Vitamin D and Vitamin D-binding Protein in Psoriasis and Effects of Treatment

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Cover illustration: Vitamin D_3 (in red) in the skin of a patient with psoriasis, depicted using the time-of-flight secondary ion mass spectrometry (ToF-SIMS) method. Cholesterol is seen in green and the cell boundaries of the keratinocytes are indicated with blue through demarcation of the phosphatidylcholine headgroup.

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To my dear uncle Constantinos, who has been like a father to me and the inspiration for pursuing this academic achievement.

To my beloved mother Anna for giving me everything.

ABSTRACT

Background: Topical vitamin D analogues constitute an established treatment for psoriasis but the role of vitamin D in the pathogenesis of the disease remains controversial. The *in loco* production and immunomodulatory effects of vitamin D need to be elucidated. Recently, the discussion about the role of vitamin D-binding protein (DBP) in inflammation and its impact on vitamin D physiology has surged.

Aims: To try to map the distribution of vitamin D metabolites in psoriatic skin using time-of-flight secondary ion mass spectrometry (ToF-SIMS) before and after ultraviolet B (UVB) phototherapy (Paper I). To compare the serum levels of DBP in psoriasis with population-based controls (Paper II) and to study the effect of phototherapy (Paper II) and etanercept (Paper III) on vitamin D status and DBP levels. To test the free hormone hypothesis for vitamin D in psoriasis (Paper IV).

Methods: Skin biopsies from a patient with psoriasis, before and after UVB phototherapy were analyzed with ToF-SIMS. A case control study including 68 patients with psoriasis who were studied before and after UVB phototherapy and compared to 105 population-based controls. Twenty bio-naïve patients with moderate to severe psoriasis were treated with etanercept for 24 weeks and 15 matched healthy controls were followed in parallel. A cross-sectional study evaluating vitamin D status in 40 bio-naïve patients with psoriasis.

Results: Vitamin D metabolites were depicted in the skin with ToF-SIMS and information about the morphology of the skin and distribution and quantity of the metabolites in the skin was obtained simultaneously. DBP serum levels were higher in patients with psoriasis compared to population-based controls. DBP levels were higher in those subjects with self-reported arthropathy compared to those without. UVB phototherapy did not affect serum DBP levels while serum DBP decreased during etanercept treatment. Patients with adequate vitamin D levels improved most in their disease on etanercept treatment. Total 25(OH)D was a reliable measure for vitamin D status.

Conclusions: ToF-SIMS is a potentially powerful tool to be used for the investigation of the vitamin D pathway in psoriatic skin and its possible local immunomodulatory effects in the inflammatory process. DBP might be a new inflammatory biomarker in psoriasis. There seems to be a synergic treatment effect between vitamin D and etanercept in psoriasis. Measurement of total 25(OH)D reflected well vitamin D status in bio-naïve patients with psoriasis.

Keywords: Psoriasis, vitamin D, vitamin D-binding protein, phototherapy, tumor necrosis factor α inhibitor.

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SAMMANFATTNING PÅ SVENSKA

Bakgrund: Vitamin D preparat är en effektiv behandling av huden vid psoriasis. Men D-vitaminets betydelse för uppkomsten och mekanismen av sjukdomen är oklar. Av särskilt intresse är vilken roll vitamin D har vid inflammation och inte minst dess bärarprotein, vitamin D-bindande protein (DBP).

Syfte: Att påvisa vitamin D innehållet i psoriasishud med hjälp av en avancerad metod, time-of-flight secondary ion mass spectrometry (ToF-SIMS), före och efter ljusbehandling. Att jämföra DBP-halten i blodet vid psoriasis med personer utan sjukdomen och studera effekten av ljusbehandling och läkemedlet etanercept på vitamin D- och DBP-halterna Att undersöka vilket prov som avspeglar adekvat vitamin D halt i blodet bäst vid psoriasis.

Metoder: Biopsier, hudbitar, togs från en patient med psoriasis, före och efter ljusbehandling och analyserades med ToF-SIMS. Sextioåtta patienter med psoriasis fick ljusbehandling och jämfördes med 105 personer ur befolkningen. Tjugo patienter med måttlig till svår psoriasis behandlades under 6 månader med etanercept, ett läkemedel som dämpar inflammationen, och jämfördes med 15 friska personer som följdes parallellt. Blodprover på 40 patienter med psoriasis avseende olika beståndsdelar av vitamin D togs.

