fig. 9. Diagram illustrating the steps involved in adipocyte differentiation and development. Preadipocytes increase in number (hyperplasia) and undergo differentiation to form mature adipocytes, which store the energy as triglycerides and increase in size (hypertrophy).

This increase in adipocyte size results in hypertrophy. When the adipocytes exceed their capacity of storing triglycerides, new adipocytes are constantly formed to store the excess energy, which results in an increase in cell number, hyperplasia (231). Eventually, both hyperplasia and hypertrophy of adipocytes result in the increase in white adipose tissue volume. Increase in adipose tissue volume further results in the development of obesity. The proposed mechanisms in the development of obesity and inflammation will be discussed under the following heading.

2.4.6 Immune cells, immune response and inflammation in adipose tissue

All of the three types of adipose tissue contain immune cells to maintain the energy homeostasis as well as to maintain the structural integrity (225). The increase in the volume of adipose tissue results in changes in the cellular composition of immune populations between lean and obese individuals.

In lean rodents, type 2 or T helper 2 (T_H2) immune populations secrete IL-33 from the epithelial cells, which are involved in maintaining the metabolism and cellular integrity. IL-33 induces the secretion of IL-5 and IL-13 from the lymphoid cells that in turn activates the eosinophils in adipose tissue (233, 234). Eosinophils

further lead to secretion of IL-4 which results in the maintenance of macrophages in a M2-polarised state (235) (anti-inflammatory or activated). Furthermore, M2-macrophages secrete IL-10 cytokine, which maintains the effects of insulin (insulin-sensitivity) and inhibits the lipolytic signals in the adipocytes, thus retaining the metabolic homeostasis (236).

In obese rodents, type 1 or T helper 1 (T_{H1}) immune populations secrete tumor necrosis factor ($TNF-\alpha$) which play a coordinated role in maintaining the structural integrity in response to the metabolic stimulus associated with excess energy accumulation. Multiple studies have demonstrated the changes occurring in macrophages in the context of obesity. Obesity in humans as well as in rodents is infiltrated by an increase in the number of macrophages in the adipose tissue (50, 237). This infiltration of M1-polarised macrophages adopts a proinflammatory environment resulting in the secretion of cytokines, $TNF-\alpha$ and interleukin (IL)-1 beta (238). In addition, macrophages are found to be present in abundance in the VAT when compared to SAT (239). This increase in macrophages in the adipose tissue along with an imbalance in the ratio between M1 (proinflammatory) and M2 (anti-inflammatory) macrophages or T_{H1}/T_{H2} imbalance marks the hallmark of adipose tissue inflammation and the development of obesity related metabolic disturbances (Figure 10) (238, 240).

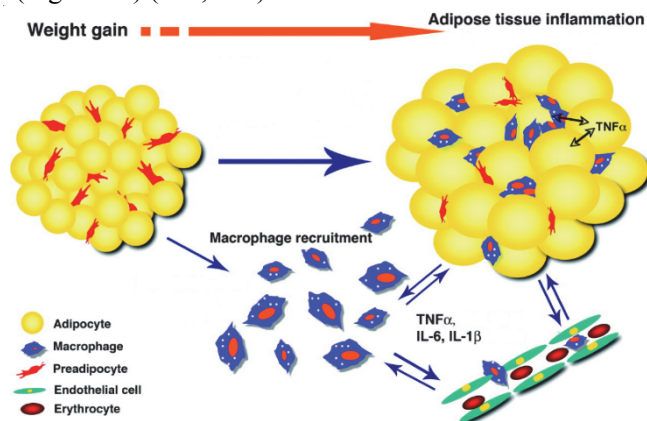


Fig. 10. Diagram representing the changes occurring in adipose tissue during the development of obesity. Adipose tissue enlarges in size and there is infiltration of M1 macrophages, and release of cytokines in the circulation (Modified with Permission from Atherosclerosis, Thrombosis, and Vascular Biology, Gustafson *et al.* 2007) (242).

This results in the formation of an inflammatory environment in obese as opposed to a protective environment in lean subjects. Insulin resistance is suggested to be

promoted by M1 macrophages, whereas the obesity-induced insulin resistance is protected by the M2 macrophages (241).

Inflammatory biomarkers in adipose tissue: The increase in the proinflammatory macrophages in the adipose tissue results in the induction of multiple inflammatory biomarkers. Inflammation in obesity was first suggested by the increased tumor necrosis factor-alpha (TNF- α) messenger RNA (mRNA) expression in the tissues of obese mice when compared to lean (243). The amount of TNF- α mRNA expressed in adipose tissue was 5- to 10-fold higher in obese vs. lean mice (243). TNF- α protein expression was further measured using ELISA. 25% and 58.3% of TNF- α protein was detected in lean (mean=61.53 pg/ml) and obese mice (mean=85.6 pg/ml) (243).

Following this, investigation of TNF- α mRNA expression in adipose tissue of obese premenopausal women (N=19) was found to be 2.5 fold higher in obese when compared to lean controls (N=18) (244). This discovery paved way for a wealth of studies examining the differences in inflammatory profile among obese and non-obese in both epidemiological and experimental study design. Recent observations have reported an involvement of a wide array of immune cells in the obese adipose tissue including, interleukins, chemokines, adhesion molecules, and acute-phase proteins (245–247). The inflammatory biomarkers secreted by the adipose tissue, their distribution and the reported association with BMI in epidemiological setting is shown in Table 8.

Table 8. List of inflammatory biomarkers measured in NFBC1966, their distribution and reported association with obesity in experimental and epidemiological observations.

Inflammatory biomarkers	Class	Distribution	Association with obesity	References
Interleukin-1-alpha (IL-1 α)	Cytokine	WAT, Monocytes, Macrophages, Endothelial cells, Vascular smooth muscle cells, B-cells, Eosinophils.	↑ in human obesity	(248)
Interleukin-1-beta (IL-1 β)	Cytokine	WAT, Monocytes, Macrophages, Natural Killer cells (NK-cells), B-cells, Eosinophils, Endothelial cells.	↑ in human obesity	(249, 250)
IL-1 receptor antagonist (IL-1RA)	Cytokine	WAT, Macrophages, WAT, Monocytes, Epithelial cells.	↑ in human obesity ↑ mice model	(251)
IL-4	Cytokine	WAT, NK-cells, Mast cells, Basophils, Th2 cells.	↑ mice model	(252)
IL-6	Cytokine	WAT, Macrophages, Fibroblasts, Endothelial cells, Skeletal muscle cells.	↑ in human obesity	(253)
IL-8	Cytokine	WAT, Macrophages, Monocytes, Fibroblasts, Endothelial cells, Epithelial cells.	↑ in human obesity	(239, 254, 255)
IL-17	Cytokine	WAT, T-helper 17 cells, Bone marrow	↑ in human obesity, ↑ mice model	(256, 257)
TNF- α	Cytokine	WAT, Macrophages	↑ in human obesity	(243, 258)
Monocyte chemoattractant protein-1 (MCP-1)/CCL2	Chemokine	WAT, Monocytes, Macrophages, Dendritic cells, Fibroblasts, Vascular smooth muscle cells.	↑ mice model, ↑ in human obesity	(255, 259–262)
Soluble CD40 ligand (sCD40L)/CD154	Chemokine	WAT, Endothelial cells, Platelets, T-helper cells, Vascular smooth muscle cells.	↑ in human obesity.	(263, 264)
C-X-C motif chemokine 10 (CXCL10)/IP-10	Chemokine	WAT, Monocytes, Endothelial cells, Fibroblasts.	↑ in human obesity	(265)

Inflammatory biomarkers	Class	Distribution	Association with obesity	References
Soluble intercellular adhesion molecule-1 (sICAM-1)	Adhesion molecules	Endothelial cells, Leukocytes, WAT.	↑ in human obesity	(266)
Soluble vascular cell adhesion molecule-1 (sVCAM-1)	Adhesion molecules	Endothelial cells, Leukocytes, Vascular smooth muscle cells, WAT.	↑ in human obesity	(266, 267)
Active plasminogen activator inhibitor-1 (activePAI-1)	Acute-phase protein	Endothelial cells, WAT.	↑ in human obesity	(267–269)
hs-CRP	Acute-phase protein	Hepatocytes, Macrophages, WAT.	↑ in human obesity	(270)
Alpha-1-acid glycoprotein (AGP)	Acute-phase protein	Hepatocytes, WAT.	↑ in human obesity	(271–273)

2.4.7 Obesity proposed mechanisms: oxidative stress and inflammation

Increase in adipose tissue volume is suggested to be associated with both low-grade inflammation as well as increased oxidative stress. It is suggested that both these mechanisms are closely interlinked. It is not clear whether oxidative stress and inflammation arise from the immune populations simultaneously in obesity, or whether oxidative stress results in secretion of inflammatory biomarkers or vice versa (Figure 11). However, both inflammation and oxidative stress in adipose tissue result in obesity-associated metabolic complications.

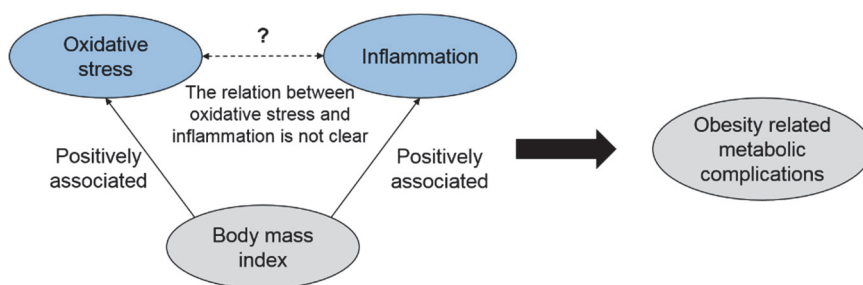


Fig. 11. Diagram illustrating the mechanisms underlying the association between BMI, inflammation and oxidative stress with metabolic complications. BMI is positively associated with increased oxidative stress and inflammation. It is not clear whether oxidative stress increases inflammation or vice versa.

A study that examined oxidative stress in normal weight (N=29), overweight (N=36) and obese (N=25) has reported an increased lipid hydroperoxide enzyme concentration in obese vs. normal weight (0.09 vs. 0.67 $\mu\text{mol/l}$) (274). However, no difference was reported with the overweight group (vs. obese) (274). Another study that compared lipid peroxidation products between normal weight (N=50) and those with BMI >40 kg/m² (N=50) has reported lower plasma malondialdehyde in normal (2.53 $\mu\text{mol/l}$) vs. subjects with BMI >40 kg/m² (4.75 $\mu\text{mol/l}$) (275). When the subjects were grouped together and analysed, BMI were inversely associated with cryoprotective enzymes, erythrocyte copper-zinc superoxide dismutase and, glutathione peroxidase (275). A study (N=1,250; country: USA; age: 60 years) investigating SAT and VAT in relation to inflammation and oxidative stress markers has reported a positive association between VAT and biomarkers, CRP, IL-6, MCP-

1 and lipid by-product isoprostanes (262). SAT was positively associated with fibrinogen only (262). The study was adjusted for sex, age, physical activity, WC, BMI, menopause, alcohol, hormone replacement therapy, and aspirin use (262).

2.4.8 Vitamin D and obesity

Vitamin D deficiency and obesity are increasing parallel to each other. Earlier RCT (1985; USA) comprising 12 obese and 14 non-obese participants first reported a lower mean serum 25(OH)D concentration in obese versus non-obese participants (20 vs. 50 nmol/L) (276). Another case-control study (1988) (N=26; USA; age: 20–35 years) reported an inverse correlation between serum 25(OH)D and body weight ($r=-0.88$) (277). Following these studies, several cross-sectional, and longitudinal studies with follow-up time from 2 years to over three decades, has reported lower 25(OH)D concentration with BMI (278–281).

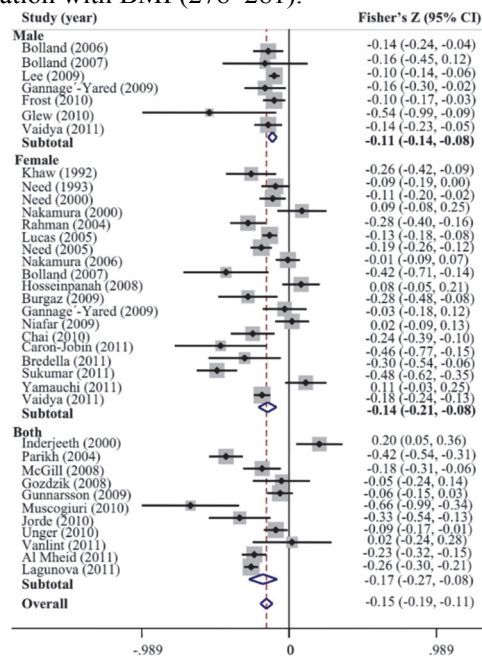


Fig. 12. Fischer's Z of correlation coefficients between 25(OH)D and BMI from 34 studies. Subgroup analysis based on sex and on studies including both sexes reported an inverse association between 25(OH)D and BMI. The meta-analysis of 34 studies showed a weak inverse association between 25(OH)D and BMI. Saneei *et al.* 2013, published by permission of Wiley and Sons, Obesity Reviews (282).

Systematic review and meta-analyses (2013) examining the cross-sectional association between BMI and vitamin D has reported a weak inverse correlation with heterogeneity among the studies (Figure 12) (282). The results persisted when sub-group analyses based on sex, study location (East vs. West) and country (developed vs. developing countries) were performed (282).

2.4.9 Possible mechanisms underlying the association between low vitamin D and obesity

The possible mechanisms behind low vitamin D status and obesity are discussed under the following sections.

Diet

The consumption of vitamin D-rich dietary sources has been reported to be lower in both obese men and women when compared to lean individuals (132). A study from Norway (N=9,252 men; N=9,662 women; age: 25–69 years), which examined the association between age groups (25–29, 30–39, 40–49, 50–59, 60–69 years) and dietary intake of vitamin D, reported lower mean intake in all age groups of obese people (>30 kg/m²) versus nonobese (≤ 30 kg/m²) in both the sexes (283). In addition, BMI was reported to inversely correlate with dietary intake of vitamin D, adjusted for age, smoking, physical activity, coffee and alcohol consumption (283).

Sequestration hypothesis

Sequestration hypothesis refers to the ability of vitamin D₂ and D₃ isoforms to dissolve into the adipose tissue compartments because of their hydrophobic nature. This results in them being unavailable to be retrieved back from their storage sites and subsequent conversion to 25(OH)D for maintaining serum 25(OH)D concentration. This may lead to vitamin D insufficiency/deficiency in obese subjects as proposed by sequestration hypothesis.

A study, which examined vitamin D deficient rats upon oral administration by radiolabelled vitamin D, has reported adipose tissue to contain the greatest quantity of radioactivity (284). This study has reported adipose tissue as the major storage site (80%) of unaltered D₃ (284). A pilot study by Blum *et al.* on obese men and women (N=17; mean BMI=50.6 kg/m²; Country: USA) examined the concentration and the association between D₃ in the serum and SAT (285). The

study has reported similar positive correlation between D3 present in the SAT with serum concentration ($r=0.68$), thus suggesting adipose tissue as major storage site (285).

Wortsman *et al.* compared 19 normal weight and 19 obese subjects (country: USA) who received whole-body UV-B irradiation or vitamin D2 supplementation (286). This study hypothesised obese subjects having a larger body surface and greater area of exposure would produce more D3 in response to UV-B irradiation (286). However, they observed a 57% lower increase in D3 concentrations in obese when compared to normal weight subjects after 24 hours UV-B irradiation (286). BMI was inversely correlated with both serum D3 after irradiation and serum D2 after D2 supplementation (286). Obesity was reported to not alter the epidermal synthesis of vitamin D in the skin, but may interfere with the release of D3 sequestered in SAT into the circulation (286).

These studies should be interpreted with caution. They are based on a limited sample size, with baseline vitamin D inadequacy, high PTH concentrations and did not include correction for any other lifestyle factors (285, 286).

Volumetric dilution

Volumetric dilution of vitamin D2 and D3 in the different adipose tissue compartments indicates that the larger the volume of total adipose tissue, the larger is the distribution of vitamin D components. This leads to decreased bioavailability of D2 and D3 in the circulation for the immediate conversion into 25(OH)D ultimately leading to lower vitamin D concentrations in the serum (deficiency/insufficiency). Drinicic *et al.* on 686 individuals (mean BMI=30 kg/m²; 25(OH)D=64.7 nmol/L) fitted a hyperbolic diffusion model (relationship between concentration and volume) to support the volumetric dilution (287). The hyperbolic model was fitted between serum 25(OH)D (concentration, nmol/L) and body weight (body size) (volume, kg) (287). The hyperbolic fit reduced the inter-individual variability in serum 25(OH)D concentrations attributable to obesity-related components (height, weight, BMI, fat mass) (287).

A recent systematic review and meta-regression analysis (N=18; published over the last 21 years) examined the reported variations after weight loss in weight, 25(OH)D, or body composition to explain the volumetric dilution (288). This review was based on the hypothesis that weight loss (decrease in volume, kg) may increase serum 25(OH)D (concentration, nmol/L) in obese subjects (288). Meta-regression analyses has reported a marginal effect of weight loss on unadjusted

weighted mean difference (WMD) in 25(OH)D concentration (288). The relationship persisted when adjusted for study quality. An increase of 6 nmol/L in the WMD of 25(OH)D was reported for every 10 kg mean weight loss (288), thus supporting volumetric dilution.

The possible mechanisms underlying the association between obesity and vitamin D deficiency are outlined in Figure 13.

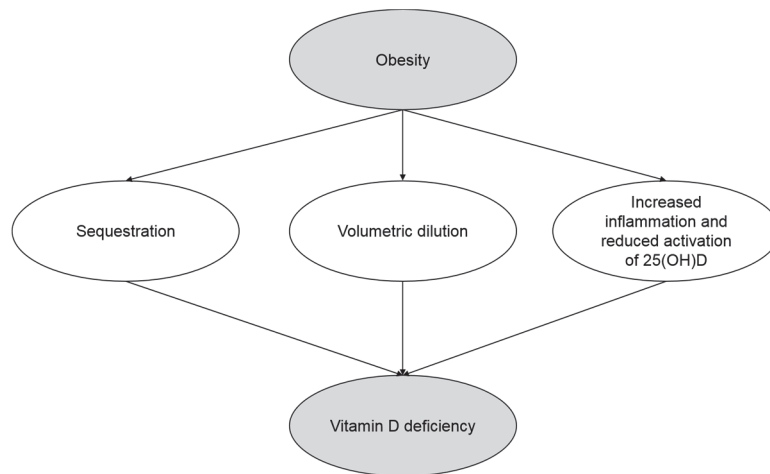


Fig. 13. Possible mechanisms underlying the association between obesity and vitamin D deficiency.

Increased inflammation and reduced activation of 25(OH)D

Another proposed mechanism to explain the low vitamin D status in obesity is adipose tissue inflammation. Hypertrophic adipocytes results in infiltration of M1 macrophages and varied immune populations, which creates a proinflammatory environment (see section 2.4.6) (48, 289, 290). To prevent inflammation arising from adipose tissue, the immune populations which express VDR receptor converts the 25(OH)D available in the circulation. Immune cells express 25-hydroxyvitamin D-1 α -hydroxylase which helps in conversion of 25(OH)D into calcitriol for regulatory properties (291). The activation of calcitriol leads to potent immunomodulation through VDR and other signalling pathways as well as higher circulating concentrations (291). The 25-hydroxyvitamin D-24-hydroxylase enzyme regulates further production of calcitriol from 25(OH)D by preventing the

feedback mechanism (291, 292). Furthermore, the enzyme catabolises both calcitriol and 25(OH)D leading to a less substrate availability for further calcitriol formation (291–293). It is suggested that constant scavenging of inflammation arising from adipose tissue may result in low vitamin D status in obesity. However, the underlying mechanisms are yet to be clearly understood. A detailed information on vitamin D and inflammation is mentioned in the following section 2.4.11.

2.4.10 Causal relationship between BMI and 25(OH)D: Mendelian Randomisation

Mendelian randomisation (MR) is a statistical technique that uses genetic information to examine the causal inference of an exposure (X) to an outcome (Y) by using instrumental variables (IV; Z) (study design: section 8, Appendix 8.2). A bi-directional MR study using data from 21 cohorts (42,024 participants) including NFBC1966 data at 31 years examined the causality and direction of the association between BMI and 25(OH)D (294). The study has reported that, for every 1 kg/m² increase in BMI, the 25(OH)D decreased by 1.15% (meta-analysis of 21 studies) (294). The study has included adjustments for age, sex, 25(OH)D batch, month of blood sampling and other variables (available from each cohort). In the evaluation of causality, a 4.2% lower 25(OH)D concentration was observed with 10% higher genetically instrumented BMI (294). The causal effect of 25(OH)D on BMI was reported to be insignificant (294).