Resultat: Vitamin D avbildades i huden och samtidigt erhölls information om hudens utseende, utbredning och mängden av vitamin D i huden. Bärarproteinet, DBP-halten i blodet, var högre vid psoriasis än i befolkningsgruppen. Bland patienter med psoriasis var DBP-halten högre hos dem med samtidig ledvärk. Ljusbehandling påverkade inte DBP-halten i blodet. Däremot vid behandling med etanercept, som minskade inflammationen vid psoriasis, så minskade DBP-halten. Patienter med högre vitamin D i blodet vid start av behandlingen blev bättre i sin sjukdom av etanercept. Mätning av vitamin D, 25(OH)D, i blodet var ett tillförlitligt mått för vitamin D i kroppen.

Slutsatser: ToF-SIMS är en bra metod för undersökning av vitamin D i huden vid psoriasis. DBP kan vara ett bra prov avseende inflammationen vid psoriasis. Vitamin D verkar förstärka effekten av behandling med etanercept vid psoriasis. Mätning av totalt 25(OH)D speglar väl vitamin D-halten i kroppen hos patienter med obehandlad psoriasis.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals I-IV:

I. **Vandikas MS**, Hellström E, Malmberg P, Osmancevic A. Imaging of vitamin D in psoriatic skin using time-of-flight secondary ion mass spectrometry (ToF-SIMS): A pilot case study. The Journal of Steroid Biochemistry and Molecular Biology. 2019;189:154-60.

II. **Vandikas MS**, Landin-Wilhelmsen K, Holmäng A, Gillstedt M, Osmancevic A. High levels of serum vitamin D-binding protein in patients with psoriasis: A case-control study and effects of ultraviolet B phototherapy. The Journal of Steroid Biochemistry and Molecular Biology. 2021;211:105895.

III. **Vandikas MS**, Landin-Wilhelmsen K, Polesie S, Gillstedt M, Osmancevic A. Impact of etanercept on vitamin D status and vitamin D-binding protein in bio-naïve patients with psoriasis. Acta Dermato-Venereologica, in press, 2021.

IV. **Vandikas MS**, Landin-Wilhelmsen K, Gillstedt M, Osmancevic A. Vitamin D-binding protein and the free vitamin D hormone hypothesis in bio-naïve patients with psoriasis. Submitted. 2021.

CONTENTS

ABBREVIATIONS

$1,25(OH)_2D$	Calcitriol
25(OH)D	Calcidiol
7-DHC	7-dehydrocholesterol
BMI	Body mass index
CASPAR	ClASsification for Psoriatic ARthritis
CDC	Centers for Disease Control and Prevention
CHD	Coronary heart disease
CLIA	Chemiluminescent assays
CRP	C-reactive protein
CYP24A1	24-hydroxylase
CYP27A1	25-hydroxylase
CYP27B1	1α-hydroxylase
CYP2R1	25-hydroxylase
DBP	Vitamin D-binding protein
DHCR7	7-DHC reductase
DLQI	Dermatology Life Quality Index
ECLIA	Electrochemiluminescence assays
ELISA	Enzyme-linked immunosorbent assays
FGF23	Fibroblast growth factor
HsCRP	High sensitivity C-reactive protein

IBD	Inflammatory bowel disease
IL	Interleukin
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
MONICA	MONItoring of trends and determinants for CArdiovascular disease
NBUVB	Narrow band ultraviolet B
PASI	Psoriasis Area Severity Index
PsA	Psoriatic arthritis
PTH	Parathyroid hormone
RA	Rheumatoid arthritis
RIA	Radioimmunoassay
SNP	Single nucleotide polymorphism
TNF	Tumor necrosis factor
TNFis	Tumor necrosis factor-alpha inhibitors
ToF-SIMS	Time-of-flight secondary ion mass spectrometry
UVB	Ultraviolet B
VAS	Visual analogue scale
VDR	Vitamin D receptor
VDRE	Vitamin D responsive elements
VDSCP	Vitamin D Standardization-Certification Program
VDSP	Vitamin D Standardization Program
WHO	World Health Organization

DEFINITIONS IN SHORT

Autocrine	Designates a mode of hormonal action in which a signal molecule binds to receptors on and affects the function of the same cell that produced it. The signal molecule moves outside the cell and binds to the surface receptors of the same cell.
Endocrine	Designates a mode of hormonal action in which the signal molecule produced by a cell is released in the bloodstream in order to be carried to target cells in distant parts of the body.
Intracrine	Designates a mode of hormonal action of self-stimulation through cellular production of a signal molecule that acts within the cell. The intracrine signal molecule does not move outside the cell.
Paracrine	Designates a mode of hormonal action in which a cell produces and releases a signal molecule that stimulates a neighboring cell.

1 INTRODUCTION

1.1 VITAMIN D

Vitamin D is a fat-soluble secosteroid. Already in the 1800s the supplementation of children with cod liver oil was practiced in industrialized countries in order to avoid rickets in children (1). In 1822, the association of lack to sun exposure and rickets was discovered by Sniadecki. However, it was not until the beginning of the 20th century that McCollum discovered that the anti-rachitic effect of cod liver oil was due to a substance that was different from vitamin A, and this substance was given the name vitamin D since the letters A, B and C were already taken (2, 3). Naming this steroid hormone as a "vitamin" (an organic compound, essential for normal growth and nutrition, required in the diet because it cannot be synthesized in the body) is misleading though, as all humans can produce vitamin D_3 after sun exposure of the skin. Some years later it was found that rickets could be prevented or treated if children were exposed to artificial UV light.

The molecular structure of vitamin D was discovered in the 1930s by Windaus and his research group in Germany (1).

In 1985, MacLaughlin *et al*. reported that cultured psoriatic fibroblasts had only partial resistance to the anti-proliferative activity of calcitriol (4), which led to the speculation that calcitriol could be used in the treatment of psoriasis. That same year, and independently from MacLaughlin, Morimoto and Kumahara observed that a patient with psoriasis who was also suffering from osteoporosis showed significant improvement in skin psoriasis while treated with 1α-(OH)D (5), which led to more clinical studies with encouraging results and even the attempt to topically treat psoriasis with calcitriol (6-9).

1.1.1 Sources and synthesis of vitamin D

There are two forms of vitamin D (also known as calciferol): vitamin D_2 (ergosterol), which comes from plants and fungi, and vitamin D_3 (cholecalciferol), which is produced in the skin and can also be obtained from animal products. In this thesis, the term "vitamin D" with no subscript will refer to both vitamin D_2 and D_3. The difference between these two forms lies on the side chain of the molecule. Their metabolism and action are otherwise similar with a slight difference in pharmacokinetics. The major source of

vitamin D in humans is through its production in the skin and a minor amount comes from dietary sources (e.g., fatty fish and fortified food).

Vitamin D_3 is synthesized in the skin from 7-dehydrocholesterol (7-DHC). Cholesterol is enzymatically transformed to 7-DHC (Kandutsch-Russell pathway) with the help of 7-DHC reductase (DHCR7). There is a biochemical equilibrium between cholesterol and 7-DHC (10, 11).

7-DHC is mainly found in the epidermis (65%) and the remaining can be found in the dermis (35%). Around 80% of 7-DHC is located in the cell membrane and 20% is located in the cytoplasm. Upon exposure to ultraviolet B (UVB) radiation (290-315 nm), the B ring of 7-DHC is photolyzed to form pre-vitamin D_3, which subsequently undergoes a thermal isomerization to vitamin D_3. Vitamin D_3 is then carried out into the circulation bound to vitamin D-binding protein (DBP) (12). Maximal vitamin D_3 synthesis occurs at suberythemogenic UVB doses and prolonged UVB exposure does not result in higher vitamin D_3 production since other inactive metabolites are produced (11). The majority of vitamin D_3 production occurs in the cell membranes in the epidermis (10).

Anything that prevents UVB to reach basal epidermis diminishes vitamin D_3 production, for example high amounts of melanin in dark skin and clothing. Use of sunscreens does not seem to notably affect vitamin D production and status according to real-life evidence (13).