2.4.11 Vitamin D and inflammation

Calcitriol exerts its immune functions through the nuclear VDR, which is present in almost all cells including dendritic cells, adipocytes, muscle, monocytes and macrophages (295).

Intracrine action: The first classical example demonstrating the immune-modulatory role of vitamin D in human monocytes was reported by Liu *et al.* (2006) (296). The toll-like receptor (TLR), a pathogen recognition receptor in innate immune response results in antimicrobial activity (296). However, in studies on *Mycobacterium tuberculosis* (*M. tb*), the TLR signalling reduced the intracellular viability of the pathogen in human macrophages and monocytes, but not in dendritic cells (DCs). This led to examining the differences in gene expression profiles between monocytes and DCs by stimulation with or without *M. tuberculosis*-derived lipopeptide. Gene upregulation was noticed in monocytes but

not in dendritic cells. Further cross checking revealed expression of VDR and 25-hydroxyvitamin D-1 α -hydroxylase genes following TLR-activation (296). To determine the functional status of VDR, primary human monocytes were further treated with 25(OH)D, which showed local synthesis of calcitriol that could then bind to and signal through the VDR. This demonstrated the potential role of vitamin D in the intracrine system for localised, VDR mediated regulation of gene expression (296). In this setting, the potential target for VDR is the gene for cathelicidin, which encodes a protein involved in promoting intracellular killing of bacteria (296). This was the first example of an intracrine action and local synthesis of calcitriol in immune cells. However, this mechanism is sensitive to the bioavailability of 25(OH)D, and simple variation in the 25(OH)D status could enhance or impair this intracrine response (296). This was further tested in the monocytes cultured in the medium supplemented with serum from vitamin D-insufficient donors (296). The vitamin D-insufficient donors showed lower cathelicidin expression following TLR activation when compared to vitamin D-sufficient donors (296). However, the precise mechanism by which pathogens such as *M. tb* enhance transcription of VDR and calcitriol via TLR are yet to be clarified.

Paracrine action: Antigen presenting cells (APCs) deliver bacterial antigens to cells from the adaptive system such as T-cells. APCs include macrophages, but antigen presentation is more effectively carried out by the dendritic cells (DCs). However, in monocytes and macrophages intracrine synthesis of calcitriol promoted bacterial killing, while in DCs intracrine synthesis acts to suppress DC maturation and promotes a more immunosuppressive APC phenotype (297). Differentiation of the DCs into mature APC phenotype is associated with increased 25-hydroxyvitamin-D-1 α -hydroxylase expression but with reciprocal decrease in VDR concentrations (298). In this setting, DCs utilise a paracrine vitamin D system, where vitamin D-rich immature DCs responding to calcitriol produced by mature VDR-depleted DCs (5). The importance of calcitriol as a modulator of the DC function has been reported from an experimental investigation (299). The T and B-cells also express VDR (47, 300). Similar paracrine mechanism may also induce direct effects on the T and B-cells (47, 300).

2.4.12 *Epidemiological observations on vitamin D and inflammation*

The association between inflammation and vitamin D status was reported in few epidemiological observations, but not in all (301). A study conducted in older adults

(N=957; age: ≥ 60 years) examining the association between 25(OH)D and cytokines (IL-6, CRP), cytokine ratios (IL-6/IL-10, CRP/IL-10, and TNF- α /IL-10) has reported a negative correlation (302). Another study conducted in women (N=69; age range: 25–82 years) including sun exposure (high and low) participants, examined in relation to cytokines reported a weak inverse association between TNF- α and 25(OH)D (303). A summary of the epidemiological observations on the reported association between serum 25(OH)D and inflammation published to date are shown in Table 9. This table was adapted from article IV and updated with recent studies reporting association between vitamin D and inflammation (Table 9).

A recent systematic review examining the impact of vitamin D status on inflammation and protein expression has reported an anti-inflammatory action of vitamin D (304). However, the major limitations of all these studies are sample size, inclusion of wide age groups, and not taking into account adjustment for potential covariates (season, smoking, alcohol, and physical activity) (301) (Table 9).

Epidemiological investigations in Table 9 have highlighted the reported association between vitamin D and inflammation and their limitations. The link between vitamin D and inflammation in adipose tissue is further demonstrated by the functional analyses that have defined the mechanisms by which cells from the immune system handle and utilise 25(OH)D. The experimental studies has revealed molecular pathways for vitamin D metabolism and signal transduction that are both similar to, but distinct from, the classical calciotropic and skeletal function of vitamin D as mentioned in section 2.4.11 and in the following section 2.4.13.

Table 9. A summary of the epidemiological studies examining the association between vitamin D and inflammation (adapted and modified from Palaniswamy et al., 2016 (article IV).

Study design	Country	Sample Size	Mean BMI (kg/m ²)	Age Range	Inflammatory biomarkers tested	Associations with serum 25(OH)D	References
Cross-sectional	Italy	Male (N=377); Female (N=480)	27.3 ± 4.0	≥ 65	IL-6, soluble IL6 receptor, TNF-α, soluble TNF receptors (sTNFR1, sTNFR2), IL-1β, IL-10, IL-18, IL-1RA, glycoprotein 130	Negative for IL-6, positive for IL-	(305)
Cross-sectional	Northern Ireland	Male (N=481); Female (N=476)	28.7 (26.5–31.7) 29.0 (25.9–33.6)	>60	IL-6, CRP, TNF-α, IL-10, IL-6:IL-10 ratio	Negative for IL-6, IL-6:10 ratio, CRP, TNF-α/IL-10 ratio and CRP/IL-10 ratio	(302)
Cross-sectional	Japan	Male (N=34); Female (N=61)	23.9 ± 2.6 21.2 ± 2.8	21–69	IL-17, IFN-γ, IL-6	Positive for IL-17	(306)
Cross-sectional	USA	Female (N=69)	24.4 ± 0.8	25–82	TNF-α, IL-10, CRP and IL-6	Negative for TNF-α	(303)
Cross-sectional	USA	Male (N=9); Female (N=10)	22.0 ± 1.6 21.0 ± 1.1	27–37	TNF-α, IFN-γ, IL-10, IL-4	Negative for TNF-α	(307)
Cross-sectional	USA	Male (N=14); Female (N=14)	24.8 ± 1.4	25–42	IL-2, IL-1β, TNF-α, IL-10, IFN-γ	Not significant	(308)
Cross-sectional	USA	Male (N=679)	27 ± 4	74 ± 6	CRP, IL-6, TNF-α, sTNFR1, sTNFR2, soluble IL6 receptor, IL-10	Negative for IL-6	(309)

Study design	Country	Sample Size	Mean BMI (kg/m ²)	Age Range	Inflammatory biomarkers tested	Associations with serum 25(OH)D	References
Cross-sectional	USA	Male (N=246); Female (N=528);	29.0 (16.5– 58.4)	18–86	50 inflammatory biomarkers were measured; Biomarker concentrations compared between vitamin D deficient (<28 nmol/L) and sufficient groups (>62 nmol/L)	No difference between the biomarkers except for granulocyte macrophage colony-stimulating factor (GM-CSF).	(310)
Cross-sectional	Kuwait, Middle-East	Female (N=118)	Not reported	19–47	TNF- α , IL-1 β , IL-4, IL-6, IL-8, IL-13, IL-17, IFN- γ , IL-10	Deficient vitamin D had high CRP and high TNF- α and IL-8	(311)
Cross-sectional	England	Male (N=2640); Female (N=3230);	Not reported	50–80	CRP and fibrinogen	Negative for CRP	(312)
Cross-sectional	USA	Male (N=669); Female (N=712)	28.1 \pm 5.2	59–67	CRP, TNF- α , sTNFR2, MCP-1, sCD40L, sICAM-1, P-selectin	Not significant	(313)

2.4.13 Vitamin D and adipose tissue inflammation

The action of calcitriol in different immune cells was described in the previous section. Before going into detail about the regulation of vitamin D in adipose tissue inflammation, I κ B kinase (IKK) enzyme complex will be described.

I κ B kinase (IKK) enzyme complex: The IKK complex is activated in response to stimulus such as, cytokines, free radicals and many others. Inhibitor κ B (I κ B) protein is a potent inhibitor of NF- κ B. The NF- κ B sequestering by I κ B makes it to be restricted within the cytoplasm in an inactive (bound) form. Phosphorylation of inhibitor I κ B α by IKK during cellular stimuli leads to NF- κ B being dissociated from I κ B and migrate freely in the circulation. This unbound NF- κ B movement leads to free translocation in to the nucleus, thereby eventually initiating mRNA expression of diverse inflammatory and transcription factors that are involved in pathogenesis (314).

Regulation of NF- κ B signalling in immune cells: The regulation of NF- κ B by VDR was first reported from mice fibroblasts (315). The mRNA expression of IL-6 and the transcriptional activity of NF- κ B that are involved in cytokine production was compared between VDR (+/-) and VDR (-/-) mice fibroblasts (315). Induction of IL-6 cytokine production by IL-1 β or TNF- α resulted in increase in both IL-6 and its mRNA expression and was reported to be higher in VDR (-/-) versus VDR (+/-) (315). In addition, the cytokines induced degradation of I κ B protein (50%) in the VDR (-/-) (315). When NF- κ B signalling pathway was examined, VDR null mice (-/-) exhibited higher NF- κ B activity, increased NF- κ B p65 translocation into the nucleus, and lack of p65 binding to VDR (315). These reports suggested the role of VDR in the regulation of NF- κ B signalling pathway, and binding of VDR to p65 resulting in down regulation of transcription and mRNA expression of IL-6, which was observed in VDR mice (+/-) when compared to VDR (-/-) (315). These mechanisms suggest sufficient 25(OH)D concentrations in the serum may play an important role in the modulation of NF- κ B pathway by upregulating both VDR as well as regulation of NF- κ B translocation into the nucleus. The action of calcitriol on the regulation of NF- κ B signalling in dendritic cells (316), keratinocytes (317), and lymphocytes (318) have also been demonstrated.

Regulation of molecular pathways: The role of vitamin D in adipose tissue inflammation through similar regulatory mechanisms has been proposed. Experimental study using bone marrow-derived human mesenchymal stromal cells (hMSCs) and adipocytes derived from biopsies were first stimulated with

lipopolysaccharide (LPS) (319). The cells were then treated with or without vitamin D. Vitamin D cotreatment decreased LPS-stimulated inflammatory cytokine, IL-6 secretion in a dose-dependent manner in both hMSCs and adipocytes (319). Vitamin D inhibited IL-6 secretion by interfering with $\text{I}\kappa\text{B}$ phosphorylation-mediated $\text{NF-}\kappa\text{B}$ translocation in the nucleus (319). During adipose tissue inflammation, cytokines are suggested to degrade $\text{I}\kappa\text{B}$ protein allowing $\text{NF-}\kappa\text{B}$ free translocation into the nucleus, thereby leading to mRNA expression of multiple inflammatory cytokines in the circulation (319). Another study reported dose-dependent inhibition of LPS-induced p38 phosphorylation, IL-6 and $\text{TNF-}\alpha$ production by human blood monocytes with vitamin D treatment (320). In addition, the expression of MAPK phosphatase-1 (MKP-1) was reported to be upregulated in human monocytes and bone marrow-derived macrophages (BMM) by vitamin D treatment (320). In BMM from MKP-1 (-/-) mice, the inhibition of LPS-induced p38 phosphorylation was completely inhibited thus reporting the interference of vitamin D in MAPK signaling pathway (320). Similar results has been reported from two other experimental studies (321, 322). Summary of the actions of vitamin D in immunomodulation is shown in Figure 14.

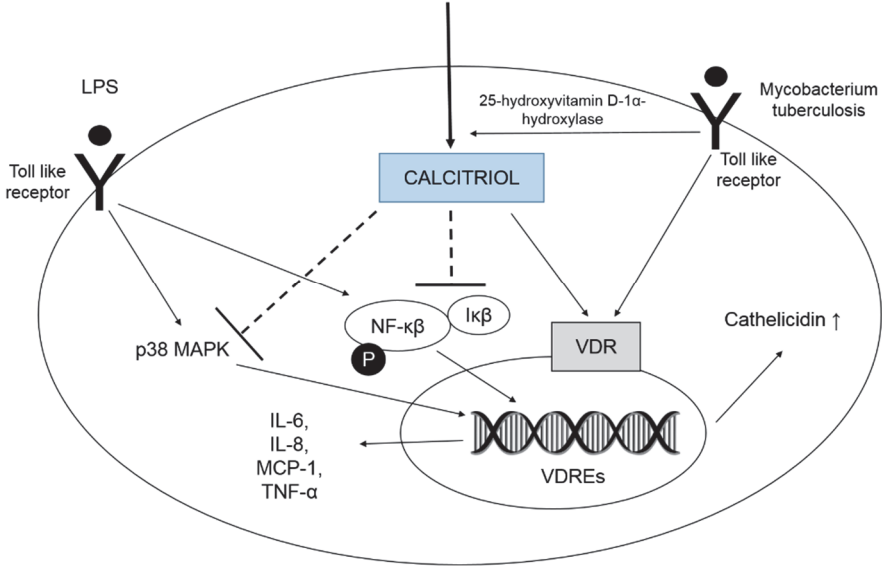


Fig. 14. Summary of the actions of vitamin D in immunomodulation as reported from epidemiological and experimental studies.

In addition to interference with regulation of signalling pathways, vitamin D is proposed to act on preventing oxidative stress arising from obesity.

Antioxidant enzymes: A study conducted in rats (N=40) fed with high-fat diet (HFD) and cosupplementation with vitamin D (HFD-vitamin D) has reported a decrease in TNF- α and MCP-1 concentrations in the HFD-vitamin D group (323). In addition, the study reported an increase in markers of oxidative enzymes, superoxide dismutase and glutathione peroxidase (GPx) in the HFD-vitamin D suggesting an anti-oxidant mechanism to decrease the inflammation in adipose tissue (323). The inflammatory biomarkers IL-6, TNF- α , and MCP-1 were inversely correlated with GPx and positively correlated with malondialdehyde (323).

2.4.14 Summary

Obesity is a pandemic public health problem. This section has outlined the prevalence of obesity, adipose tissue and its roles, its different compartments, adipogenesis and development of obesity, differences in immune cells and inflammatory profile between lean and obese individuals.

BMI and its association with inflammatory environment and secretion of pro-inflammatory biomarkers has been reported in both observational and experimental investigations. The suggested mechanisms behind the adipose tissue inflammation were also discussed. However, investigation of a comprehensive inflammatory profile in relation to BMI is still lacking in young general population.

Calcitriol is proposed to act through the vitamin D receptor and an inverse association between vitamin D and inflammatory biomarkers has been reported in few epidemiological investigations (Table 9). However, the information on the association between vitamin D and inflammation remains largely conflicted, with the majority of the investigations carried out in older populations, wide age groups, obese, and not taking into account adjustment for potential covariates.

If vitamin D could be proposed as a modifiable factor to reduce BMI associated inflammation, it should be taken into consideration that BMI has been reported to be associated with low vitamin D status. There are various reasons (sequestration, volumetric dilution, increased inflammation, and reduced activation) proposed to explain the low vitamin D status in obesity. In addition, a causal relationship between BMI and low 25(OH)D has been reported.

In the context of obesity, experimental studies on human preadipocytes and adipocytes has reported the inhibition of inflammation arising from adipose tissue

by vitamin D with interference with NF- κ B and MAPK signalling pathways. In addition, an inverse association of vitamin D with oxidative stress markers has been reported.

Study III will examine the association of vitamin D and BMI with inflammatory biomarkers and, test whether the association of BMI with inflammatory biomarkers is mediated through serum 25(OH)D.

Literature review (IV) objective

1. The review explores the current evidence for an association between vitamin D status in pregnancy and maternal and fetal health status.
2. To review current evidence on the reported associations between vitamin D and inflammation in the general population (Table 9).

Study I objectives

1. To examine the determinants (environmental, anthropometry, lifestyle, socioeconomic and sex-specific) of D2, D3 and total 25(OH)D concentrations.
2. To investigate the risk factors for vitamin D insufficiency.

Study II objectives

1. To explore the association between 25(OH)D and leukocyte telomere length adjusting for confounders (the season of blood sampling, age, sex, 25(OH)D batch, BMI, SEP, physical activity, diet, smoking status, alcohol consumption).
2. To assess the association between BMI and LTL, and to examine whether this association is independent of 25(OH)D.
3. To examine the mediating role of inflammatory biomarker, high sensitivity C-reactive protein in the association of both BMI and 25(OH)D with leukocyte telomere length (Figure 16).

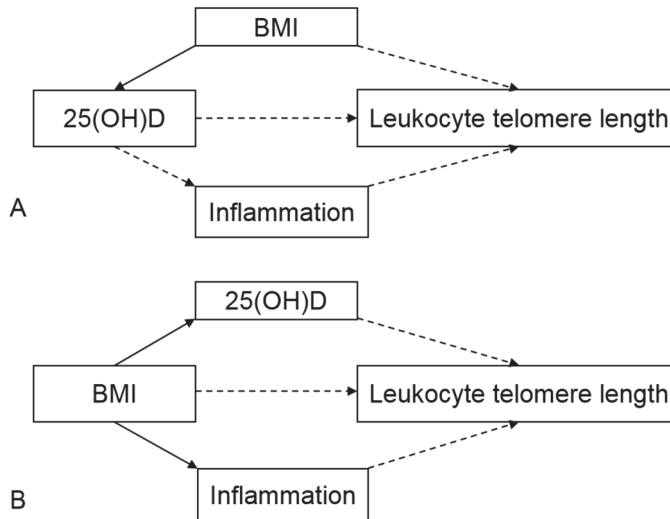


Fig. 16. The objectives tested in Study II are depicted as directed acyclic graphs. The solid arrows show the established causal relationships. The dashed arrows show the hypothesised direction of causality. (A) Serum 25(OH)D concentration is considered as a determinant of longer LTL. BMI is considered as a confounder in the association between 25(OH)D and LTL. Higher BMI is causally associated with lower 25(OH)D; and a determinant of LTL [association independent of effects on serum 25(OH)D]. Inflammation may mediate the association between 25(OH)D and LTL; based on the anti-inflammatory mechanism of action that inflammation shortens LTL. (B) BMI is considered as a determinant of shorter LTL. The association between BMI and LTL may be mediated by the causal relation of BMI with 25(OH)D or inflammation, or through other mechanism (a direct association is also possible from BMI to LTL).

Study III objectives

1. To explore the association between BMI and 16 inflammatory biomarkers adjusting for confounders (sex, smoking status, alcohol consumption, smoking, physical activity and SEP).
2. To investigate the association between 25(OH)D and 16 inflammatory biomarkers adjusting for confounders (25(OH)D batch, the season of blood sampling, sex, SEP, smoking, alcohol consumption and physical activity).
3. To examine the relationship between BMI and 25(OH)D and, to test whether the association of BMI with inflammatory biomarkers is mediated through 25(OH)D concentration (Figure 17).

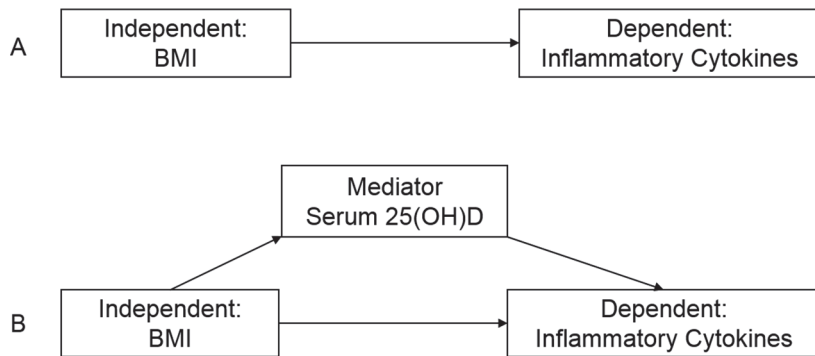


Fig. 17. Graphical representation of proposed mediation through 25(OH)D in the association of BMI with inflammatory biomarkers. (A) The association of BMI with inflammatory biomarkers. (B) Mediation analysis to test whether the association of BMI with inflammatory biomarkers may be mediated or modified through 25(OH)D. I realise the sample size restriction, but for training purposes, causal analyses were conducted.