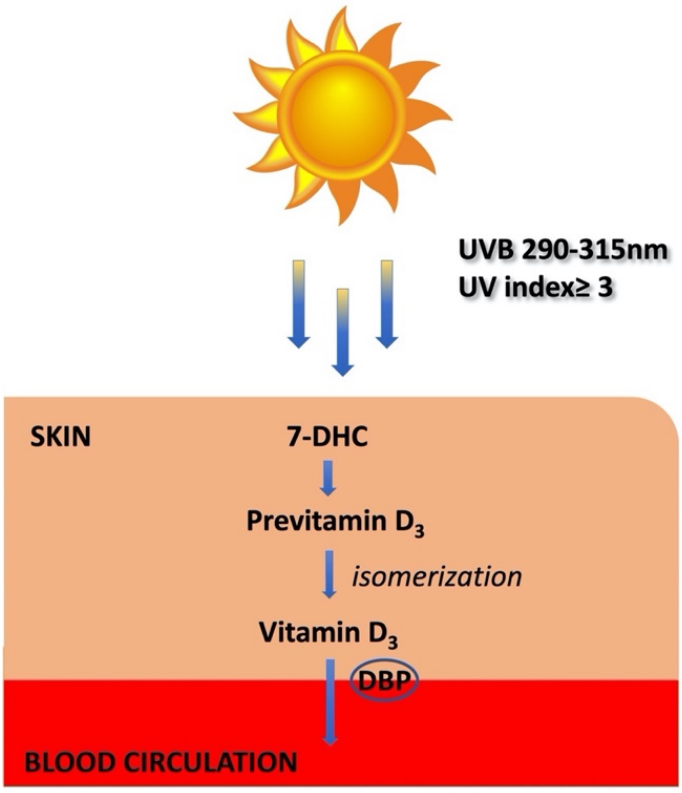


Figure 1. Vitamin D synthesis in the skin.

1.1.2 Vitamin D metabolism

Calciferol, or vitamin D, is biologically inert. Most vitamin D actions are mediated through calcitriol [1,25 $(OH)_2D$], the biologically active hormone. The formation of calcitriol is enzymatic and occurs through two hydroxylation steps that are differentially regulated.

The first step, 25-hydroxylation, is substrate-dependent (on the amount of calciferol) and is performed by 25-hydroxylase. There are different enzymes with 25-hydroxylase activity but the most important ones are CYP2R1, found primarily in the liver but even in other tissues like the skin, and CYP27A1. The metabolite obtained after the first step is 25(OH)D (calcidiol), which is the major circulating metabolite of vitamin D.

The second hydroxylation to form calcitriol is performed by 1α-hydroxylase, CYP27B1. The activity of this enzyme in the kidney (in the mitochondria of the proximal renal tubules) is strictly regulated. Kidney 1α-hydroxylase is stimulated by the parathyroid hormone (PTH) and inhibited by both fibroblast growth factor (FGF23) and calcitriol itself. 1α-hydroxylase in the kidney is responsible for endocrine production and the formation of the majority of circulating calcitriol.

However, 1α-hydroxylase is also found in most of the body's tissues and cells (*in loco* production for autocrine/paracrine use) and there, the regulation of its activity is substrate-dependent [the amount of 25(OH)D] and cytokine-regulated, e.g., by tumor necrosis factor (TNF) and interferon-γ (14, 15).

The catabolism of both calcidiol and calcitriol occurs mainly by the enzyme 24-hydroxylase (CYP24A1) that is upregulated by calcitriol itself.

Mutations in each of the enzymes responsible for vitamin D synthesis and catabolism cause different diseases such as rickets and hypercalcemia.

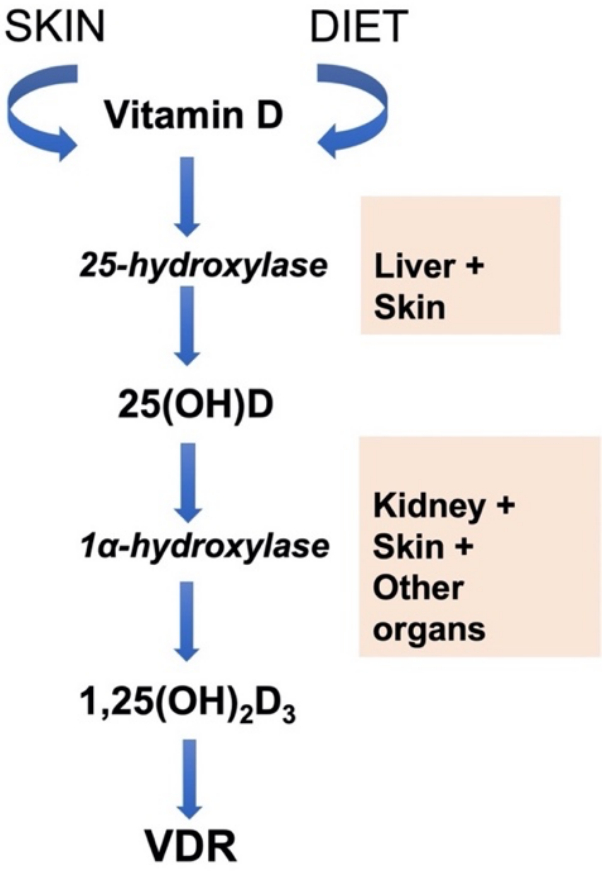


Figure 2. *Vitamin D metabolism.*

1.1.3 The Vitamin D Receptor (VDR)

Calcitriol exerts its genomic actions via binding to the nuclear receptor, vitamin D receptor (VDR), which is a gene transcription factor. After binding to VDR, a heterodimer with the retinoid X receptor is formed that interacts with the vitamin D responsive elements (VDRE). VDR is ubiquitously expressed in many tissues of the body. Around 3% of the human genome is under direct or indirect control by calcitriol.

Non-genomic actions are believed to be mediated through the newly discovered transmembrane VDR and through binding to membrane receptors in the caveolae (15, 16).

A strong indicator of the action of calcitriol after binding to VDR is the induction of the expression of 24-hydroxylase (CYP24A1) and the production of the inactive metabolite 24,25$(OH)_2$D (17).

1.1.4 Vitamin D and Parathyroid Hormone

Both 25(OH)D and calcitriol [1,25$(OH)_2$D] can suppress the production of PTH via entry into the parathyroid cells and binding to VDR (18, 19).

When the levels of 25(OH)D fall, serum levels of PTH increase. An increase in PTH serum levels is noted already when 25(OH)D levels drop below 75 nmol/L (30 ng/ml) (20, 21). Hypocalcemia stimulates also the production of PTH through calcium-sensing receptors (18). Increased PTH stimulates kidney 1α-hydroxylase (CYP27B1) to produce calcitriol.

1.1.5 Endocrine actions of vitamin D

The classic role of the active metabolite calcitriol is calcium and phosphorus homeostasis and bone mineralization. This is achieved by stimulation of intestinal calcium and phosphorus absorption and increased calcium reabsorption in the kidneys as well as mobilization of calcium from the bones and stimulation of osteoblast differentiation for bone mineralization (22). Calcitriol also suppresses PTH production as described above. Finally, it stimulates 24-hydroxylase and FGF23 activity (15).

1.1.6 Autocrine/paracrine actions of vitamin D

What supports the existence and importance of the autocrine/paracrine production and action of vitamin D is the wide expression of its metabolic enzymes and VDR in the human body.

The active metabolite of vitamin D can be produced in different tissues and cells outside the kidney (extra-renal production), such as the brain, heart, skeletal muscles, breast, bowel, pancreas, skin, and immune system, by local 1α-hydroxylase. The local production serves the individual needs of these tissues and cells in an autocrine and/or paracrine way and is responsible for the abundance of its non-skeletal effects including that of immunomodulation (23-26).

Given that the local 1α-hydroxylase activity is substrate-dependent, the amount of $1,25(OH)_2D$ produced locally in the tissues would be negatively affected if the concentrations of 25(OH)D in the serum are low (21).

25(OH)D deficiency has been associated with different adverse health outcomes like cancer as well as cardiovascular, autoimmune, and neurological diseases but a causal association (causation) has not been verified. The possibility of reverse causality was discussed, i.e. that low vitamin D levels might be the result of chronic inflammation and even in some cases because of malabsorption and inadequate acquirement of vitamin D from the intestine. Preclinical and epidemiological studies have suggested that maintaining sufficient vitamin D levels might help prevent autoimmune diseases like diabetes, multiple sclerosis, and rheumatoid arthritis (RA), but large supplementation studies could not verify this (15). However, the majority of the participants in these supplementation studies, had sufficient baseline 25(OH)D levels. This is a possible explanation to why they failed to prove a positive effect (27).

The strongest evidence exists on the prevention of upper respiratory tract infections where daily or weekly (but not bolus) doses of vitamin D, especially in individuals with low baseline levels, were effective in reducing the number of infections (28). Metanalyses have also shown a positive effect of vitamin D supplementation in the reduction of the severity of Crohn's disease (29).

An inverse correlation between levels of 25(OH)D and all-cause mortality was shown in some studies and a U-shaped curve regarding overall mortality and cardiovascular mortality was found in two studies (27).