4 Materials and methods

4.1 Study population

The present study utilised the data from a population-based birth cohort, the Northern Finland Birth Cohort 1966 (NFBC1966). All mothers who were pregnant and residing in Northern Finland – Provinces of Oulu and Lapland – with their expected delivery dates falling in 1966 were enrolled in the study (N=12,055). Women (approximately 80%) made their first attendance to antenatal clinics on average on the 16th gestational week and were enrolled in the study if pregnancy continued after the 24th week of gestation. In total, 12,231 children were born, of which 12,058 alive and 173 stillborn. According to the Finland's central office of statistics, NFBC1966 birth cohort has a high coverage of 96.3% of all eligible births in the study area during 1966. The children were then followed up regularly after birth to ages 1, 14 and 31 years. The adult data utilised in this thesis was from the cross-sectional examination at 31 years of age. In 1997 (31 years), cohort participants who were alive and with known address residing in Northern Finland, who had moved to Helsinki, or who were living abroad (N=11,541) were contacted and sent a postal questionnaire. The postal questionnaire data was received from N=8,767 (response rate: 75.3%). The participants residing only in Finland was then simultaneously invited for a clinical examination and the data was received from N=6,033 (response rate: 71.3%). Clinical examination at 31 years were performed by trained nurses, which involved collection of blood samples, anthropometric measurements and several other examinations.

4.2 Participants and study design

Figure 18 shows the flowchart of data collection in NFBC1966 and the study design (I–III). From 6,033 participants who attended clinical examination at 31 years, 25(OH)D data was available from N=5,657 for the analysis. These measurements were then combined with their available anthropometric, biomarker measurements, LTL and postal questionnaire data. Participants who had missing information on vitamin D, BMI, inflammatory biomarkers, LTL were excluded from the analysis. The studies (I–III) utilised three datasets for the analyses. This was performed to maximise sample size and increase the statistical power. The first data set was restricted to only those individuals who had a complete set of information on

25(OH)D₂, 25(OH)D₃ and 25(OH)D and other factors tested in relation to vitamin D (N=4,758). The second data set was restricted to those who had data on 25(OH)D, BMI, hs-CRP, LTL and other confounders examined (N=5,598). The third data set was restricted to individuals with measurements of 25(OH)D, BMI, inflammatory biomarker and other covariates adjusted (N=3,586; main source of attrition was exclusion on N=607 with women using contraceptives) (Figure 18).

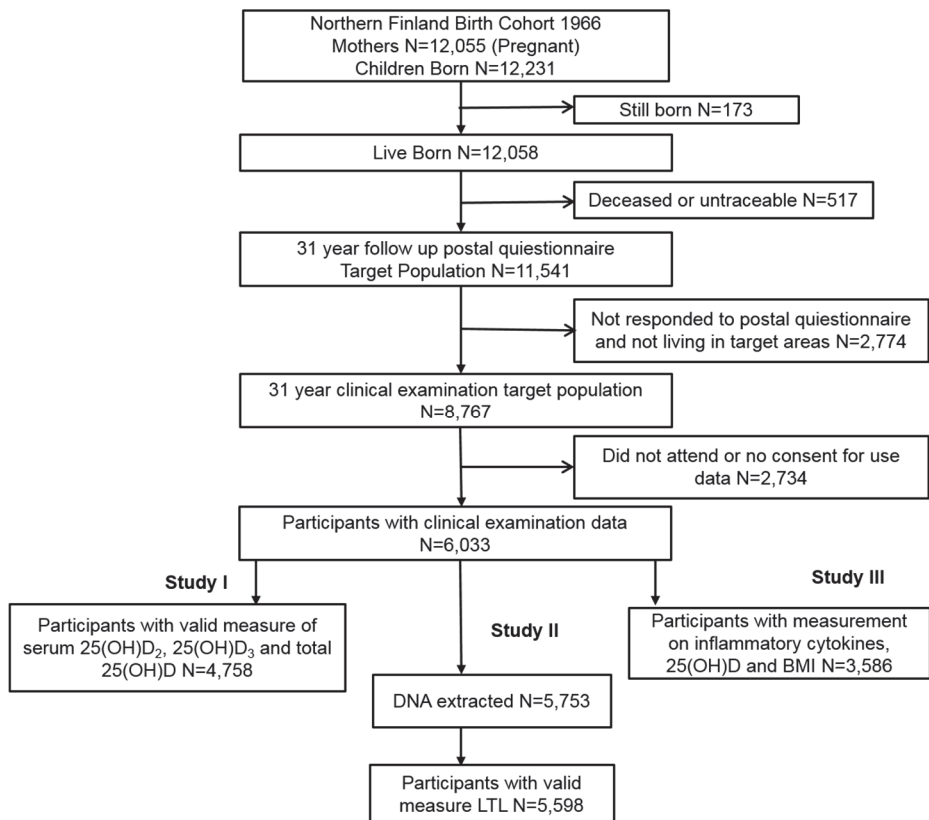


Fig. 18. Flowchart of NFBC1966 data collection and study design (I–III).

The difference between complete and incomplete data set for each study was examined by the Kruskal-Wallis test and Pearson’s Chi-square test. The selected sample was representative of the comprehensive NFBC1966 participants at 31 years. No difference between the complete and incomplete dataset was observed, except for small numerical differences in Study II as shown in Table 10.

Table 10. Characteristics of the participants included in Study II and those excluded because of missing information on one or more variables.

Variables	Included (N=5,096)		Excluded (N=937)		p-value ¹
	Median (IQR)	%	Median (IQR)	%	
Age, years ²	31.1 (0.35)		31.1 (0.34)		0.12
Female sex		51.8		53.7	0.27
Body mass index (kg/m ²)	24.0 (21.9–25.6)		24.1 (21.9–26.9)		0.32
Socioeconomic position					0.58
Professional		23.8		23.3	
Skilled worker		31		29.8	
Unskilled worker		25.6		25.3	
Farmer		15.8		18.1	
Other		3.6		3.5	
Physical activity, MET hours/week	11.3 (3.8–20.3)		10.5 (3.8–22.5)		0.72
Diet quality ³					0.41
0		7.5		7.1	
1		24.3		25.8	
2		30.4		27.9	
3		26.6		25.9	
4 or 5		11.3		13.3	
Ever smoking, yes/no		63.2		64.1	0.64
Current smoking (n=1,838), cigarettes/day	10 (5–20)		12 (7–20)		0.28
Alcohol consumption, g/day	4.2 (1.1–11)		3.6 (0.9–8.7)		0.002
Oral contraceptive (women only)		23.6		21.3	0.26
C-reactive protein, mg/L	0.75 (0.36–1.87)		0.78 (0.38–2.14)		0.17
LTL, T/S ratio	1.13 (0.91–1.41)		1.16 (0.95–1.46)		0.03

Abbreviations: IQR, interquartile range, MET, metabolic equivalent of task.

¹p-value for difference between excluded and included participants, based on a 2-tailed t-test, Kruskal-Wallis test, or χ^2 test.

² Values are presented as mean (standard deviation) because of the normal distribution of the variable.

³ Lower values indicate a healthier diet.

4.3 Variables used in the thesis, the cutoffs and categorisation

Anthropometry

Participants wore light clothing and were barefoot during the clinical examination. Trained nurses measured body weight (kg), height (cm), and waist circumference (cm). Body mass index (BMI) was calculated using the formula, body weight in kilograms divided by height in meters squared (kg/m^2) (4). BMI was then categorised as underweight (<18.5), normal weight ($18.5\text{--}24.9$), overweight ($25.0\text{--}29.9$) and obese (≥ 30.0) (WHO classification) (220).

Waist circumference (WC) was measured at the level midway between the lowest rib and the iliac crest. WC was categorised as elevated when it is ≥ 94 cm in men and ≥ 80 cm in women (IDF, abdominal obesity) (91).

Environmental aspects (the season of blood sampling and latitude)

A month of participants attendance to the clinical examination was used to examine the seasonal influence on serum 25(OH)D concentration. During the data collection, the Finnish holiday period marks a break, although the data was collected throughout the year. The season of blood sampling was categorised as high sunlight (summer and autumn: June–October) and low sunlight season (winter and spring: November–May) according to the Finnish Meteorological Institute (FMI) (82). This classification was made to examine the impact of natural high and low vitamin D periods throughout the year. Information on the participant's residence was obtained from the population register office and was used to calculate the latitude for each individual. The following categorisation was made as residing in the city of Oulu (65°N); in other northernmost provinces of Oulu and Lapland ($>65^\circ\text{N}$); and in Helsinki (60°N).

Alcohol consumption

Multiple questions on the consumption of various types of alcoholic beverages (beer, wine and spirits), their frequency of consumption during the last year, amount and duration were asked. The frequency of the consumption were first quantified as grams per day (31). WHO sex-specific classification of alcohol consumption was then used to classify as low risk drinker (≤ 20 g/day and ≤ 40 g/day for women

and men) or at risk drinker (>20 g/day and >40 g/day for women and men, respectively) (324).

Smoking status

Smoking included a query on current smoking status, if they had ever smoked, if they had quit smoking, the number of cigarettes per day, use of alternative types of tobacco including cigars, pipes, snuff or chewing tobacco. Current smoking status were categorised as a) non-smoker, b) former/occasional smoker, and c) active smoker. Current use or non-use of alternative tobacco products were categorised as a) no, b) other cigarettes, c) cigars, d) pipes, e) snuff and chewing tobacco.

Physical activity

Physical activity queries included the frequency and the duration of light and keep-fit (brisk) exercises during leisure time. The duration and frequency were calculated as metabolic equivalent of task (MET) hours per week (325) and categorised into quartiles of physical activity. An intensity value of 3 METS was used as light activity and 5 METS was used for brisk physical activity (326).

Leisure time computer use

The frequency of computer use during the leisure time was categorised as a) never, b) no more than once per week, c) on 2–5 days per week, and d) on more than 5 days per week.

Dietary intake (Food frequency questionnaire)

The postal questionnaire included queries on 32 products categorised under grain products, milk products, vegetables, meat, fruits and others (chocolates, packaged meals and sweets). The participants were asked to consider their habitual food consumption during the previous six months, and to choose a suitable classification on a structured six-point scale (less than once a month or never, once or twice a month, once a week, a couple of times a week, nearly every day, and once a day or more often). The postal questionnaire on food consumption was based on Finland population based surveys of health behaviour (327, 328). In this case, data on the frequency of consumption of foods rich in fibre (such as rye bread or crisp bread,

fresh vegetables and salads, berries or fruit) and foods high in saturated fats (such as sausages) were obtained (31). A 6-category diet quality variable was calculated [ranging from healthiest quality (0) to unhealthiest quality (5)]. An unhealthy diet was taken to be one that included daily or almost daily consumption of sausages, and less frequent (twice a week or less often) consumption of rye bread or crisp bread, fresh vegetables and salads, berries or fruit, one point being assigned on each of these counts, so that a sum of four-five points indicated an unhealthy diet (4–5 points) and three or less (≤ 3 points) a healthy diet (31).

Contraception status

The use of contraception was categorised as a) no contraception, b) other types of contraception than oral contraceptive, and c) oral contraceptive pills (OCP). The group ‘other types of contraception’ comprised of hormone intrauterine device (IUD), other IUD, chemical contraception (foam, suppository) and any other method of contraception used by women.

Socioeconomic position (SEP)

Socioeconomic position was calculated based on the occupation and employment queries reported in the postal questionnaire. SEP was categorised from I (high) to IV (low), plus farmers and others as in Sovio *et al.* (2007) (329). The following categorisation was used: a) I+II (Professional), b) III (Skilled worker), c) IV (Unskilled worker), d) V (Farmer), and e) VI (other). The group ‘other’ consists of students, pensioners, long-term unemployed, or undefined.

Laboratory measurements

Blood sampling: During clinical examination at 31 years, overnight fasted blood samples (≥ 12 hours) were taken between 8.00 AM and 11.00 AM from the antecubital vein in a sitting position. The samples were then transferred into a cryotube for long-term deep freezing, labelled and stored at -70°C until further analyses. They were later utilised for measurement of serum 25(OH)D and plasma inflammatory biomarkers, respectively. Total blood DNA was extracted from 5,753 individuals in EDTA tubes and was used to measure leukocyte telomere length. Aliquoting participants samples, extraction of DNA by phenol-chloroform method, quality control and bio banking were performed at The National Institute for Health

and Welfare, Biomedicum Helsinki, Finland (Detailed information on blood sampling, section 8, Appendix 8.3).

25-hydroxyvitamin D assay

Serum 25(OH)D₂ and 25(OH)D₃ concentrations were measured by the liquid chromatography tandem mass spectrometry (LC-MS/MS) system. The quality and accuracy of the 25(OH)D analyses were validated on an ongoing basis by participation in the vitamin D External Quality Assessment Scheme (DEQAS, Elstree, Hertfordshire, UK). Participating laboratories receive a certificate if 80% of their results from the five quarterly samples are within 30% of the all-laboratory trimmed mean (ALTM). The values comparing the laboratory submitted samples to DEQAS and the DEQAS ALTM, identified one obvious outlier. The method showed <10% positive bias against DEQAS ALTM at the time of the measure (2008–2009). The coefficient of variation (CV) were less than 16% across the working range of the assay for 25(OH)D₂ and 25(OH)D₃. The ng/ml to nmol/L conversion of vitamin D is mentioned in section 4.4. The detailed analytical procedure is mentioned in section 8, Appendix 8.4.

Leukocyte telomere length (LTL) measurement (outline)

Mean relative LTL was measured from DNA samples extracted from whole blood leukocytes using a monochrome multiplex quantitative real-time polymerase chain reaction method (MMqPCR) (330), with certain modifications (331). The measurement was performed at the Imperial College London. Telomere length measured from peripheral leukocytes acts a proxy for the average telomere length of all immune cell populations in a sample (i.e., cells derived from both lymphoid and myeloid progenitor: monocytes, lymphocytes, neutrophils, basophils and eosinophils). The multiplex qPCR technique employed was based on the polymerase chain reaction technique. Polymerase chain reaction amplifies genomic DNA (ng) targets. Each DNA sequence is amplified by two set of primer pairs, one pair for telomere sequence (T) and a pair for single copy gene sequence (S). The amount of T and S is measured as reference to standard curves produced from five serial dilutions of reference DNA samples spanning 5–30 ng of qPCR reaction. This method provides a ratio between telomere repeat copy number (T) and single-copy gene copy number (S) in each test sample (T/S method). The T/S ratios represent the average telomere length measured from each participant in the cohort. The

detailed analytical procedure of MMqPCR reaction and T/S ratio calculation is mentioned in section 8, Appendix 8.5.

Inflammatory biomarkers measurement

In 2013, biomarker concentrations in the stored plasma samples with no previous freeze-thaw cycles were analysed by multiplex array method using human cytokine/chemokine magnetic bead panel (HCYTOMAG-60K-12, Merck Millipore, USA) and non-magnetic human CVD/cytokine panel (SPR349, Merck Millipore, USA). The multiplex cytokine/chemokine array included IL-17, IL-1 α , IL-1 β , IL-4, IL-6, IL-8, CXCL10/IP-10, IL-1RA, MCP-1/CCL2, vascular endothelial growth factor (VEGF), soluble cluster of differentiation antigen 40 ligand (sCD40L/CD154), TNF- α , active PAI-1, and soluble cell adhesion molecules, E-selectin, sVCAM-1, and sICAM-1. All biomarker assays were performed according to the manufacturer's instructions. The assay range and the intra- (within samples) and inter-assay CV are listed in Table 11.

Table 11. Manufactures assay range and the inter- and intra-assay CV.

Inflammatory biomarkers	Assay range	Inter CV %	Intra CV %
Interleukin-17 (IL-17) (pg/ml)	16–10,000	8.4	2.5
Interleukin-1 alpha (IL-1 α) (pg/ml)	16–10,000	11.0	4.0
Interleukin-1 beta (IL-1 β) (pg/ml)	16–10,000	9.5	3.0
Interleukin-6 (IL-6) (pg/ml)	3.2–10,000	7.7	2.6
Tumor necrosis factor-alpha (TNF- α) (pg/ml)	16–10,000	7.2	2.6
Interleukin-1 receptor antagonist (IL-1RA) (pg/ml)	16–10,000	13.0	4.6
Interleukin-4 (IL-4) (pg/ml)	40–10,000	10.4	3.0
Interleukin-8 (IL-8) (pg/ml)	3.2–10,000	7.6	2.2
C-X-C motif chemokine 10 (CXCL10/IP-10) (pg/ml)	40–10,000	8.3	4.5
Monocyte chemoattractant protein-1 (MCP-1/ CCL2) (pg/ml)	3.2–10,000	6.7	3.1
soluble CD40L (sCD40L/CD154) (pg/ml)	3.2–10,000	14.9	3.7
soluble intercellular adhesion molecule-1 (sICAM-1) (ng/ml)	0.08–250	15.9	8.3
soluble vascular adhesion molecule-1 (sVCAM-1) (ng/ml)	0.08–250	13.9	7.9
active plasminogen activator inhibitor-1 (active PAI-1) (ng/ml)	0.08–250	18.6	6.8
High sensitivity C-reactive protein (hs-CRP) (mg/L)	0.08–29	5.2	4.2

Inflammatory biomarkers (VEGF, sE-selectin) with CV>20% were excluded from the analysis to lessen the intervariability among samples measured.

High sensitivity C-reactive protein (hs-CRP)

Immunoenzymetric assay was used to measure hs-CRP concentration (Medix Biochemica, Espoo, Finland). The assay range is 0.08–29.0 mg/L. The intra- and inter-assay CVs were 4.2% and 5.2% respectively (332).

Alpha-1-acid glycoprotein (AGP)

Alpha-1-acid glycoprotein was extracted along with a group of 123 metabolites in NFBC1966. High throughput nuclear magnetic resonance spectroscopy (¹H NMR spectra) was used to measure the serum acute-phase inflammatory marker, alpha-1-acid glycoprotein (mmol/L) (333). The NMR metabolomics platform (334) was applied for absolute metabolite quantifications and is based on three molecular windows (lipoprotein, low-molecular-weight metabolite data and lipid) for each sample (333). The CV was 1.1% (333).

4.4 Variable conversions used in Study I–III

Conversions of serum 25(OH)D: To include all the participants with D2 measurements, and those with values below the limits of detection (LOD) were assigned a value of 1.25 nmol/L. This is because of the limitation in the analytical procedures to precisely measure the small concentration in the serum samples. Furthermore, the D2, D3 and total 25(OH)D measured in units of ng/ml is converted into nmol/L by multiplying with 2.496. Serum 25(OH)D concentration will be mentioned as units of nmol/L in all the studies. The conversion of measurement units into nmol/L was made to facilitate direct comparison with the IOM recommendations and other population studies examining vitamin D status. The serum concentrations of 25(OH)D₂ and 25(OH)D₃ were summed to reflect total 25(OH)D concentration of an individual. Total 25(OH)D concentration is considered as an absolute measure of circulating vitamin D (2).

Season-adjusted 25(OH)D variable

Serum 25(OH)D was adjusted for the season of blood sampling and a new continuous variable (25(OH)D-season adjusted) was created taking into account the influence of sunlight across the seasons in Study II. Linear regression trigonometric sine and cosine functions were utilised to model 25(OH)D against

the date of blood sampling at 31 years. The purpose of using this function is that the model assumes that the seasonal variation follows a sinusoidal pattern (335). In addition, this model may be flexible for adjusting the seasonal variation with a single measure of 25(OH)D as a continuous variable and does not require categories to be added as dummy variables in the linear regression analyses (336). The following function was used where the estimated regression parameters are α , β_h , and θ_h and date of blood sampling is t .

$$f(t) = \alpha + \sum_{h=1}^4 \beta_h \sin(2h\pi t) + \sum_{h=1}^4 \theta_h \cos(2h\pi t)$$

To produce the best fit of the data, the number of terms (β_h and θ_h) was included only after investigation using Wald tests and graphical plots (337). During the data collection, the Finnish holiday period marks a break, although the data were collected all throughout the year. Therefore, the model was applied separately as two sections, with participant's assessment period before and after the break. The season adjusted 25(OH)D variable to be used in the analysis were then obtained as residuals from these models. In addition, in Study II, 25(OH)D has also been tested as a season unadjusted variable.

Conversions of inflammatory biomarkers: Inflammatory biomarker concentrations, which are below the lower limits of detection, were reported with the lowest detection value of each biomarker tested.

General and study specific exclusion criteria

The general exclusion criteria consists of participants with non-fasted samples, no consent for use of the data and missing information on one or more variables of interest (complete case dataset). In Study III, underweight subjects ($<18.5 \text{ kg/m}^2$), participants who reported having fever during clinical examination, hs-CRP concentration ($>10 \text{ mg/L}$; to exclude for acute inflammation), thyroid problems and lipid medication users (interference with vitamin D metabolism) were excluded. We also excluded women who were pregnant and those using oral contraceptive (interference with vitamin D and inflammation pathways).