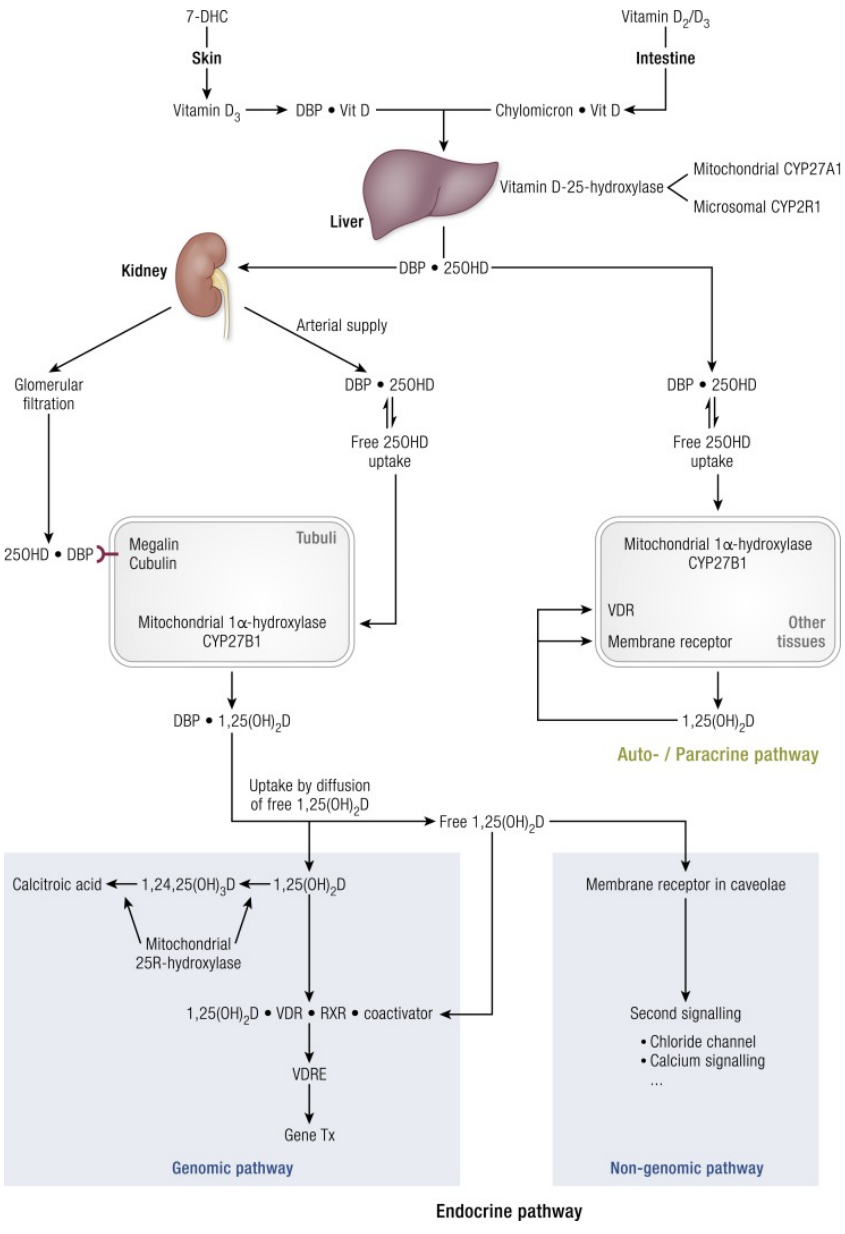


***Figure 3**. Metabolism and action of vitamin D and its metabolites, with special focus on renal and extrarenal production of 1,25(OH)2D and the genomic or nongenomic pathways of vitamin D action. Bouillon et al., Skeletal and Extraskeletal Actions of Vitamin D, Endocrine Reviews, August 2019, 40(4):1109-1151.*

1.1.7 The skin as source and target of vitamin D

The keratinocytes in the skin possess all the necessary enzymes for vitamin D metabolism: 7-DHC reductase, 25-hydroxylase, 1α-hydroxylase, and 24-hydroxylase. This makes the skin the only organ of the body that can autonomously synthesize, metabolize, and degrade vitamin D (30).

The skin also expresses VDR, the main mediator of vitamin D's biological action, which makes it also a target tissue for vitamin D (30, 31). In synergy with calcium, the locally produced calcitriol is important for cell differentiation in the epidermis. Mice that lack the VDR gene present with defects in epidermal differentiation due to low levels of loricrin, involucrin and profilaggrin (11, 32). A similar phenotype presents also in mice with a gene mutation in 1α-hydroxylase, thereby emphasizing the importance of the locally produced calcitriol. Binding of calcitriol to VDR is also involved in the production of antimicrobial peptides, wound healing, and suppression of tumorigenesis in the skin (11, 15, 31).

1.1.8 Assays for measurement of 25(OH)D

Measurement of serum total 25(OH)D levels [the sum of 25(OH)D_2 and 25(OH)D_3, both protein-bound and free] is today the internationally accepted marker for estimating the vitamin D status of an individual. This decision is based on the following:

i. 25(OH)D is found in higher amounts compared to the other metabolites,
ii. it has a long half-life, 2-3 weeks,
iii. the enzyme responsible for the synthesis of 25(OH)D, 25-hydroxylase, is substrate-regulated which means that rate of 25(OH)D production and the amount of 25(OH)D found in the blood is directly associated to the supply of vitamin D_3 or D_2 to the body via skin synthesis or dietary intake, respectively,
iv. its serum levels are relatively stable, and
v. the epidemiological and clinical studies conducted have shown a relationship between 25(OH)D insufficiency and different adverse outcomes and not a relationship with the active metabolite 1,25$(OH)_2$D, which is more strictly regulated (33, 34).