4.5 Statistical analyses

The statistical analyses and the software used in the Studies I, II and III are explained under the following headings.

4.5.1 Descriptive analyses

The variables that were selected for the statistical analyses were first examined for any possible error. The distribution of the variables was checked with histograms to look for the presence of any outliers. The variables were then transformed based on their distribution. The distributions of the continuous variables were described by means, geometric means, median, confidence intervals (CI), interquartile range (IQR) and standard deviation (SD). The categorical variables are presented as number of observations (N) and percentages. SAS procedure proc MEANS and proc FREQ used in SAS version 9.4 (SAS Institute Inc. 2010) were used to perform the descriptive analyses. This gives the characteristics of the study population in Study I, II and III. The difference between sexes was examined by Student's *t*-test for normally distributed variables and Wilcoxon Mann-Whitney U-test for non-normally distributed variables. For class variables, Pearson's Chi-Square test or Fischer's exact test were used. In Study I and II, the distribution of continuous variables across the tertiles of serum 25(OH)D and fifths of 25(OH)D distribution was analysed by one-way analysis of variance (ANOVA).

Selection of variables in the study

Spearman correlation was employed to check collinearity among the variables examined in each study. Collinearity is a condition in which some independent variables are highly correlated (linear combination of one another). The linear association between dependent and independent variable and the possible non-linear associations were also tested by using scatter plots and by plotting as graphs.

While performing multiple regression analyses, the unadjusted regression coefficient between the dependent and independent variable was first examined. At each step, one variable was then added to this model and the change in regression coefficients and model fit/estimates were carefully interpreted. When collinearity occurs in regression analyses, it does not reduce the reliability of the model or the predicted outcome. However, the problem arises in estimating the regression coefficients of other individual predictors in the model. The regression model with the highly correlated variables can indicate how the group of variables predicts the outcome variables. However, it will not provide any valid interpretation on the contribution of individual predictor. The variables, which cause collinearity, were then dropped from the model and interaction analysis was further conducted.

Confounders in the analyses are associated with both dependent and independent variable, but does not lie in the causal pathway. Controlling for confounders will prevent underestimation or overestimation of the association between dependent and independent variable. Potential confounders to be included in the statistical analyses were selected carefully based on the previously published literature and for any other possible confounders in relation to the study. In addition, the association of confounders with both the exposure and the outcome was tested. Confounding was controlled in the analyses by stratification and by using multi-variable linear regression models in Study I–III. The magnitude of confounding in analyses were examined by comparing crude estimates with the adjusted estimates after adding confounder in the model. The confounders included in Study I–III were smoking, physical activity, alcohol consumption, oral contraceptive, batch effects and socioeconomic position.

4.5.2 Regression analyses

The overview of the variables used in Studies I–III is given in Table 12.

Table 12. Overview of the variables used in Study I–III and the exclusion criteria.

Studies	Study I	Study II	Study III
Participants (<i>n</i>)	4,758	5,096	3,586*
Inclusion year	1997	1997	1997
Measurements	Serum 25(OH)D ₂ and 25(OH)D ₃ Serum 25(OH)D Anthropometry	Serum 25(OH)D Leukocyte telomere length Body mass index (kg/m ²) C-reactive protein	Serum 25(OH)D 16 inflammatory biomarkers Anthropometry
Exclusion criteria	Postal questionnaire Non-fasted samples, pregnant women, no consent for data use	Postal questionnaire Non-fasted samples, pregnant women, no consent for data use	Postal questionnaire Non-fasted samples, pregnant women, no consent for data use, lipid lowering drugs, hs-CRP >10 mg/L, participants with fever, Underweight (<18.5 kg/m ²), thyroid problems, oral contraceptive. * The major exclusion from oral contraceptive users (N=607)

Study I

Serum 25(OH)D₂, 25(OH)D₃, total 25(OH)D and tertiles of 25(OH)D were selected as the main outcome. D₂, D₃ and 25(OH)D were natural logarithm transformed to reduce skewness and were used on the standard score (z-scores) (mean=0; standard deviation (SD)=1). SAS procedure proc GLM was used for the analyses. First, the association between determinants (environment, anthropometry, lifestyle, socioeconomic, and sex-specific) and D₂, D₃, 25(OH)D concentrations were analysed by regression analyses. To investigate whether sex was an effect modifier, an interaction term (sex x outcome variable) was incorporated in the unadjusted analyses. Multivariable analyses were performed aiming to examine mutually adjusted (independent) associations of different exposures (environment, anthropometry, lifestyle, socioeconomic, and sex-specific) with the outcome variables. They were the season of blood sampling, latitude, BMI, WC, SEP, smoking status, alcohol consumption, leisure time computer use, physical activity, diet score and contraception status. In addition, the analyses were also conducted with exclusion of women using oral contraceptive pills. Univariable and multivariable regression results are presented as regression coefficients (β) with 95% CIs.

In addition, the risk factors associated with being in lower tertile of 25(OH)D (reference: tertile III) were analysed by multinomial ordinal logistic regression analyses. Proc GENMOD was used for the analyses. The classification of 25(OH)D into tertiles was made owing to different cutoffs (see section 2.1.6) used in defining the optimal vitamin D status. The unadjusted and adjusted results are presented as odds ratio (OR) with 95% CIs.

Study II

The LTL measurements were selected as the main outcome. Telomere T/S ratio was right skewed and hence natural logarithm transformed to achieve normal distribution. Linear regression analyses was used to estimate the effect size of association between season adjusted 25(OH)D and BMI with LTL taking into account adjustment for covariates and mediating factors. To investigate whether there is any sex-specific associations between 25(OH)D-LTL and BMI-LTL, interaction terms (sex x outcome variable) were tested. In females, oral contraceptive pills may interfere with both 25(OH)D (Study I results) and LTL (for

example, by modification of inflammatory biomarkers), so the interaction were also tested in the analyses.

There were several regression models fitted to examine the 25(OH)D-LTL and BMI-LTL association. For 25(OH)D-LTL association, model 1 was adjusted for sex, 25(OH)D batch and age. Model 2 included model 1 and additional adjustment for further potential confounders (BMI, SEP, smoking status, alcohol consumption, diet and OCPs). Model 3 was in addition, adjusted for hs-CRP. The regression results are presented as percent change in LTL (T/S ratio) per 1 nmol/L increase for 25(OH)D with 95% CIs. The regression analyses were also repeated without adjustment for the season of blood sampling. This was made in order to obtain similar and comparable results based on a previous publication (338) which was relevant to our hypotheses. The study has not included adjustment for season (338). In addition, to test whether there are any pronounced differences in mean LTL values between cutoffs of 25(OH)D, 25(OH)D was categorised as <50 nmol/L, between 50-75 nmol/L, and >75 nmol/L and then analysed.

To examine BMI-LTL association, model 1 was adjusted for sex and age. Model 2 was further adjusted for confounders (SEP, smoking, alcohol consumption, physical activity, diet and OCPs). Model 3 was further adjusted for 25(OH)D and 25(OH)D batch. Model 4 involved additional adjustment for hs-CRP. The results are presented as percent change in LTL (T/S ratio) per unit increase for BMI with 95% CIs. The results are presented as regression coefficients (β) with 95% CIs.

Study III

The inflammatory biomarker measurements were selected as the main outcome. Inflammatory biomarker data was skewed and hence appropriate transformation procedures were applied to achieve normal distribution. Unsupervised hierarchical clustering analyses were performed to examine the clustering pattern of inflammatory biomarkers. Correlations were then calculated using Proc CORR procedure to assess the Pearson correlation coefficients of plasma biomarker concentrations.

There were several regression models fitted to examine the BMI-inflammation and 25(OH)D-inflammation associations. BMI, 25(OH)D and inflammatory biomarkers were used on their standard scores (z-score) (mean=0; SD=1). For BMI-inflammation association, model 1 was unadjusted. Model 2 was further adjusted for sex. Model 3 included additional adjustment for covariates (SEP, smoking, physical activity and alcohol consumption). The regression coefficients

can be interpreted as the change in 1 SD units in inflammatory biomarker per 1 SD increase in BMI with 95% CIs.

For 25(OH)D-inflammation association, model 1 was adjusted for 25(OH)D batch. Model 2 was further adjusted for the season of blood sampling and sex. Model 3 included additional adjustment for covariates (alcohol consumption, smoking status, physical activity and SEP). Model 4 included additional adjustment for BMI. The regression coefficients are interpreted as the change in 1 SD units in inflammatory biomarker per 1 SD increase in 25(OH)D with 95% CIs. Adjusted *p*-values (multiple testing) from regression analyses was performed using the Benjamini and Hochberg (1995), BH or called as “False discovery rate (FDR)” approach, separately for each biomarker cluster (339).

Mediation analysis: To test whether the association of BMI with inflammatory biomarkers is mediated through 25(OH)D, the Baron and Kenny Mediation (1986) method (340) as well as genetic instrument using the Mendelian randomisation approach was utilised. The results of the latter analyses (MR) will not be described in the thesis. Figure 16 (see section 3, aims and objectives) shows the principle of the Baron and Kenny mediation model tested in Study III. For detailed information of the methodology see section 8, Appendix 8.6.

Before testing mediation, an important criteria to perform is to check for the interaction (moderation effect) of the exposure and covariates with the mediator serum 25(OH)D. If the beta coefficient is decreased by >10% according to Baron and Mediation method (340), then a partial mediation is considered (341). However, when the association between the exposure and the outcome is attenuated by the addition of mediator variable, then complete mediation is considered. All the mediation models were adjusted for covariates (sex, alcohol consumption, smoking status, physical activity, and SEP). The mediation results are presented as regression (β) coefficients with 95% CIs.

The Sobel test was used to examine the significance of the mediation effect (342). Sobel *et al.* (1981) hypothesised that the association between the exposure and the outcome variable is considered to be present through an indirect effect, which occurs by the influence of the third variable (mediator). When the mediator is added in the model (step iv: mediation model; previous paragraph) the effect of the association between exposure and the outcome variable should be reduced illustrating a mediation. Online platform for testing the significance of mediation by Sobel test was used (342).

In the recent years, more sophisticated statistical procedures have been developed for testing mediation. Baron and Kenny mediation (Sobel’s test) causal

steps approach have been criticised on multiple grounds. More precisely, it has been reported from simulation studies, that testing for mediation reduces the power in this method. New methods, for example, bootstrapping and *M*-test are proposed. Although these analytical procedures are robust in calculating the mediation, it is way beyond the scope of the thesis to compare Baron and Kenny mediation results with other new analytical procedures.

4.5.3 Software

The statistical software used in this thesis were SAS Proprietary Version 9.4 (2002-2012 SAS Institute Inc., Cary, NC, USA), SAS Enterprise Guide 7.1 (2012 SAS Institute., Cary, NC, USA), R version 3.3.1 (2016, The R foundation for Statistical Computing) and STATA statistical software (StataCorp. 2013. Release 13. College Station, TX: StataCorp LP). The level of statistical significance was set to $p < 0.05$ and correction for p -values (multiple testing) with Benjamini and Hochberg (BH, 1995) or called as “false discovery rate (FDR)” approach was performed in the analysis as necessary (339). Appropriate power calculations to examine the effect size of the study has been performed (343).

4.6 Ethical considerations

The different time points of data collection in NFBC1966 were based on voluntary participation by the participants and their mothers (initial data collection). The purpose and the use of data collected have been well acknowledged to the participants. During the 31-year data collection, cohort participants were asked to give additional written informed consent for 1) research on data in an unidentifiable manner within the University of Oulu, 2) data sharing (collaboration), and 3) for procuring additional hospital register data to appendix the various data collected. Participants were also briefed about the possibility to withdraw or refute permission for using their data at any stage of data collection or study.

The data obtained from NFBC1966 participants have been stored and accessed securely at the NFBC Cohort Center, Faculty of Medicine, University of Oulu, Finland. A declaration form is signed by the researcher using the data to maintain confidentiality. It is also restricted from passing the data to unauthorised sources apart from those mentioned in research plan. The NFBC1966 study has been reviewed and approved by the Ethical Committee of the University of Oulu and

Northern Ostrobothnia Hospital district. The study was performed in accordance with the Helsinki Declaration and University of Oulu.

5 Study specific results and discussion

In this chapter, the main results followed by discussion of the findings from Studies I–III are presented under separate headings. The results related to review paper (IV) are presented in section 2.4.12 (Table 9). More information of the results are presented in the Studies I, II and III (papers) as well as in review paper (IV). An overall discussion combining all the studies is provided in the next chapter.

5.1 Potential determinants of vitamin D status (Study I)

The factors associated with serum 25(OH)D₂, 25(OH)D₃ and total 25(OH)D concentrations and the risk factors for being in lower tertile of vitamin D status were assessed in Study I. The variables used in Study I–III and the exclusion criteria employed in each study is shown in previous section 4.5.2 (Table 12).

5.1.1 Descriptive analyses

Vitamin D status in NFBC1966: The mean serum total 25(OH)D concentration in the study population was 68.4 nmol/L (95% CI: 67.6–69.2) (Table 13). Women had overall marginally higher concentration of 25(OH)D₂ when compared to men ($p < 0.01$). There was no difference in mean 25(OH)D and 25(OH)D₃ concentrations between sexes.

Table 13. Vitamin D status in NFBC1966 at 31 years.

Variable	Total (N=4,758) Mean (95% CI)	Male (N=2,374) Mean (95% CI)	Female (N=2,384) Mean (95% CI)	<i>p</i> -value ¹
Vitamin D status, nmol/L				
Serum 25(OH)D ₂	4.2 (3.9–4.3)	3.9 (3.6–4.2)	4.4 (4.1–4.7)	<0.01
Serum 25(OH)D ₃	64.8 (63.9–65.6)	65.6 (64.4–66.7)	64.0 (62.8–65.1)	0.45
Serum total 25(OH)D	68.4 (67.6–69.2)	68.9 (67.7–70.1)	67.9 (66.7–68.9)	0.78
Without OCP (nmol/L)				
Serum 25(OH)D ₂			4.2 (3.9–4.5)	0.05
Serum 25(OH)D ₃			60.9 (59.8–62.2)	<0.01
Serum total 25(OH)D			64.6 (63.3–65.8)	<0.01

¹The differences in mean 25(OH)D between males and females assessed using Student's *t*-test.

*Serum total 25(OH)D concentration may differ slightly from the actual sum of D₂+D₃, because of the substitution of the lower limits in D₂. For detailed procedure, see materials and methods section 4.4.

When the analysis was carried out in women, excluding for oral contraceptive pill users, the difference between sexes became more pronounced for total 25(OH)D and D3 concentration ($p < 0.01$). According to the IOM criteria for optimal vitamin D status, 3.3% of the participants were vitamin D deficient (≤ 30 nmol/L), 24.2% were insufficient (30–50 nmol/L), and 71.5% were sufficient (≥ 50 nmol/L).

Table 14 shows the common descriptive characteristics of the study population for all the three studies under this heading. Other specific variables examined in each study are mentioned separately in respective sections.

Table 14. Characteristics of the study population.

Factors	Total population n % or mean (95% CI) (N=4,758)	Males n % or mean (95% CI) (N=2,374)	Females n % or mean (95% CI) (N=2,384)	p-value ¹
Season of blood sampling ²				
High sunlight period	2953 (62.1)	1501 (63.2)	1452 (60.9)	0.087
Low sunlight period	1805 (37.9)	873 (36.8)	932 (39.1)	
Latitude ^{2,3}				
65°N	891 (28.7)	460 (29.3)	431 (28.1)	0.582
>65°N	3105 (71.3)	1571 (70.7)	1534 (71.9)	
Anthropometry				
Body mass index (kg/m ²)	24.8 (24.7–25.0)	25.2 (25.0–25.3)	24.4 (24.2–24.6)	<0.001
Waist circumference (cm)	83.8 (83.5–84.2)	88.9 (88.5–89.3)	78.8 (78.3–79.2)	<0.001
Socioeconomic position ²				
I+II (professional)	1134 (23.8)	653 (27.5)	481 (20.2)	
III (skilled worker)	1483 (31.2)	433 (18.2)	1050 (44.0)	
IV (unskilled worker)	1228 (25.8)	856 (36.1)	372 (15.6)	<0.001
V (Farmer)	165 (3.5)	111 (4.7)	54 (2.3)	
VI (other)	748 (15.7)	321 (13.5)	427 (17.9)	
Smoking ²				
Non-smoker	2128 (44.7)	952 (40.1)	1176 (49.4)	
Former/occasional smoker	1214 (25.5)	600 (25.3)	614 (25.7)	<0.001
Active smoker	1416 (29.8)	822 (34.6)	594 (24.9)	
Alcohol consumption (g/day) ²				
Abstainer	426 (8.9)	191 (8.1)	235 (9.9)	
Low-risk drinker	4053 (85.2)	2026 (85.3)	2027 (85.0)	<0.001
At-risk drinker	279 (5.9)	157 (6.6)	122 (5.1)	

Factors	Total population n % or mean (95% CI) (N=4,758)	Males n % or mean (95% CI) (N=2,374)	Females n % or mean (95% CI) (N=2,384)	p-value ¹
Physical activity (MET hours/week) ²	15.0 (14.6–15.4)	14.9 (14.4–15.6)	15.0 (14.5–15.6)	<0.001
Diet score ²				
0–1	1461 (30.7)	453 (19.1)	1008 (42.3)	
2–3	2739 (57.6)	1531 (64.5)	1208 (50.6)	<0.001
4–5	558 (11.7)	390 (16.4)	168 (7.1)	
Contraception status ^{2,4}				
No contraception			1154 (49.1)	
Other kinds of contraception except OCP's			591 (25.1)	
Oral contraceptive pills			607 (25.8)	

¹p-value for significance is analysed by Student's *t*-test (normal distribution), Wilcoxon Mann-Whitney U-test (non-normal distribution) and Pearson's chi-square test for categorical variables.

²The categorisation of the variables (season, latitude, and all the others) are mentioned in section 4.3.

³Data included only on samples taken during all seasons from Oulu city and other provinces of Oulu and Lapland. Data not included on N=343 in men and N=419 in women with samples taken during winter months from Helsinki region.

⁴Data available on N=2352 women; N=32 missing with contraception status.

5.1.2 Factors associated with 25(OH)D₂, 25(OH)D₃ and 25(OH)D concentration

Unadjusted and adjusted multiple linear regression analyses were employed to examine the association between potential explanatory factors and D₂, D₃, 25(OH)D concentrations (Table 15; for additional results see original article I; Table 3). In unadjusted analyses, being a women (*vs.* men), residing in higher latitudes (>65°N), low sunlight months, being obese (≥30 kg/m²), higher waist circumference (M≥94 cm, F≥80 cm) and being in lower quartiles of physical activity were associated with both serum 25(OH)D₂ and 25(OH)D₃ concentrations. Dietary consumption of unhealthy diet (red meat and sausages) and active smoking were associated with lower 25(OH)D₂ concentration. Lower socioeconomic position, being a skilled or unskilled worker were positively associated with D₃ concentration, i.e. D₃ concentration were higher than the reference group (only limited results are shown in Table 15, for additional results see original article I; Table 3).

In multivariable analyses, women (*vs.* men) were positively associated with 25(OH)D₂ and negatively with 25(OH)D₃ concentrations. To assess whether there is any impact of oral contraceptive pills on 25(OH)D₂ and 25(OH)D₃ concentrations, the analyses were repeated with exclusion of women using OCPs. The negative association between sex (females) and 25(OH)D₃ concentrations still persisted, however, was attenuated for D₂. The season of blood sampling (low sunlight exposure period) was positively associated with 25(OH)D₂ and negatively with 25(OH)D₃ concentrations.

Higher 25(OH)D₃ concentrations were found in individuals classified as low-risk drinkers and high-risk drinkers (*vs.* abstainers). In addition, frequent use of computer during leisure time and dietary consumption of unhealthy diet were associated with decreased D₃.

The data were further analysed with stratification by sex. The results showed similar direction of association and magnitude with 25(OH)D₂ and 25(OH)D₃ concentrations. Serum 25(OH)D concentration showed comparable associations with 25(OH)D₃ concentration. Women using oral contraceptive pills (*vs.* no contraception users) had 0.17 nmol/L and 0.48 nmol/L greater 25(OH)D₂ and 25(OH)D₃ concentrations, respectively (Table 15; for more results, see article I).

Table 15. Factors associated with D2 and D3 concentrations (N=4,758)*.