The available and most commonly used assays for serum 25(OH)D measurement can be categorized into immunoassays and chemical assays (34). Immunoassays can be further classified into radioimmunoassays (RIA), enzyme-linked immunosorbent assays (ELISA), and chemiluminescent assays (CLIA). Immunoassays use a labeled antibody that will specifically bind to the antigen of interest, in this case 25(OH)D.

The major downsides of immunoassays are:

i. the low affinity of the labelled antibodies for 25(OH)D_2 which might lead to underestimation of total 25(OH)D levels,
ii. cross-reaction of the antibody with other metabolites than 25(OH)D, for example 24,25$(OH)_2$D, thereby resulting in high variability of the measured amount of total 25(OH)D levels,
iii. the results in some automated immunoassays might be affected by the amount of DBP in the serum if 25(OH)D is not adequately released from DBP, and
iv. matrix-specific interference resulting from different clinical conditions (pregnancy, critical illness, and hemodialysis) may occur (33-36).

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is the gold standard method for the measurement of 25(OH)D levels. The separation of the different metabolites is done according to their mass-to-charge ratio (m/z) and the detection and quantification of the different metabolites is performed with mass spectrometry. LC-MS/MS has a high analytical sensitivity and newly developed methods can simultaneously detect and measure up to 13 different vitamin D metabolites (37).

The international Vitamin D Standardization Program (VDSP) was established in 2010 in order to overcome the problem of variability in results of 25(OH)D measurements when using different assays, and the subsequent generated bias in research results (38).

1.1.9 Measurement of free 25(OH)D

Measurement of free 25(OH)D levels in serum is challenging because the levels are very low. Until recently, determination of serum free 25(OH)D levels involved a difficult, expensive, and time-consuming procedure, e.g., the centrifugal ultrafiltration assay (39). An assay that directly measures serum

free 25(OH)D levels has now been developed (Future Diagnostics) using a two-step immunosorbent assay (ELISA) performed with a commercial kit.

1.1.10 Factors affecting serum vitamin D levels

There are many factors that can affect the levels of 25(OH)D in the serum and these can be categorized into:

i. factors that affect the production of vitamin D in the skin (skin type, clothing, sun exposure and sun habits, season of the year and latitude),
ii. diet,
iii. medication that leads to higher catabolism of vitamin in the body (e.g., anticonvulsants),
iv. high body weight and high body mass index (BMI),
v. diseases that lead to lower absorption of vitamin D in the intestine (e.g., fat malabsorption), impaired production of 25(OH)D in the liver (e.g., liver cirrhosis), high urine loss (e.g., nephrotic syndrome),
vi. physical activity, and
vii. genetic factors, including genes involved in the synthesis, metabolism, catabolism, and transport of 25(OH)D.

A schematic presentation of the factors that have been taken into account in this thesis as confounders for vitamin D status, is shown in *Figure 4*.

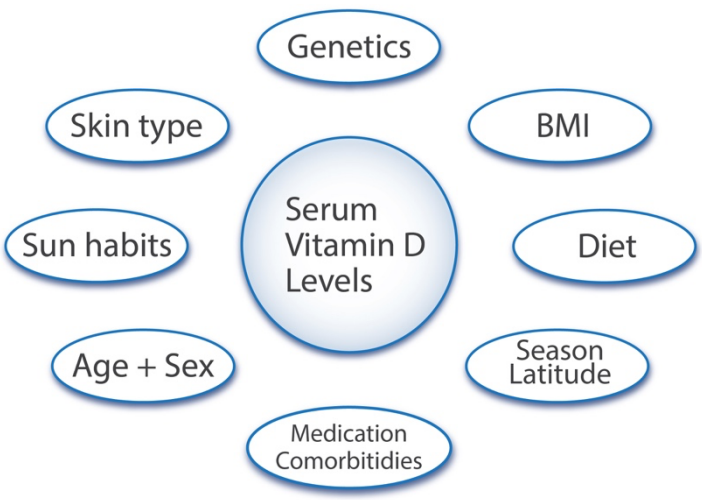


Figure 4. *Factors that affect serum vitamin D levels. BMI, body mass index.*

1.1.11 Defining hypovitaminosis D

There is internationally no consensus on how to define vitamin D insufficiency and sufficiency. However, there seems to be an agreement that levels below 12 ng/ml or 30 nmol/L are definitely deficient and that levels above 30 ng/ml or 75 nmol/L are definitely sufficient (27). The disagreement in definition regards the range between 12 to 30 ng/ml (30 to 75 nmol/L). According to the Institute of Medicine, levels of 20 ng/ml (50 nmol/L) should be considered sufficient (40) while the Endocrine Society recommends a level greater than or equal to 30 ng/ml or 75 nmol/L (20).