Factors	Serum 25(OH)D ₂ , nmol/L† (β (95% CI))		Serum 25(OH)D ₃ , nmol/L† (β (95% CI))	
	Univariable	Multivariable‡	Univariable	Multivariable‡
Sex (Reference: males)				
Females	0.10 (0.04, 0.16)	0.12 (0.06, 0.18)	-0.06 (-0.12, -0.003)	-0.09 (-0.14, -0.04)
global <i>P</i> value	0.0008	0.0001	0.038	0.0005
Season of blood sampling (Reference: High vitamin D months)				
Low vitamin D months	0.57 (0.51, 0.63)	0.29 (0.21, 0.36)	-1.03 (-1.08, -0.98)	-0.43 (-0.49, -0.36)
global <i>P</i> value	<0.0001	<0.0001	<0.0001	<0.0001
BMI (kg/m ²) (Reference : Normal (18.5–24.99))				
Underweight (<18.5)	-0.05 (-0.25, 0.15)	-0.06 (-0.24, 0.13)	-0.08 (-0.27, 0.12)	-0.06 (-0.22, 0.10)
Overweight (25–29.99)	-0.10 (-0.17, -0.04)	-0.01 (-0.08, 0.06)	0.02 (-0.04, 0.08)	-0.001 (-0.06, 0.06)
Obese (≥30)	-0.13 (-0.24, -0.03)	-0.01 (-0.14, 0.11)	-0.19 (-0.30, -0.09)	-0.16 (-0.27, -0.06)
global <i>P</i> value	0.0035	0.94	0.0008	0.0035
Alcohol consumption (g/day) (Reference: Abstainer)				
Low risk drinker	0.04 (-0.06, 0.14)	0.07 (-0.03, 0.16)	0.17 (0.07, 0.27)	0.12 (0.04, 0.20)
At-risk drinker	0.03 (-0.12, 0.18)	0.07 (-0.08, 0.21)	0.13 (-0.02, 0.28)	0.19 (0.06, 0.31)
global <i>P</i> value	0.71	0.39	0.0043	0.0041
Quartile of physical activity (MET-hours per week)				
(Reference: QI: 0.0–3.79)				
QII: 3.80–11.29	0.08 (0.0003, 0.16)	0.05 (-0.03, 0.12)	-0.02 (-0.10, 0.06)	0.003 (-0.06, 0.07)
QIII: 11.30–21.99	0.10 (0.02, 0.18)	0.05 (-0.03, 0.12)	0.02 (-0.06, 0.10)	0.05 (-0.01, 0.12)
QIV: >22.0	0.11 (0.03, 0.20)	0.08 (-0.002, 0.16)	0.15 (0.07, 0.23)	0.14 (0.07, 0.20)

Factors	Serum 25(OH)D ₂ , nmol/L† (β (95% CI))		Serum 25(OH)D ₃ , nmol/L† (β (95% CI))	
	Univariable	Multivariable‡	Univariable	Multivariable‡
global <i>P</i> value	0.022	0.29	<.0001	<.0001
Diet score (Reference: Healthy diet)				
Unhealthy diet	-0.12 (-0.21, -0.03)	-0.06 (-0.15, 0.02)	-0.07 (-0.15, 0.02)	-0.07 (-0.15, -0.0004)
global <i>P</i> value	0.009	0.14	0.15	0.049
Females (N=2,384)				
Contraception status (Reference: No contraception)				
Other kinds of contraception than OCP's	0.01 (-0.09, 0.11)	0.03 (-0.07, 0.13)	0.10 (0.01, 0.20)	0.06 (-0.02, 0.14)
Oral contraceptive pills	0.21 (0.11, 0.31)	0.17 (0.08, 0.27)	0.47 (0.37, 0.57)	0.48 (0.41, 0.56)
global <i>P</i> value	<.0001	0.0018	<.0001	<.0001

* The values are regression coefficients (β) and *p*-values from linear regression models by entering each variable separately in univariable analysis and by entering all the variables in multivariable analyses.

† 1 SD increase/decrease in 25(OH)D₂, 25(OH)D₃ and 25(OH)D nmol/L per 1 unit or category change in explanatory variable.

‡ Analysis performed on N=3,996 (total). Blood drawn only in winter on N=343 males & N=419 in females residing in Helsinki were excluded.

^{||}Multivariable analysis done on N=1,933 (females). Blood drawn only in winter on N=419 in females residing in Helsinki were excluded. In addition, N=32 individuals missing with contraception status in females.

5.1.3 Factors associated with lower tertile of vitamin D status

The next step in the analyses was to test the factors associated with lower tertile of serum 25(OH)D using multinomial ordinal logistic regression analyses (Figure 19).

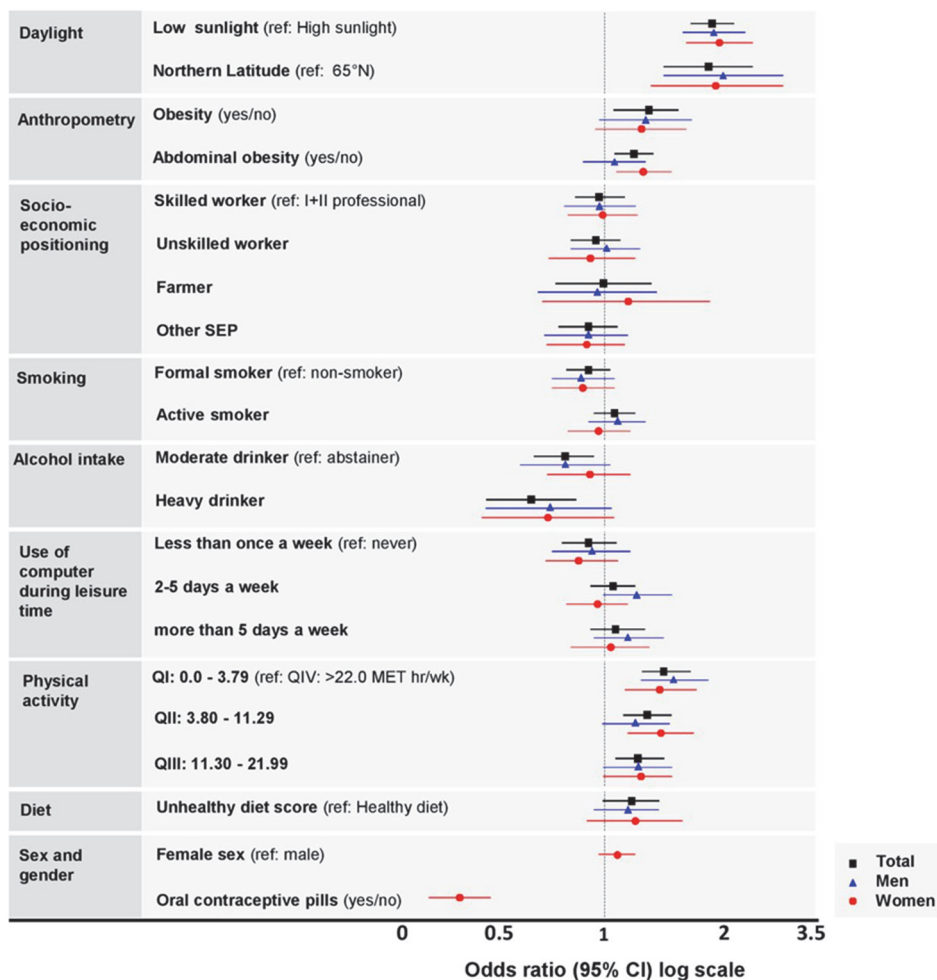


Fig. 19. Forest plots showing the multinomial ordinal logistic regression analyses of the risk factors associated with low vitamin D status in the total population and by sex. Reprinted with permission from BMJ Publishing group, BMJ Open, Palaniswamy *et al.* 2017.

Higher odds of being in lower tertile of serum 25(OH)D were found in individuals whose blood samples were collected during low sunlight months (winter/spring), residence in latitudes ($>65^{\circ}\text{N}$), elevated waist circumference ($M \geq 94$, $F \geq 80$ cm) and consumption of an unhealthy diet (Figure 19). Further, the data analyses was conducted stratified by sex. Females who used oral contraceptive (*vs.* nonusers) were associated with being in higher tertile of 25(OH)D (Tertile III).

5.1.4 Summary and discussion

The regression analyses demonstrated the association between various factors (environmental, anthropometric, lifestyle, socioeconomic and sex-specific) and serum 25(OH)D₂, 25(OH)D₃, total 25(OH)D concentrations. In concordance with previous literature, the season of blood sampling, residing in northern latitudes, reduced physical activity, increased utilisation of computer during leisure time, being obese and elevated waist circumference were associated with low vitamin D status (73, 74). There are various hypotheses linking obesity and reduced vitamin D status (344). Sequestration or volumetric dilution or the interplay with autocrine factors produced by the adipose tissue may explain the lower 25(OH)D status in obesity (344). A bi-directional Mendelian Randomisation (MR) study exploring the causality of association between BMI and 25(OH)D by using BMI related genetic variants as an instrumental variable, has reported a causal relationship (294). A 10% increase in BMI was associated with 4.2% reduced vitamin D and not vice versa (294).

A positive association between 25(OH)D and women who used oral contraceptives was reported in the study. However, there was only a marginal increase in D₂ and D₃ concentrations, which show little to no clinical significance. An epidemiological study, which examined the effect of oral contraceptive during vitamin D supplementation, has reported the influence of exogenous estrogen in increasing the response to supplementation (345). However, previous findings using the same data from NFBC1966 has reported an increase in inflammatory biomarker (CRP) and alteration in metabolic profile in women who used oral contraceptives (142, 346). A study that investigated the association between 25(OH)D and CRP has reported nil association, when OCP was adjusted in the analysis (347). These results may suggest analysing the relationship between oral contraceptives, vitamin D and inflammation simultaneously in future investigations. In addition, further work is needed to investigate how estrogens

(post/perimenopausal hormone therapy) may affect 25(OH)D concentration and inflammation over the life course.

The present study replicated the differential associations between the season of blood sampling and D2, D3 concentrations as reported in a previous study from UK (348). The factors associated with 25(OH)D influenced similarly the D3 concentrations. D3 was the major contributor of 25(OH)D and was positively associated with latitude and the season of blood sampling. In addition, the increased D2 concentration observed during winter is yet to be understood. The national recommendation for vitamin D fortification was introduced in Finland in 2003, which is after the NFBC1966 sample collection at 31 years (1997). In 1997, vitamin D supplements were more likely D2-based. Currently, only D3 is used for the fortification of food products in Finland from 2003 and D3 is the predominant form in supplements as well (75).

The present study was in concordance with previous literature and highlights the need for a better understanding of various factors associated with D2 and D3 concentrations. The study also supports the differential associations between D2 and D3 concentrations: D2 (the season of blood sampling, waist circumference) and D3 (the season of blood sampling, latitude, being obese, alcohol consumption, leisure time computer use, physical activity, and diet score) (see Chapter 7, Figure 23A). Understanding the association between multiple factors and different vitamin D isoforms could help in better interpretation of the vitamin D status in clinics, research and public health recommendations.

Vitamin D deficiency is pervasive worldwide. Apart from the well-known determinants of vitamin D, deeper insights on the other determinants and its association with vitamin D isoforms are indispensable and significant. This could help in formulating appropriate cutoffs for vitamin D sufficiency (optimal status) and for developing meaningful interventions to reduce the prevalence of vitamin D deficiency. Vitamin D, body mass index and leukocyte telomere length (Study II) The association between 25(OH)D and LTL were examined in Study II. In addition, the study tested whether the previously reported BMI-LTL association from Buxton *et al.* (2014) is independent of 25(OH)D concentration. Further, the study tested whether 25(OH)D-LTL and BMI-LTL associations were independent of inflammatory pathways (hs-CRP was studied) (Figure 16).

5.1.5 Descriptive analyses

The mean LTL (T/S ratio) was 1.13 (95% CI: 0.91–1.41) and mean hs-CRP concentration was 0.75 mg/L (95% CI: 0.36–1.87) in the studied population. When participants characteristics were examined by fifths of 25(OH)D distribution, comparable results were observed as previously reported in Study I (for detailed results see article II, supplementary file Table 1).

5.1.6 Association between 25(OH)D and leukocyte telomere length

The association between 25(OH)D and LTL was analysed by three models (see section 4.5.2). First, the results of the association between season adjusted 25(OH)D and LTL from multivariable regression models is summarised in Table 16. There was no association observed between 25(OH)D and LTL in any of the models.

Table 16. Associations between 25(OH)D and LTL.

Models	Mean difference in LTL, %*	95% CI	p-value
Model 1 : adjusted for age, sex and 25(OH)D batch	-0.01	-0.20, 0.18	0.94
Model 2 : Model 1 + BMI, SEP, physical activity, diet, smoking, alcohol and use of contraceptives (females only)	-0.01	-0.20, 0.20	0.91
Model 3 : Model 2 + CRP	-4×10^{-5}	-0.20, 0.20	0.97

*Relative percent change in LTL (T/S ratio) per 1-nmol/L increase in 25(OH)D

Secondly, the analysis was repeated without adjustment for the season of blood sampling. However, the results did not differ from the previous observations. Third, the results on testing whether there were any pronounced differences in LTL across the categories of 25(OH)D [<50 nmol/L and 50–75 nmol/L with 25(OH)D >75 nmol/L as a reference category] are summarised in Table 17. LTL did not differ across lower categories of 25(OH)D (<50 nmol/L or 50–75 nmol/L) when compared with the highest category (>75 nmol/L) (Table 17).

Table 17. Mean differences in LTL across 25(OH)D categories (<50 nmol/L, N=2,550; 50–75 nmol/L, N=2,261; and >75 nmol/L, N=285).

Models	Mean difference in LTL comparing those with 25(OH)D=50 to75 nmol/L to those with 25(OH)D >75 nmol/L		Mean difference in LTL comparing those with 25(OH)D<50 to those with 25(OH)D >75 nmol/L	
	β (95% CI)	<i>p</i> -value	β (95% CI)	<i>p</i> -value
Model 1 : adjusted for age, sex, 25(OH)D batch	0. 10 (-4.0, 4.4)	0.97	0.2 (-4.1, 4.7)	0.92
Model 2 : Model 1 + BMI, SEP, physical activity, diet, smoking, alcohol intake, and use of oral contraceptive	-0. 10 (-4.0, 4.4)	0.94	0.1 (-4.2, 4.7)	0.96
Model 3 : Model 2 + hs-CRP	-0. 20 (-4.0, 4.4)	0.91	0.0 (-4.3, 4.6)	0.99

*Beta coefficients and 95% CI represent mean percent difference in telomere length between 25(OH)D groups.

5.1.7 Association between BMI and leukocyte telomere length

The results of the association between BMI and LTL are summarised in Table 18. An inverse association observed between BMI and LTL in model 1, adjusted for age and sex. The strength of the associations remained unchanged with further adjustment for SEP, physical activity, diet, smoking and alcohol consumption and 25(OH)D in model 2 and 3. When the model was further adjusted for hs-CRP (model 4), the association between BMI and LTL was slightly attenuated, but remained significant ($p=0.01$).

Table 18. Associations between BMI and LTL.

Models	Mean difference in LTL, %*	95% CI	<i>p</i> -value
Model 1 : adjusted for age and sex	-0.40	-0.59, -0.19	0.001
Model 2 : Model 1 + SEP, physical activity, diet, smoking and alcohol consumption	-0.40	-0.59, -0.20	0.001
Model 3 : Model 2 + 25(OH)D, 25(OH)D batch	-0.40	-0.59, -0.22	<0.001
Model 4 : Model 3 + CRP	-0.30	-0.60, -0.10	0.01

*Relative percent change in LTL (T/S ratio) per unit increase in body mass index.

There was no interaction observed between 25(OH)D and BMI in associations with LTL (p -value=0.55). In addition, there was no interaction of either exposures [25(OH)D, BMI] with LTL by sex or oral contraceptive (subsample analysis, women only, all p -values \geq 0.39).

5.1.8 Summary and discussion

The findings of the study did not demonstrate an association between serum 25(OH)D and LTL in young adults. However, this study contrasts with two other previous findings (338, 349). A study from United Kingdom on 2,160 female twins (age: mean=49.4 years) examining 25(OH)D-LTL associations has reported an association between 25(OH)D-LTL after adjusting for age, the season of blood sampling, menopausal status, physical activity and family structure (349). However, this study has not allowed for an adjustment of other potential confounders including BMI, socioeconomic status, smoking, alcohol and CRP concentration (349). Another study conducted in 1,227 female nurses from the United States (mean age=59 years) has reported a positive association between 25(OH)D-LTL after adjusting for age, smoking, BMI and physical activity (338). When compared with previous investigations, our study was performed on 5,096 participants which included both men and women and the large sample size of our population meant that we are better powered to detect a magnitude of association between 25(OH)D and LTL, as those reported from study in women alone (338). Power analysis when compared with previous studies (338, 349) demonstrated that the effect size was large and consistent enough to detect an association (343). However, it is not clear why our findings differ from other previous observations (338, 349).

This study has included both sexes, but there were no difference in association between 25(OH)D-LTL by sex. One plausible reason for a null observation between 25(OH)D and leukocyte telomere length may be that the population is of younger age (mean age=31 years) in comparison with previous studies (338, 349). Furthermore, if the previous findings were not by chance, we may hypothesise that the association between 25(OH)D-LTL may emerge later in the life course.

This study has also extended the previous investigation, which tested the cross-sectional association of BMI with LTL in NFBC1966 (205). Here, the potential mediation of 25(OH)D and hs-CRP in the association of BMI-LTL were examined. The negative association between BMI and LTL observed in our study were consistent with some previous studies, but not with all examined to date (350). The partial attenuation of BMI-LTL association by adjustment of C-reactive protein

suggested that, inflammation could partly mediate the association between adiposity and potentially increased telomere degradation. Furthermore, adjustment with CRP provides information on only one aspect of the inflammation involved rather than the contribution of multitude of inflammatory pathways that may link adiposity with telomere length. Future studies are required to examine the BMI-LTL association taking into account wider contribution of other inflammatory markers.

5.2 Body mass index, 25(OH)D and inflammatory biomarkers (Study III)

The association of BMI and 25(OH)D with inflammatory biomarkers and the mediation through 25(OH)D in the association of BMI with inflammatory biomarkers were investigated in the Study III.

5.2.1 Descriptive analyses

Mean BMI and 25(OH)D concentrations of the participants was 24.8 kg/m² (95% CI: 24.7–25.0) and 50.3 nmol/L (95%CI: 49.8–50.7), respectively. Table 19 shows the characteristics of the study population. The sample size is smaller (N=3,586) when compared to study I and II. Major exclusion criterion in Study III was women using oral contraceptives (N=607) and the other criterion is listed in section 4.5.2.

The inflammatory biomarker measurements differed between men and women ($p<0.05$), except for hs-CRP concentrations (Table 19). All the other biomarker concentrations were lower in women ($p<0.005$), except IL-17 which was lower in men ($p<0.005$) (Table 19).

Table 19. Plasma inflammatory biomarkers concentrations in the study population.

Inflammatory biomarker	Total population (n=3,586)	Males (n=2,073)	Females (n=1,513)	<i>p</i> -value ¹
IL-17 (pg/ml)	56.2 (49.4–63.0)	56.1 (50.2–62.0)	56.3 (42.3–70.2)	0.006
IL-1 α (pg/ml)	73.5 (63.4–83.7)	89.0 (73.4–104.7)	52.3 (41.6–63.1)	<0.001
IL-1 β (pg/ml)	6.7 (5.1–8.2)	7.8 (5.1–10.5)	5.1 (4.2–5.9)	0.001
IL-6 (pg/ml)	19.0 (14.7–23.2)	21.0 (17.1–24.8)	16.2 (7.6–24.8)	<0.001
TNF-alpha	9.5 (8.4–10.6)	10.8 (9.0–12.6)	7.8 (7.0–8.6)	<0.001
IL-1RA(pg/ml)	67.9 (52.5–83.2)	78.1 (55.3–100.9)	53.8 (35.2–72.3)	<0.001
IL-4 (pg/ml)	30.4 (25.8–35.0)	35.7 (28.4–43.0)	23.1 (18.7–27.4)	<0.001
IL-8 (pg/ml)	38.8 (33.7–44.0)	39.9 (36.7–43.1)	37.3 (25.9–48.7)	<0.001
IP-10/CXCL10 (pg/ml)	434.7 (422.4–447.0)	448.7 (432.4–465.0)	415.5 (396.9–434.1)	<0.001
MCP-1/ CCL2 (pg/ml)	319.6 (315.5–323.8)	349.4 (343.8–355.1)	278.9 (273.4–284.3)	<0.001
sCD40L/CD154 (pg/ml)	545.5 (525.0–565.9)	588.9 (560.8–616.9)	486.0 (456.7–515.3)	<0.001
sICAM (ng/ml)	147.2 (145.3–149.1)	156.6 (154.0–159.3)	134.2 (131.5–136.9)	<0.001
sVCAM (ng/ml)	1692.3 (1679.5– 1705.1)	1766.8 (1750.0– 1783.7)	1590.3 (1571.8– 1608.7)	<0.001
activePAI-1 (ng/ml)	22.2 (21.2–23.1)	26.2 (24.8–27.5)	16.7 (15.7–17.7)	<0.001
hs-CRP (mg/L)	1.16 (1.11–1.21)	1.15 (1.09–1.22)	1.18 (1.10–1.26)	0.10
Alpha-1-acid glycoprotein (AGP) (mmol/L)	1.36 (1.35–1.37)	1.39 (1.38–1.40)	1.32 (1.31–1.33)	<0.001

¹*p*-value by Student's *t*-test for normally distributed variables and Wilcoxon Mann-Whitney U-test for non-normally distributed variables.

5.2.2 Correlation of inflammatory biomarkers

Figure 20 shows the dendrogram for the clustering pattern and the Pearson correlation coefficients of inflammatory biomarkers. Four clustering patterns of inflammatory biomarkers were observed. Cluster 1-all interleukins and TNF- α ; cluster 2-adhesion molecules (sICAM-1, sVCAM-1); cluster 3-acute phase proteins (activePAI-1, hs-CRP, AGP); and cluster 4-chemokines/CD154 (IP-10/CXCL10, MCP-1/CCL2, sCD40L/CD154).

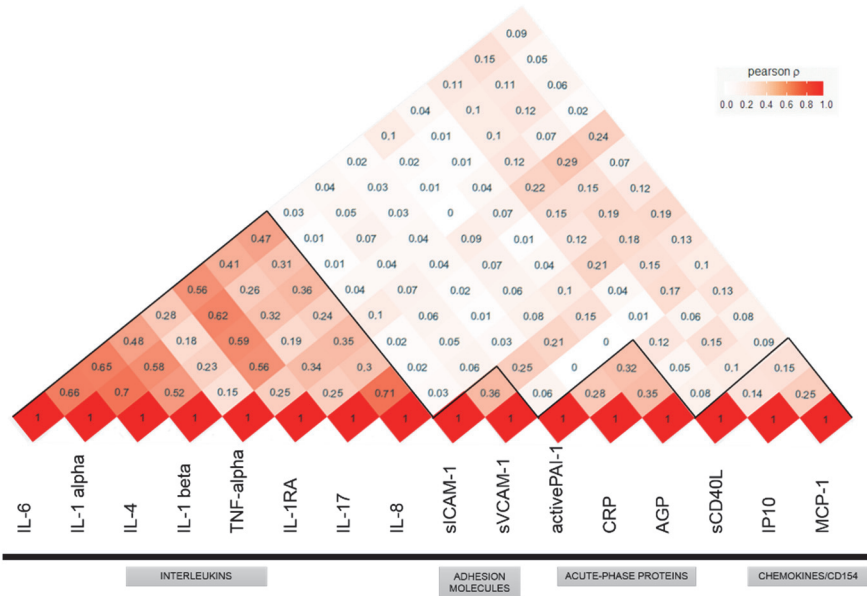


Fig. 20. Correlation structure of inflammatory biomarkers (Pearson correlation analysis).

5.2.3 Association between BMI and inflammatory biomarkers

Figure 21 shows the results of the association between BMI and inflammatory biomarkers analysed by multivariable linear regression analyses. In the fully adjusted model (model 3, FDR corrected) BMI was positively associated with IL-6 ($\beta=0.05$; 95% CI: 0.02, 0.09) and IL-1RA in cluster 1 ($\beta=0.06$; 95% CI: 0.03, 0.09); sICAM-1 ($\beta=0.12$; 95% CI: 0.09, 0.15) in cluster 2; activePAI-1 ($\beta=0.41$; 95% CI: 0.38, 0.44), CRP ($\beta=0.39$; 95% CI: 0.36, 0.43) and AGP ($\beta=0.27$; 95% CI: 0.24, 0.30) in cluster 3; and with sCD40L ($\beta=0.04$; 95% CI: 0.008, 0.07), IP-10 ($\beta=0.09$; 95% CI: 0.05, 0.12) and MCP-1 ($\beta=0.03$; 95% CI: 0.002, 0.07) in the cluster 4. Only sVCAM-1, in cluster 2 was negatively associated with BMI ($\beta=-0.03$; 95% CI: -0.07, -0.002).

5.2.4 Association between 25(OH)D and inflammatory biomarkers

Figure 21 shows results from the multivariable regression analysis of association between 25(OH)D and each biomarker. In the fully adjusted model (model 3, FDR corrected) we observed inverse association between 25(OH)D and IL-8 ($\beta=-0.07$;

95% CI: -0.11, -0.04), sICAM-1 ($\beta=-0.05$; 95% CI: -0.08, -0.008), CRP ($\beta=-0.04$; 95% CI: -0.08, -0.004) and AGP ($\beta=-0.09$; 95% CI: -0.13, -0.06). The association between 25(OH)D and inflammatory biomarkers was further tested with adjusted for BMI (Study III; supplementary table 3). We did not observed any interaction between 25(OH)D and BMI therefore fulfilling the criteria for mediation analyses (data not shown).

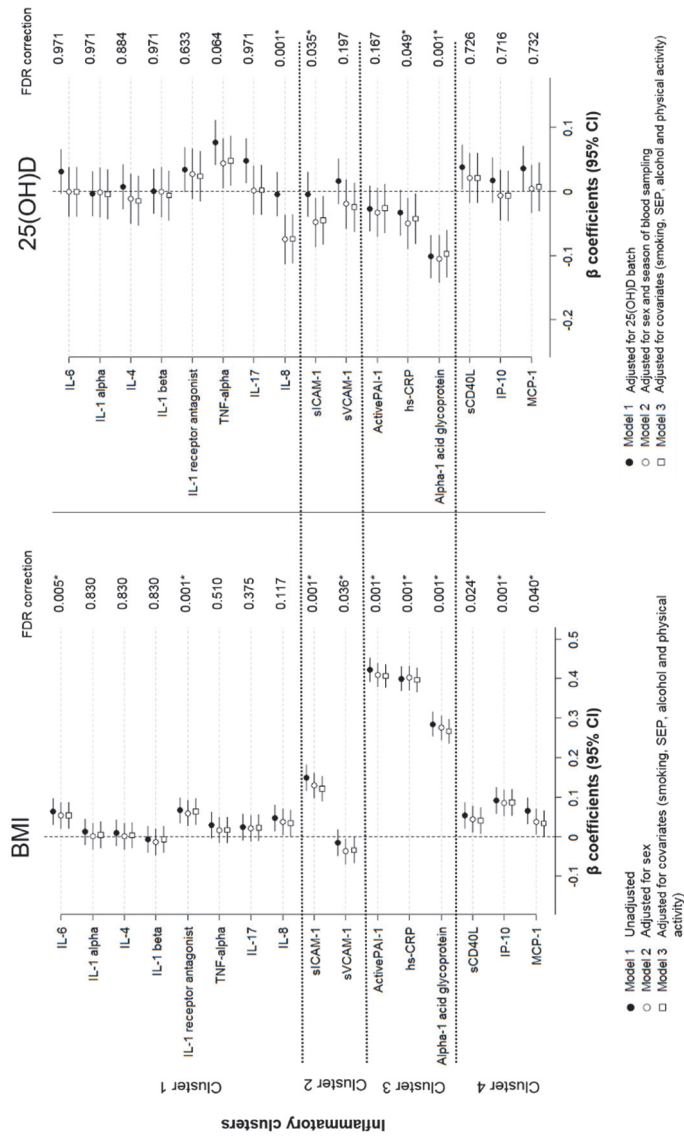


Fig. 21. Association of both BMI and 25(OH)D with inflammation. The results are presented as β coefficients and 95% CIs. Correction of p -values was made by BH-FDR approach for both BMI and 25(OH)D. *indicates statistical significance after multiple testing.

5.2.5 Mediation analyses

Table 20 shows the mediation analysis results. Multivariable regression analyses results demonstrated positive association between BMI and inflammatory biomarkers and inverse association between 25(OH)D and biomarkers. Three biomarkers sICAM-1, hs-CRP and alpha-1-acid glycoprotein fulfilled the criteria for mediation analysis as shown in Figure 17 (see section 3). The steps fulfilling the criteria for the Baron and Kenny mediation analyses are described in detail in section 8, Appendix 8.6.

Table 20. Mediation analyses of 25(OH)D in the relationship between BMI and inflammatory biomarkers, sICAM-1, hs-CRP and alpha-1-acid glycoprotein¹.

Inflammatory biomarkers	β_c^2 Total effect	β_a^2 BMI to Vitamin D	β_b^2 Vitamin D to inflammation	$\beta_c'^2$ Direct effect	% mediation (indirect)	Sobel test (p -value) ³
sICAM-1 (ng/ml)	0.12 (0.09, 0.15)	-0.05 (-0.08, -0.02)	-0.04 (-0.07, -9.7x10 ⁻⁶)	0.12 (0.09, 0.15)	1.5	0.08
hs-CRP (mg/L)	0.39 (0.37, 0.43)	-0.05 (-0.08, -0.02)	-0.02 (-0.05, 0.02)	0.39 (0.37, 0.43)	0.2	0.4
Alpha-1-acid glycoprotein (mmol/L)	0.27 (0.24, 0.30)	-0.05 (-0.08, -0.02)	-0.08 (-0.11, -0.04)	0.26 (0.23, 0.29)	1.5	0.006

¹The results are expressed as beta coefficients and 95% CI.

²All the models are adjusted for covariates sex, the season of blood sampling, 25(OH)D batch, alcohol consumption, SEP, physical activity and smoking status.

³Soble-test p -value for mediation.

Body mass index was inversely associated with 25(OH)D ($\beta_a=-0.05$, $p=0.0005$). BMI was positively associated with sICAM-1 ($\beta_c=0.12$ $p=<0.001$) and alpha-1-acid glycoprotein ($\beta_c=0.27$; $p=<0.001$). The association of BMI with AGP ($\beta_c'=0.26$, Sobel p -value=0.006) was partly reduced when adjusted for mediator. Similarly, the association of BMI with sICAM-1 ($\beta_c'=0.12$, 95% CI: 0.09, 0.15, Sobel p -value=0.08) was also reduced when adjusted for mediator. The association of BMI with CRP was marginally reduced when adjusted for mediator, although it was not statistically significant (p -value>0.05).

5.2.6 Summary and discussion

The key findings and more discussion that is detailed will be in article III. The main strengths and weakness of all Studies I–IV will be discussed in the next chapter 6 (general discussion).

Inflammatory biomarkers: The inflammatory biomarkers correlated with each other forming four molecular clusters that organised into interleukins, adhesion molecules, acute-phase proteins and chemokines. The clustering pattern of inflammatory biomarkers observed in our study was in line with their biological response or organisation which occurs during an inflammatory stimulus.

BMI and inflammatory biomarkers: BMI alone was positively associated with nine inflammatory biomarkers from all the molecular clusters suggesting an inflammatory load in the circulation. In addition, the magnitude of the association between BMI and the inflammatory biomarkers remained robust, taking into account adjustment for multiple covariates (sex, SEP, alcohol, physical activity, smoking). The inflammatory clusters we delineated were found independent from each other. This may suggest that at least four independent molecular and, or, organ specific pathways are related to BMI. For example, cytokine TNF- α may be directly related to BMI-related insulin resistance pathway (243) or in conjunction activate the production of other inflammatory mediators (IL-6, MCP-1 and acute-phase proteins) through involvement of transcriptional mediated molecular pathways.

25(OH)D and inflammatory biomarkers: In contrast, 25(OH)D was only associated with certain inflammatory biomarkers in three of the observed clusters. After adjusting for environmental, lifestyle and technical (batch effects) covariates, IL-8, sICAM-1, CRP and AGP was inversely associated with 25(OH)D supporting its immunomodulatory role. The observed results were in line with another study which reported dose-dependent downregulation of IL-8 secretion by 25(OH)D co-treatment (322). Furthermore, 25(OH)D was reported to impact the NF- κ B and MAPK signalling pathways resulting in the downregulation of biomarkers, IL-8 and sICAM-1 (319-322).

Observations from RCTs: It has been suggested that serum 25(OH)D status may modulate the inflammatory profile in young adults with obesity because of vitamin D's role in immunomodulation (351). However, BMI was reported to be causally associated with lower 25(OH)D status and vitamin D reduction in relation to higher BMI might contribute to increased BMI-related inflammation (294). In this case, vitamin D supplementation might be required in obese subjects to replete both lowered 25(OH)D concentration and decrease the BMI-associated inflammation

(352). However, RCTs investigating vitamin D supplementation in adults with overweight or obesity have reported little to no evidence on the effects on inflammatory status (Study III: see supplementary Table 1a and b) (353–355).

Analysing the mediation: The measured estimates for IL-8, sICAM-1, CRP and AGP, showing opposed effect between BMI and vitamin D, supported the hypothesis of mediation of the effect of BMI on these inflammatory factors by the reduction of vitamin D. The mediation analysis suggested that the reduction of serum 25(OH)D associated with a high BMI could mediate a small part of the effect. The mediating effect was, however, very small, i.e. only 1.5% of the effect of BMI suggesting that very high concentration of vitamin D would be needed to reduce the effect of BMI. A possible explanation for the lack of mediation between 25(OH)D and CRP might be because of the low mean concentration of 1.16 mg/L in our study, which is notably smaller when compared to earlier studies (356, 357).

In conclusion, the complex association between BMI and the inflammatory load suggesting that obesity may influence multiple independent inflammatory pathways was described in detail. The current evidence from this study does not support the hypothesis that a low vitamin D status co-occurring with obesity may causally aggravate the inflammatory status. We acknowledge some limitations from our finding that warrant replication and further studies. It is noteworthy that 55 registered trials on vitamin D supplementation in obesity are still on-going and we recommend to analyse in greater details the inflammatory response for IL-8, sICAM-1, and AGP.

6 General discussion

In this chapter, the findings from Studies I, II, III and review (IV) will be discussed taking into account their strengths and limitations as to the study material and methodology used.

6.1 Summary of the results and discussion

Review of literature: In summary, the review of literature (paper IV) addressed the importance of vitamin D during pregnancy and the regulatory role of vitamin D in inflammation. Table 9 mentioned in section 2.4.12 has described the reported associations between 25(OH)D and inflammation from epidemiological studies. In addition, Table 9 has been updated in the thesis with recent studies that examined the above-mentioned association. The review recognised the gaps regarding the association between vitamin D and inflammation reported in multiple epidemiological studies. This formed as a preliminary step in investigating the association between vitamin D and inflammatory biomarkers in NFBC1966 (Study III).

Study I: In our population, 3.3% of the participants were vitamin D deficient (≤ 30 nmol/L), 24.2% were insufficient (30–50 nmol/L), and 71.5% (≥ 50 nmol/L) were sufficient with the criteria of optimal status defined by IOM. The information about the vitamin D isoforms, 25(OH)D₂ and 25(OH)D₃ was collected using the gold standard technique. LC-MS/MS is considered as the reliable reference method because of its ability to distinguish vitamin D isoforms, low batch-to-batch variation and lower limit of detection (measure even the smallest concentration) (64). Our study was performed before the introduction of VDSP (2010) and internal Standard Reference Materials for serum 25(OH)D measurement (2012) (68). However, the laboratory has participated in the routine DEQAS evaluations during 25(OH)D measurement which is recognised by the VDSP as one of the accredited external quality assessment scheme (68).

Multiple determinants (environmental, anthropometry, lifestyle, socioeconomic, and sex-specific) were examined in relation to vitamin D isoforms. The differential association of the season of blood collection with vitamin D isoforms has been replicated in our study (348). Oral contraceptives associated with increased concentrations of both vitamin D isoforms. However, the marginal increase in D₂ and D₃ concentrations has little to no clinical significance. Previous findings using the same data from NFBC1966 has reported an increase in

inflammatory biomarker (CRP) in women who used oral contraceptives (142). A study which investigated the association between 25(OH)D and CRP has reported nil association, when OCP was adjusted in the analysis (347). These results suggest analysing oral contraceptives in relation to inflammation and vitamin D simultaneously in future investigations. In addition, further work is needed to investigate how estrogens (post/perimenopausal hormone therapy) may affect 25(OH)D concentration and inflammation over the life course.

The previously reported risk factors (low daylight period, being obese ($\text{BMI} \geq 30 \text{ kg/m}^2$), and low physical activity levels) of low vitamin D status were also demonstrated in our study. The factors such as heavy drinking, oral contraceptive were demonstrated to associate with being on a higher 25(OH)D tertile. The plausible mechanisms behind all these associations have to be examined in future investigations.

Study II: Telomere shortening is an independent risk factor for metabolic health outcome. When tested whether serum 25(OH)D concentration may prevent telomere attrition and maintain longer LTL, 25(OH)D was not associated with LTL in young adulthood. The study included adjustment for multiple confounders, the season of blood sampling, age, sex, 25(OH)D batch, BMI, SEP, physical activity, diet, smoking, alcohol consumption and use of oral contraceptives. Previous investigations did not correct for the season of blood collection (338) or other confounders. Despite no association demonstrated between 25(OH)D and LTL, it is plausible that these association may be more pronounced with advancing age as reported from previous investigations in middle-aged (mean age: 49 years) (349) and old age adults (mean age: 59 years) (338). In addition, the inverse association of BMI with telomere length was also demonstrated to be independent of 25(OH)D concentration. When examined whether inflammation may mediate the association of both BMI and 25(OH)D with leukocyte telomere length, CRP modestly mediated the association of BMI with LTL; no association was demonstrated with 25(OH)D-LTL. When compared to previous investigations, our study was performed on 5,096 participants which included both men and women and the large sample size of our population meant that we are better powered to detect a magnitude of association between 25(OH)D and LTL, as those reported from study in women alone (338). Power analysis revealed that our effect size was large enough and consistent enough, to be really important to detect an association (343). However, it is not clear why our findings differ from other previous observations (338, 349).

Study III: The inflammatory biomarkers analysed in the present study correlated with each other forming four molecular clusters that organised into

interleukins, adhesion molecules, acute-phase proteins, and chemokines. The clustering pattern of biomarkers we observed in our study was in line with their biological response or organisation which occurs during an inflammatory stimulus. BMI alone were positively associated with nine biomarkers, IL-6, IL-1RA (interleukins), sICAM-1 (adhesion molecules), activePAI-1, hs-CRP, AGP (acute-phase proteins), and sCD40L, IP-10, MCP-1 (chemokines) suggesting an inflammatory load in the circulation. The inflammatory clusters were found independent from each other in our study. This may suggest that at least four independent molecular and, or, organ specific pathways are related to BMI. The magnitude of the association between and inflammatory biomarkers remained robust taking into account the adjustment for multiple covariates (sex, smoking status, alcohol consumption, physical activity, and SEP).

Serum 25(OH)D was only associated with certain inflammatory biomarkers in three of the observed clusters. After adjusting for environmental, lifestyle and technical (batch effects) covariates, IL-8, sICAM-1, CRP and AGP was inversely associated with 25(OH)D supporting its immunomodulatory role. The observed results were in line with another study which reported dose-dependent downregulation of IL-8 secretion by 25(OH)D co-treatment (322). Furthermore, 25(OH)D was reported to impact the NF- κ B and MAPK signalling pathways resulting in the downregulation of biomarker, IL-8 (319–322).

It has been suggested that serum 25(OH)D status may modulate the inflammatory profile in young adults with obesity because of vitamin D's role in immunomodulation (351). However, BMI was reported to be causally associated with lower 25(OH)D status and vitamin D reduction in relation to higher BMI might contribute to increased BMI-related inflammation (294). In this case, vitamin D supplementation might be required in obese subjects to replete both lowered 25(OH)D concentration and decrease the BMI-associated inflammation (352). However, RCTs investigating vitamin D supplementation in adults with overweight or obesity have reported little to no evidence on the beneficial effects on inflammatory status (Study III: see supplementary Table 1a and b) (353–355). In mediation analyses, 25(OH)D was found to mediate a small part of the effect of BMI with biomarker, alpha-1-acid glycoprotein. The magnitude of the mediation effect was, however, too small, *i.e.* only 1.5% of the effect of BMI suggesting that a very high concentration of vitamin D would be needed to reduce the effect of BMI. However, there are no comparable studies to replicate these findings. It is noteworthy that 55 registered trials on vitamin D supplementation in obesity are still on-going and we recommend analysing in greater detail the inflammatory

response for IL-8, sICAM-1, and AGP to provide a better interpretation on the role of vitamin D in obesity.

6.2 Strengths of the study

This thesis was based on the data from a population-based birth cohort study, the Northern Finland Birth Cohort 1966. NFBC1966 data has been collected from prenatal period until the 31 years of age (1997). The phenotypic data have been collected extensively at every stage of the data collection. In addition, the blood samples collected from participants at 31 years were used to examine comprehensively the biomarkers, for example, 25(OH)D, LTL and inflammation. The clinical examination data has been collected by well-trained nurses with proper attention to well-standardised protocols of data collection. The serum and plasma samples were stored at -70°C until the analysis.

NFBC1966 data could be considered as a nationally representative sample of the young adult population living in Finland at the time of the measure. Figure 22 shows the prevalence of overweight and obesity reported in the latest study (358) from NFBC1966. The prevalence of obesity follows the similar trend as reported by the National Institute for Health and Welfare (1978-2016, THL, Finland) thus showing that overweight/obesity increases with age (section 2.4.2, Figure 8).

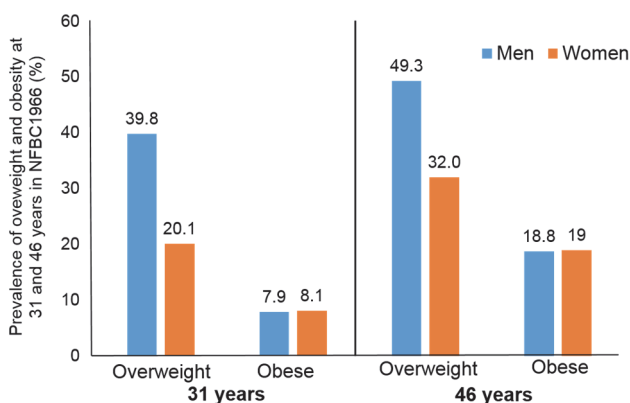


Fig. 22. Illustration of overweight and obesity prevalence in NFBC1966 at two time points, 31 years (1997) and 46 years (2012). The obesity prevalence have thus doubled in men and women, respectively. The NFBC1966 data represents similar prevalence and increase in obesity rates as reported from the National Institute for Health and Welfare (1978–2016, see section 2.4.2, Figure 8) (223).

Appropriate public health strategies are required to prevent the health risks and health care costs associated with overweight and obesity. The THL data were collected using postal questionnaire from randomly selected participants (population register, 20–64 year olds) residing in all regions of Finland from 1978–2016 (223).

Comparison of our data with fortification (First and second): Serum 25(OH)D concentration examined in this thesis were collected (1997) before the introduction of nationwide fortification of vitamin D in Finland (75). The data thus provides an overview of the naive vitamin D status in Northern Finland prior implementing any fortification strategy. The serum 25(OH)D concentration reported in our study was observed to be higher than in the study performed during 2000–2001 (68.4 vs. 45.3 nmol/L, mean age:50.7 y) (145). It is unclear why our average serum 25(OH)D concentration was higher at the time of measure when compared to later studies (145). However, a study from Norway (N=2,505; age: 19-55 y) from similar latitude (64°N) has reported average serum 25(OH)D to be 59 nmol/L, which is comparable to our population (115). We are unable to discount the possibility of storage-related interference by unknown metabolites in our samples affecting measures obtained by LC-MS/MS. A possible source of error may be the use of in-house calibrators and internal standardisation in the LC-MS/MS procedure, which, in part may account for a positive bias (359). Our study was performed before the VDSP (2011) and introduction of internal standard reference materials in vitamin D measurement (2012). More recent studies (76, 77) have reported the mean concentration of serum 25(OH)D to increase from 48 to 65 nmol/L between years 2000 and 2011 (77). Now the dietary intake by fortification with vitamin D (D3) has been increased from 0.5 to 1.0 µg per 100 g for all fluids and 10 to 20 µg per 100 g of spreadable fats at two time points in 2003, and its revision in 2010 (75). However, the comparison between studies is difficult due to various analytical issues and changes in quality assessment scheme. In addition, the populations are different and differences in the dietary intake might explain a part of the differences (76, 77). The latest follow-up data collection for the NFBC1966 at 46 years was completed in 2014. NFBC1966 longitudinal data will be a novel tool to follow-up the same participants 15 years apart (31–46 years), which may show the success of fortification in maintaining/increasing 25(OH)D concentration. In Study I, 25(OH)D₃ contributed to 95% of total serum 25(OH)D concentration. The increase in vitamin D status after the second degree of fortification shows the efficacy of D3 in increasing the serum 25(OH)D concentration (77).

The serum samples used for 25(OH)D measurement and plasma samples used for biomarker measurement have been stored at -70°C for 11 years before laboratory analysis (2008–2009). A study by Hollis *et al.*, has reported that long term storage ($>10\text{y}$) of pooled human 25(OH)D internal controls led to no detectable degradation of 25(OH)D, and concentrations of 2(OH)D in frozen serum have been shown to be stable for $\leq 24\text{ y}$ (360). In addition, serum 25(OH)D concentration was reported to remain intact when serum samples were frozen at -70°C , although they underwent multiple freeze-thaw cycles (361, 362). However, there was no previous freeze thaw cycles with both the serum and plasma samples used for 25(OH)D and biomarker measurement and was only thawed for the respective analysis, which ensures the reliability of the measurements.

The findings reported from a previous research article (348) on the differential association of the season of blood sampling with D2 and D3 concentrations were also replicated in our study population. In addition, rather than using the season as a categorical variable, the influence of season on 25(OH)D was also computed as a linear season-adjusted 25(OH)D variable and its association with LTL was assessed. However, we did not demonstrate any association between 25(OH)D in either (season-adjusted, season unadjusted or 25(OH)D cut-offs) and LTL. Previous investigation (338) which examined 25(OH)D-LTL association has reported a positive association, however, did not include adjustment for the season of blood collection. When compared to previous investigations, our study was performed on 5,096 participants which included both men and women and the large sample size of our population meant that we are better powered to detect a magnitude of association between 25(OH)D and LTL, as those reported from study in women alone (338). Power analysis revealed that our effect size was large enough and consistent enough, to be really important to detect an association (343). However, it is not clear why our findings differ from other previous observations (338, 349).

The review article IV reported the current evidence on the association between 25(OH)D-inflammation and their limitations. 25(OH)D was inversely associated with certain biomarkers IL-8, sICAM-1, CRP and AGP after adjustment for multiple covariates. No previous study has included adjustment for wide-range of covariates (review IV; see section 2.4.12, Table 9). Moreover, earlier studies which reported the association of vitamin D with inflammatory biomarkers were performed mostly on older adults, inclusion of wider age population samples, smaller population, and without adjustment for covariates (301). To our knowledge this is the first study (III) to investigate a comprehensive panel of inflammatory biomarkers in relation to 25(OH)D and BMI.

A complete case analyses procedure was used in the dissertation. The missing occurs only due to the exclusion criteria employed in each study, so no imputation analysis was performed. In Study III, the main source of attrition was exclusion on women using contraceptives (N=607). So, the distribution between the missing and non-missing data was examined to account for any differences between the groups in each study. The selected sample was representative of the data collected from all the participants at 31 years (see section 4.2). Potential confounders were investigated while designing the study and adjustment for confounders were performed in all the studies. The interaction of sex with exposures in relation to the outcome variables was also examined in all the studies. We found no interaction between sex x exposure and any of the outcomes. In addition, effect modification was also assessed as a basic criteria to perform the mediation analysis.

6.3 Limitations of the study

There are certain limitations in this study. In the Study I, information on the dietary intake of vitamin D-rich foods such as mushrooms, eggs and fish products were not quantifiable. This could have provided information on the contribution of diet, which is the main source of vitamin D during the winter period in Finland. The study also lacks data on the outdoor/indoor physical activity, a precise measure of UV-B exposure (summer months), sun protection habits and of supplement use. However, in the Health 2000 survey, the use of vitamin D supplements was 10% at that time of the examination, so before the year 2003 the use of supplements was not common in Finland unless physician advised it (145). Data on biochemical parameters such as serum calcium and PTH concentration would have added some information on the physiology of vitamin D, but these were not available in the present study.

The BMI-LTL association was not adjusted for the distribution of WBC cell types (neutrophils, basophils, eosinophils, monocytes, lymphocytes), which may affect telomere length. This is because LTL measured from peripheral leukocytes is calculated as an average comprising all the WBC cell types. Although the WBC subsets are reported to be correlated with each other, different WBC cell types have different telomere length (363). In addition, the distribution of different WBC cell types in circulation at the time of measure may confound the association between 25(OH)D-LTL and BMI-LTL association. For example, if a person is sick (acute illness), he/she is more likely to have greater proportion of neutrophils due to the natural defense mechanism. The distribution of immune cells may vary during a

defense mechanism. Neutrophils have been reported to have longer telomere length when compared to lymphocytes (363). If a person's blood sample is obtained during acute illness, he/she may have longer average LTL estimate, because of the increase in proportion of neutrophils due to illness in the circulation. Therefore, even though the participant may have an average longer LTL estimate when compared to other participant, they are prone to metabolic disease.

BMI and 25(OH)D was not associated with inflammatory markers, either TNF- α or IL-6 or with both as reported from previous studies (301). This may be because of the low circulating concentration of these biomarkers in plasma samples of the young adults. However, our biomarker concentrations were comparable to those reported in previous investigation (364). In addition, we have included adjustment for multiple covariates when compared to previous studies (301).

Cross-sectional design is used to potentially examine the association of multiple exposures and multiple outcomes at the particular time point. Cross-sectional designs are inappropriate to examine the causal relationships. In Study I, taking into account the risk factors associated with 25(OH)D there may be chances of reverse causality. There may be participants who have sun avoidance behaviour or involving in fewer outdoor activities that may lead to noticeable low vitamin D status. Residual confounding may have occurred while recalling and reporting the information in the self-reported questionnaire (for example, alcohol consumption, smoking during the past six months). Some individuals may have over reported or under reported their physical activity levels, dietary intake information which may have led to residual confounding. However, careful adjustment with multiple confounders may have decreased the risk of confounding overall.

7 Summary and implications

This dissertation included four publications. The review article IV reported the current evidence on the association between vitamin D and inflammation and their limitations in the general population. This formed as a preliminary step, and the same objective was tested in Study III. The Studies I, II and III provided novel results as well as replicated findings that were reported previously.

7.1 Summary

The main findings of the thesis resulting from Study I, II and III are summarised in Figure 23 A, B and C.

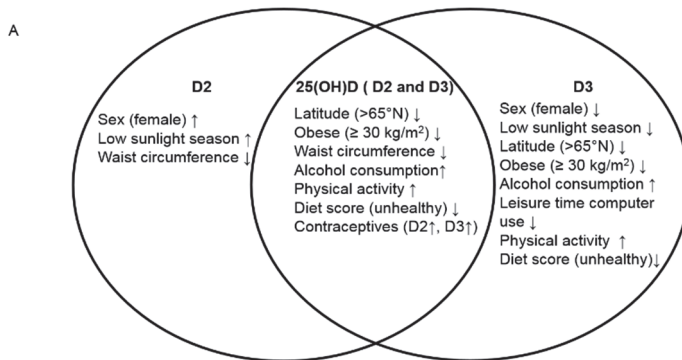


Fig. 23. (A). Summary of the determinants associated with D2, D3 and 25(OH)D concentrations in multivariable analyses.

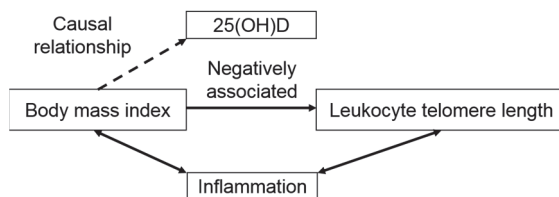


Fig. 23. (B). Body mass index was inversely associated with LTL, after adjustment for 25(OH)D, CRP and covariates (age, sex, SEP, physical activity, alcohol consumption, smoking, diet). There was no association between 25(OH)D and LTL in the young adulthood. (For aims and objectives see section 3, Figure 16.)

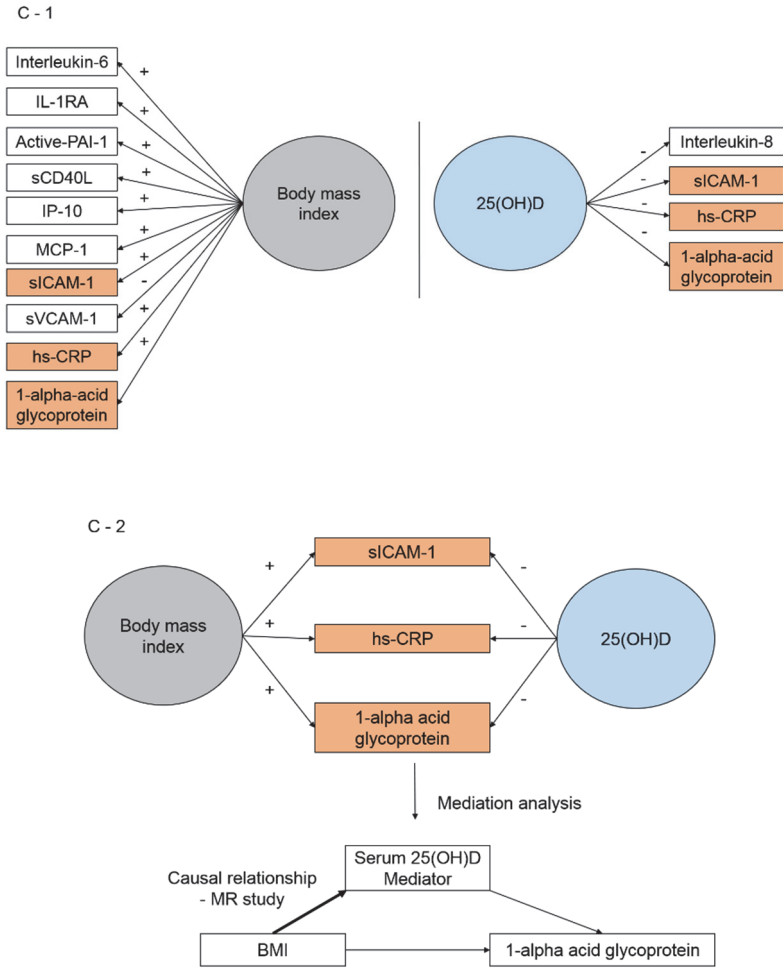


Fig. 23. (C-1). BMI was positively associated with nine inflammatory biomarkers from four inflammatory clusters (interleukins, adhesion molecules, acute-phase proteins and chemokines). 25(OH)D was inversely associated with biomarkers, IL-8, sICAM-1, hs-CRP and alpha-1-acid glycoprotein, that is, higher 25(OH)D, lower levels of inflammatory markers listed here. **(C-2).** BMI was positively associated with sICAM-1, hs-CRP, and alpha-1-acid glycoprotein concentrations. 25(OH)D was inversely associated with sICAM-1, hs-CRP, and AGP. In mediation analysis the association of BMI with alpha-1-acid glycoprotein is modestly mediated through lower 25(OH)D concentration. (For aims and objectives see section 3, Figure 17.)

7.2 Implications and further study interests

Vitamin D as an essential micronutrient can be obtained from natural source, sun (UV-B radiation) and through consumption of vitamin D-rich dietary sources.

Academic research: From an academic point of view, the research included in this thesis has addressed the association of environmental, anthropometric, lifestyle, socioeconomic and sex specific factors with vitamin D isoforms (D2 and D3). Particularly, the association of oral contraceptive with increase in D2 and D3 has been addressed for the first time from NFBC1966. However, the marginal increase in D2 and D3 has little to no clinical significance. Previous findings using the same data from NFBC1966 has reported an increase in inflammatory biomarker (CRP) and alteration in metabolic profiles in women who used oral contraceptives (142, 346). More clear understanding on the relationship between OCPs, inflammation and vitamin D are warranted in future investigations. The seasonal influence on D2 and D3 concentrations replicated in our study are also noticeable. The results may suggest safe sun exposure during summer months and dietary intake of vitamin D-rich sources during winter to maintain optimal serum 25(OH)D concentration. In Finland, only D3 is used for the fortification of food products in Finland from 2003. In addition, the supplements predominantly contain D3 rather than D2. The efficacy of fortification with D3 in increasing serum 25(OH)D concentration in Finland has been reported recently (76, 77). The efficacy of D3 in increasing/maintaining 25(OH)D concentration in the same study population could be tested in future in the longitudinal follow-up (31–46 years).

BMI was inversely associated with telomere length. In addition, the association of BMI with LTL was modestly attenuated, when adjusted for C-reactive protein. C-reactive protein concentration may mediate the association between BMI and telomere length (219). However, 25(OH)D was not associated LTL in young adult population. It is plausible that these associations may be pronounced in middle age adults and could be tested longitudinally in NFBC1966.

BMI was associated positively with molecular clusters (interleukins, adhesion molecules, acute phase proteins and chemokines). The identification of inflammatory biomarkers related to BMI may act as candidates in future epidemiological studies to evaluate the effect of inflammation or inflammatory pathways contributing to BMI related metabolic health outcomes. 25(OH)D was inversely associated with certain biomarkers after correcting for multiple confounders. The mediating effect of reduced 25(OH)D in BMI associated inflammation (AGP) was very small, *i.e.* only 1.5% of the effect of BMI suggesting

that very high concentration of vitamin D would be needed to reduce the effect of BMI. In addition, RCTs investigating vitamin D supplementation in adults with overweight or obesity have reported little to no evidence on the effects on inflammatory status (Study III: see supplementary Table 1a and b) (353-355). The results warrants for replication in further studies.

Non academic research: There are multiple cut-offs (IOM, endocrine society etc.) available to define optimal 25(OH)D status. Understanding the influence of various factors on vitamin D status will help determine optimal vitamin D cut-offs in relation to metabolic health outcomes.

Public health: Study I has also demonstrated the risk factors for low vitamin D status, the season of blood sampling in winter/spring, living in northern latitudes, increased waist circumference, low physical activity levels and unhealthy diet. The public health aspects of the thesis include promotion of safe sun exposure during summer months to allow natural vitamin D synthesis on the skin. Finland, which lies above 60°N, where vitamin D is not synthesised during the long winter period, dietary intake of vitamin D-rich sources such as oily fish, fortified food products (see section 2.1.3, Table 1), and supplementation would maintain optimal vitamin D status. This information is implemented in the public health policy of Finland, which suggest that the data from Study I is in support with the recommendations.

Results from Study I and III suggest that BMI plays an important role in the reduction of vitamin D status as well as enhance inflammatory biomarkers. Although recent studies from Finland has reported the increase in average serum 25(OH)D concentration, the studies did not provide any additional information on the factors influencing vitamin D such as BMI, sun exposure, lifestyle and other factors (76, 77). Individuals who have a BMI \geq 30 kg/m² (obese) could consult a physician to assess their vitamin D status and for additional vitamin D intake, if necessary. The additional intake of vitamin D in obese was suggested in the Endocrine society guidelines (55), but not in any other recommendations. With respect to BMI-inflammation-vitamin D, it is noteworthy that 55 registered trials are still on-going on vitamin D supplementation in obesity and we recommend to analyse in greater details the inflammatory response for IL-8, sICAM-1, and AGP. The results may provide a better interpretation on the role of vitamin D supplementation in obesity-induced inflammation.

Social Impact: Earlier findings from NFBC1966 has reported reduced risk of type I diabetes and schizophrenia with vitamin D supplementation during the first year of life (21, 22). In Study III, 25(OH)D was inversely associated with certain

inflammatory biomarkers. This demonstrated the effect of vitamin D in the immune system apart from the classical calcitropic and skeletal function of vitamin D.

The human genome consists of 2776 binding sites and 229 genes that are activated with respect to calcitriol stimulation (52). This suggests the role of vitamin D in regulation of molecular pathways beyond its role in bone metabolism. However, there is no concrete evidence available yet in the scientific literature on the functions of vitamin D in metabolic health beyond the calcium homeostasis.

The present thesis adds to the current scientific literature regarding the factors associated with vitamin D isoforms (D2, D3), the risk factors for vitamin D deficiency, the association of vitamin D and BMI with leukocyte telomere length and inflammation, and tested the possible role of vitamin D as a modifiable target to reduce obesity induced inflammation.

Future prospects: Study II did not account for adjustment of multiple inflammatory biomarkers as examined in Study III. This is because CRP was the only available marker during the BMI-LTL and 25(OH)D-LTL investigation. The analysis of other biomarkers than reported here from the same plasma samples was performed recently. In the future, we are aiming to analyse the association between BMI and LTL taking into account adjustment for biomarkers mentioned in Study III.

Replication, causal relationship: The efficacy of fortification in maintaining/increasing serum 25(OH)D concentration could be tested in the NFBC1966 longitudinal data 15 years apart (1997, 2012). The relationship between oral contraceptives, inflammation and vitamin D and its association over the life course could be tested. 25(OH)D was not associated LTL at 31 years. The plausible association between 25(OH)D and LTL in middle age adults (46 years) as reported from other investigations could be tested. In addition, future studies are required to replicate the results on the association between 25(OH)D, BMI and inflammation and could also be tested longitudinally follow-up in NFBC1966.

The latest follow-up data collection for the NFBC1966 at 46 years was completed in 2014. The plan for analysing vitamin D and other biochemical parameters are currently under way. NFBC1966-31 to 46 years data collection will be a novel tool to analyse the longitudinal transition of vitamin D and its relation to metabolic health from young adulthood to middle-aged adults.

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Appendices

The detailed analytical procedure of all the assays mentioned in Chapter 4 will be described below.

7.3 Appendix 1

Table 1. Description of the key biological terms.

Biological terms	Description
Mitosis	Part of cell-cycle mechanism by which a cell that has undergone replication of its chromosomes separates the chromosome into two identical twins.
Senescence	Also known as biological ageing is a gradual deterioration of the living forms that could be either cellular senescence (cellular level) or the organismal senescence (whole organism).
Apoptosis	Process of programmed cell death that occurs with changes in cell morphology followed by death.
Telomere	Noncoding repetitive nucleotide sequences that are located at the chromatid ends of the chromosome.
Telomerase	Ribonucleoprotein, enzyme that adds DNA sequences to the 3' region of the telomeres and maintains the length of the telomere.
Telomere attrition	The process by which the ends of the chromosomes become shorter due to factors such as oxidative stress, inflammation, lifestyle factors (smoking) among others.
Oxidative stress	Accumulation of reactive oxygen species (ROS) within the cells; an abnormality of the normal scavenging process which results in imbalance between increased ROS and the body's ability to detoxify the toxic effects resulting in damage of cell constituents, lipids, DNA and proteins.

7.4 Appendix 2

Mendelian Randomisation study design

Mendelian randomisation (MR) is a statistical technique that uses genetic information to examine the causal inference of an exposure (X) to an outcome (Y) by using instrumental variables (IV; Z). The instrumental variable is a method of statistical estimation. Figure 1 shows the schematic representation of Mendelian randomisation approach used in examining the causality and direction of the association between BMI and 25(OH)D from Vimalleswaran KS *et al.*, 2013 (294).

The BMI-allele score were created using twelve BMI-related SNPs and 25(OH)D-allele score using two 25(OH)D-related SNPs reported from Wang *et al.* 2010 (157) (see section 2.2.6). The BMI allele score were positively associated with BMI and inversely associated with 25(OH)D [-0.06 %, per unit increase; 95% CI: -0.10 to -0.02; $p=0.004$] (294). 25(OH)D-allele score were strongly associated only with 25(OH)D, not with BMI. The study results are explained in more detail in section 2.4.11.

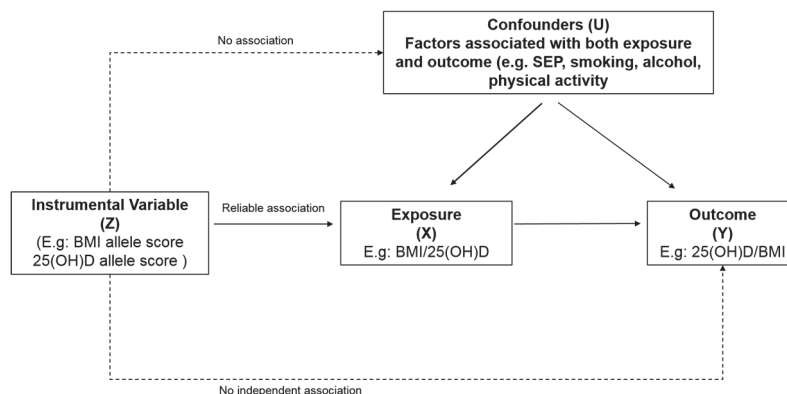


Fig. 1. Schematic representation of the Mendelian Randomisation approach. The three principles of MR are as follows: (1) IV (Z) must associate with the exposure (X), (2) IV (Z) must not associate with confounders (U) and, (3) No pathway from IV (Z) to the outcome (Y) without the exposure (X). Here, MR analysis was first used to assess the causal effect between BMI and 25(OH)D with BMI allele score (IV). Reciprocal MR was used to investigate the causal effect between 25(OH)D and BMI with 25(OH)D allele score.

7.5 Appendix 3

Blood sampling

During clinical examination at 31 years, overnight fasted blood samples (≥ 12 hours) were withdrawn between 8.00 AM and 11.00 AM from the antecubital vein in a sitting position. The blood samples were then stored in different sets of tubes designated for measurement of either serum or plasma sample measurements. The serum samples were stored in serum separating (gel) tubes and plasma samples were stored in Lithium-Heparin tube. 1.5 ml of serum or plasma sample was centrifuged, transferred into a cryotube for long-term deep freezing, labelled and

stored at -70°C until further analyses. The samples were then used for measurement of serum 25(OH)D and plasma inflammatory biomarkers, respectively. Total blood DNA was extracted from 5,753 individuals in EDTA tubes and was used to measure leukocyte telomere length. Aliquoting participant's samples, extraction of DNA by phenol-chloroform method, quality control and, bio banking were performed at the National Institute for Health and Welfare, Biomedicum Helsinki, Finland.

7.6 Appendix 4

Liquid chromatography tandem mass spectrometry (LC-MS/MS) system

- a) In 2008, the stored samples were thawed, 25(OH)D₂ and 25(OH)D₃ concentrations were measured by using a high-performance liquid chromatography-mass spectrometry technique (Elstree, Hertfordshire, UK).
- b) Waters SunFire C18 (3.5µm 100mm x 2.1mm id) analytical column fitted with a guard column was used for chromatographic separation. 10mm C18 guard column was fitted to improve column life.
- c) The temperature of the column was maintained at 40°C. A Waters Premier XE with a Z spray source, in Electrospray Ionization positive multiple reaction monitoring mode with a source temperature maintained at 130°C. Desolvation temperature was set at 250°C, gas flow of 950L/h, and argon collision gas set at 0.3mL/min was used for mass detection.
- d) The relative retention time for peak identification and peak area ratios with internal standardisation for quantitation was calculated using the QuanLynx software.
- e) Mobile phase A contained 2mmol/l ammonium acetate (VWR) in 0.1% formic acid (Fluka Chemical Company). Mobile phase B contained 2mmol/l ammonium acetate in methanol containing 0.1% formic acid.
- f) A binary step gradient was used to clean the column of any late eluting peaks. Elution of the vitamins was achieved using 84% B at 0.4 ml/min for 3.5mins then switching to 100% B for a further minute before returning to 84% B.
- g) Injection interval was set to 5.5 mins, allowing 1 min for re-equilibration of the column before the next injection. Solvent divert was used to allow data acquisition to take place between 1.2 and 3.5 mins.

h) Eluent was then introduced into a Waters Premier XE tandem mass-spectrometer fitted with an electrospray ionisation source. The sample tray area was maintained at 15°C.

i) Quantitation multiple reaction monitoring transitions were 401.1>383.2, 413.2>395.2 and 407.1>107.1 for 25(OH)D₃, 25(OH)D₂ and d6 25(OH)D₃, respectively.

j) Data was measured in four stages, first in March 2008 and the last in August 2009. Internal Quality Assurance samples were included at the front and back of each assay. The performance of the LC-MS/MS based on internal standards was fair with % CV for 25(OH)D₂ 12.1% at 9.2 nmol/l, 9.1% at 24.8 nmol/l, 4.8% at 52.8 nmol/l, and 3.9% at 158.5 nmol/l. For 25(OH)D₃ % CV was 15.5% at 6.1 nmol/l, 5.8% at 19.6 nmol/l, 4.3% at 58.1 nmol/l, and 3.6% at 124.5 nmol/l. The CV were less than 16% across the working range of the assay for 25(OH)D₂ and 25(OH)D₃. The quality and accuracy of the 25(OH)D analyses are validated on an ongoing basis by participation in the vitamin D External Quality Assessment Scheme (DEQAS, Elstree, Hertfordshire, UK). Participating laboratories receive a certificate if 80% of their results from the five quarterly samples are within 30% of the all-laboratory trimmed mean (ALTM). The values comparing the laboratory submitted samples to DEQAS and the DEQAS ALTM, identified one obvious outlier. The method showed <10% positive bias against DEQAS ALTM at the time of the measure (2008–2009).

k) Serum 25(OH)D₂ and 25(OH)D₃ (ng/ml) concentrations were finally measured from the LC-MS/MS system (ng/ml to nmol/L =ng/ml*2.496).

7.7 Appendix 5

MMqPCR reaction

1. The single copy gene reference used was Human beta-globin (hbg).
2. Duplicate qPCR reactions were carried out in a total volume of 12.5 µl. This comprised of 20 ng of template DNA or varying amount of reference DNA for standard curve, with final concentrations of 1x iQTM SYBR® Green Supermix (Cat no: 1708882, Bio-Rad Laboratories, Hemel Hempstead, UK), 900 nM of telg and telc primers, and 500 nM of single copy gene primers (hbgd and hbgu) (Table 2).

Table 2. Primer Sequence.

Primer	Nucleotide sequence (5'-3')
telG - F	5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3'
telC - R	5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3'
hbgU - F	5'-CGGCGGCGGGCGGCGGGCTGGGCGGAAATGCTGCACAGAATCCTTG-3'
hbgD - R	5'-GCCC GGCCCGCCGCGCCCGTCCC GCGGAAAAGCATGGTGCCTGTT-3'

3. Five serial dilutions of a single common reference sample (leukocyte DNA from a 42-year-old female) spanning 5–30 ng were run in triplicate on each plate, in addition to one or more control sample.

4. A qPCR master mix with the following quantities are prepared.

For 1 reaction	6.25 µl iQ™ SYBR® Green Supermix 2 µl primer mix 2.25 µl nuclease free distilled water
For a 384 well plate	2750 µl iQ™ SYBR® Green Supermix 880 µl primer mix 990 µl nuclease free distilled water

5. All qPCR reactions were run with CFX-real time PCR detection system (CFX384, Bio-Rad Laboratories) (Figure 2).

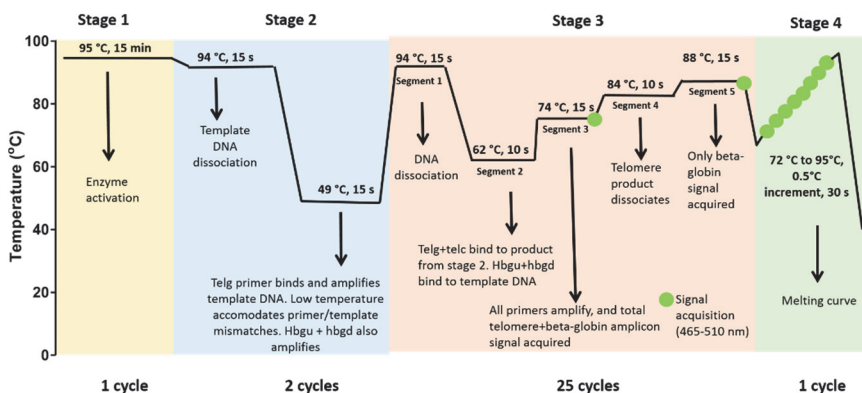


Fig. 2. PCR cycling profile. Schematic representation of the MMqPCR cycling profile, describing each stage. The cycles and the segments are marked as referenced on the CFX-real time PCR detection system (CFX384). The dark coloured circles represent fluorescence signal acquisitions.

6. The thermal cycling profile was initiated at 95°C for 15 minutes (enzyme activation). This was followed by 2 cycles at 94°C (15 seconds) and at 49°C (15 seconds). Following this, 25 cycles at 94°C (15 seconds), 62°C (10 seconds), 74°C (15 seconds), 84°C (10 seconds), and 88°C (15 seconds). The signal was acquired at 74°C and at 88°C. After all the cycles, a melting curve program was run from 72°C to 95°C at 0.5°C intervals, 30 seconds in each temperature with signal acquisition.

7. After amplification and data collection, the CFX manager software (Bio-Rad Laboratories) was used to generate standard curves from the reference DNA dilutions, one for the telomere signal and one for the single-copy gene signal.

8. The overall mean CV for T/S values of duplicate test samples on the same plate was 5% and the mean inter-assay CV for selected samples was 6.2%.

T/S ratio calculation

The telomere amplicon signal (T) to single-copy gene control amplicon (S) ratios for each sample was calculated as T/S , the amount of reference DNA that matches the experimental sample for copy number of the telomere template, divided by S , that which matches the copy number of the single-copy gene template (Figure 3).

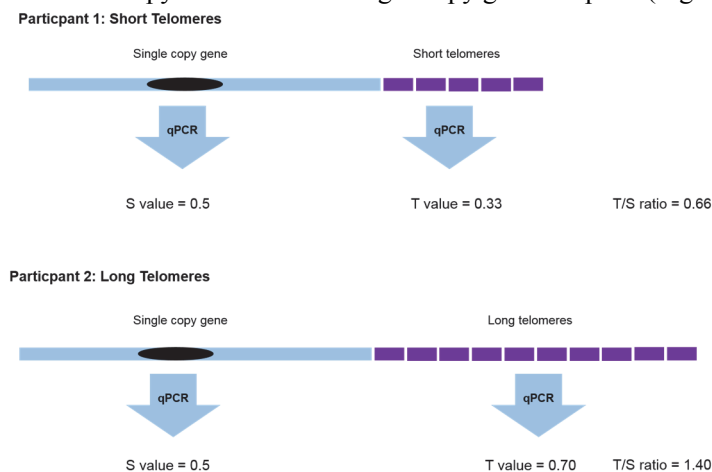


Fig. 3. Representation of telomere length measurement between participants. Participant 1 has a T/S ratio=0.66 and Participant 2 has a T/S ratio=1.40. For each participant in the cohort, T/S values are measured separately. The average of T/S ratio valued measured from all the participants provides the mean LTL of the cohort population.

LTL measurements were obtained from 5,620 individuals, of which 22 outliers (≥ 3 SD) were excluded, leaving 5,598 individuals for use in data analysis.

7.8 Appendix 6

Baron and Kenny Mediation method

According to the Baron and Kenny method, mediation is considered to be present when the following steps are fulfilled: i) exposure associated with the mediator (β_a), ii) exposure associated with the outcome without adjustment for mediator (β_c), iii) mediator associated with the outcome variable (β_b), and iv) the association coefficient ($\beta_{c'}$) between exposure and outcome is reduced upon addition of the mediating variable. Figure 4 shows the principle of this approach and the mediation model tested in Study III.

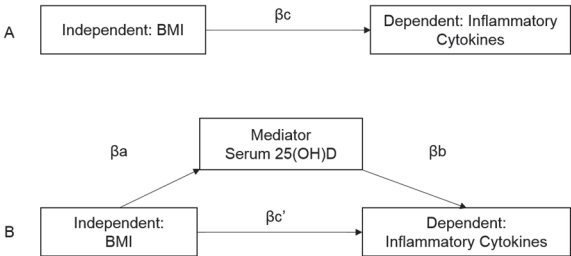


Fig. 4. Graphical representation of hypothesised mediation through 25(OH)D in the association of BMI with inflammatory biomarkers. (A) Figure represents the total effect of BMI with inflammatory biomarkers, without the adjustment for mediator (path β_c). (B) Figure represents the direct effect of BMI with inflammatory biomarkers, taking into account adjustment for mediator (path $\beta_{c'}$). Path β_a represents the regression coefficients for the association between BMI and 25(OH)D. Path β_b represents the regression coefficients for the association between 25(OH)D and cytokines.

7.9 Oxidative stress (Basics)

Increase in adipose tissue volume is suggested to be associated with oxidative stress. Reactive oxygen species are highly reactive molecules with a loss of an electron. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) comprises of

hydrogen peroxide (H_2O_2), nitric oxide (NO), hydroxyl radical (OH \cdot), superoxide ($\text{O}_2^{\cdot-}$), peroxynitrite (ONOO \cdot), and hypochlorous acid (HOCl) (365). To keep the ROS/RNS metabolism in the balance state, tissues contain antioxidant molecules and antioxidant enzymes, catalase, superoxide dismutase (SOD), peroxiredoxins (Prx), glutathione reductase, glutathione peroxidase (GPx), and glutathione S-transferase (365). ROS produced in limited concentrations are involved in the regulation of processes including signal transduction, cellular homeostasis, immune-mediated defence against pathogens and gene expression (366). Normal physiological processes such as mitochondrial oxidation, respiratory chain reactions results in the production of free radicals internally and the external sources include smoking and pollution. The imbalance between the oxidative and the antioxidative mechanisms results in the formation of oxidative stress (365). ROS when present in greater quantities causes damage to lipids, DNA and proteins.

Oxidative stress is measured by the end-products of oxidative damage caused in lipids (e.g., F2-isoprostanes, malondialdehyde (MDA)), in nucleic acid (8-hydroxy-2'-deoxyguanosine (8-OHdG)), and in proteins and amino acids (3-nitrotyrosine, protein carbonyls, advanced glycosylation end products (AGEs) and advanced oxidation protein products (AOPPs) (366).

Original publications

This academic dissertation is based on four publications, which are referred throughout the text by their Roman numerals (I–IV):

- I Palaniswamy S, Hyppönen E, Williams DM, Jokelainen J, Lowry E, Keinänen-Kiukaanniemi S, Herzig KH, Järvelin MR & Sebert S (2017) Potential determinants of vitamin D in Finnish adults: a cross-sectional study from the Northern Finland Birth Cohort 1966. *BMJ Open* 2017;7:e013161.
- II Williams DM*, Palaniswamy S*, Sebert S, Buxton JL, Blakemore AI, Hyppönen E & Järvelin MR (2016) 25-Hydroxyvitamin D concentration and leucocyte telomere length in young adults: findings from the Northern Finland Birth Cohort 1966. *Am J Epidemiol* 183(3):191–8. *Equal contribution.
- III Palaniswamy S, De Silva NM, Lowry E, Jokelainen J, Karhu T, Mutt SJ, Dehghan A, Chasman DI, Timonen M, Viinamäki H, Keinänen-Kiukaanniemi S, Hyppönen E, Herzig KH, Sebert S & Järvelin MR (2018) Does vitamin D mediate the association of body mass index with chronic inflammation? – Review of Randomised controlled trials, observational, and Mendelian Randomisation approach. Manuscript.
- IV Palaniswamy S, Williams D, Järvelin MR & Sebert S (2016) Vitamin D and the promotion of long-term metabolic health from a programming perspective. *Nutr Metab Insights* 8 (Suppl 1):11–21 (review article).

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Original publications are not included in the electronic version of the dissertation.

- I452. Capra, Janne (2018) Differentiation and malignant transformation of epithelial cells : 3D cell culture models
- I453. Panjan, Peter (2018) Innovative microbioreactors and microfluidic integrated biosensors for biopharmaceutical process control
- I454. Saarela, Ulla (2018) Novel culture and organoid technologies to study mammalian kidney development
- I455. Virtanen, Mari (2018) The development of ubiquitous 360° learning environment and its effects on students' satisfaction and histotechnological knowledge
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- I457. Tervaskanto-Mäentausta, Tiina (2018) Interprofessional education during undergraduate medical and health care studies
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- I460. Klemola, Tero (2018) Flexible hallux valgus : Results of a new surgical technique
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- I462. Käkälä, Juha (2018) Family history of mental disorders and long-term outcome in schizophrenia
- I463. Xu, Qi (2018) Role of Wnt11 in kidney ontogenesis and development of renal organoid based models to identify candidate oncogenes
- I464. Lunkka, Nina (2018) Making sense of hospital change project actuality
- I465. Isojärvi, Henri (2018) Association of glucose metabolism, physical activity and fitness with peripheral nervous system function in overweight people
- I466. Matinolli, Hanna-Maria (2018) Nutrition and early life programming of health : Focus on preterm birth and infant feeding in relation to energy-balance and related traits in adulthood
- I467. Tikanmäki, Marjaana (2018) Preterm birth and parental and pregnancy related factors in association with physical activity and fitness in adolescence and young adulthood

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