Regarding the skeletal effects of vitamin D, levels of 20 ng/ml (50 nmol/L) or higher are most probably adequate but it is unclear whether this applies to the extra-skeletal effects of vitamin D. Regarding this part of vitamin D physiology and more specifically that of immunomodulation, it has been discussed whether higher levels of 25(OH)D should be pursued (41, 42).

1.2 VITAMIN D-BINDING PROTEIN

DBP belongs to the albuminoid family together with albumin, α-fetal protein and afamin. It is 58 kD in size. It is mainly produced in the liver but there are other tissues that also express DBP in lower amounts. In rats, the DBP gene was highly expressed in the liver and moderately expressed in the kidney, abdominal fat, testes, and yolk sac (43, 44).

1.2.1 Structure and polymorphisms

DBP consists of three domains: the vitamin D-binding site, the binding site for C5a/C5a des Arg, and the actin-binding site. Arachidonic acid competes with 25(OH)D for the same binding site (45).

DBP is a highly polymorphic protein. There are more than 120 variants described. Two single nucleotide polymorphisms (SNPs) are responsible for the three most common variants: GC1f, GC1s and GC2. These variants have a racial distribution, e.g., GC1f is more common in Africans and GC1s and GC2 are more common in Caucasians. Furthermore, these polymorphisms might affect the binding affinity for vitamin D metabolites with GC1f having the highest and GC2 the lowest affinity for vitamin D metabolites, though this is controversial. The binding affinity is today believed to be variant and may vary in different clinical conditions (physiological and pathological) or in the presence of various substances in the blood like polyunsaturated fatty acids (46, 47).

The different polymorphisms might affect the total circulating levels of vitamin D and eventually the amount of free 25(OH)D but this is disputed. The total amount of DBP is believed to be the strongest determinant for the vitamin D levels in serum and in clinical conditions where the amount of circulating DBP is affected, total vitamin D levels are also affected. Characteristically, in cirrhosis, impaired production of DBP leads to low levels of total 25(OH)D and higher levels of free 25(OH)D and in pregnancy, higher concentrations of DBP result in lower concentrations of free 25(OH)D (48).

1.2.2 DBP and vitamin D

DBP is the major carrier of vitamin D metabolites. DBP binds around 85% of total 25(OH)D with high affinity but can even bind the other vitamin D metabolites, such as cholecalciferol and calcitriol, though with lower affinity.

Around 15% of vitamin D is bound on the low affinity carrier, albumin. Less than 0.1% of 25(OH)D circulates as free (46).

DBP circulates in abundance compared to its ligands and therefore only a very small percentage of the ligand-positions are occupied at any given time. This is believed to be a possible protective mechanism against vitamin D intoxication (21).

The supply of the important prohormone 25(OH)D to the body is not stable as it is affected by many factors like the season, sun exposure, and diet, as mentioned previously. DBP helps maintain stable vitamin D levels in the blood and contributes in a way to ensure access to 25(OH)D even in periods of low supply. DBP has a storage function and also protects vitamin D metabolites from urinary loss, prolonging in this way the half-life of all of the vitamin D metabolites. DBP together with its ligands is reabsorbed in the proximal tubular cells in the kidney through the megalin/cubilin receptor. DBP-null mice have extremely low vitamin D levels and are very vulnerable to vitamin D depletion conditions (45).

DBP facilitates the transport of the lipophilic vitamin D in the aquagenic plasma environment and regulates the availability of 25(OH)D to target cells and tissues for the subsequent *in loco* production of the active metabolite calcitriol (31). Some tissues and cells have access to the DBP-bound 25(OH)D, like those of the kidney, placenta, mammary cells, and parathyroid gland, as they express the megalin/cubilin receptor. In these tissues and cells, DBP is endocytosed and degraded and the prohormone is released from its transporter to be subsequently metabolized to calcitriol. However, for most of the body's other tissues and cells, that is those that do not express megalin/cubilin, the biologically relevant substrate is the free 25(OH)D that is able to passively cross the cell membrane and enter the cell (21, 31, 45). This is also the case for the skin and for immune cells.

DBP may negatively influence vitamin D's action in an indirect way. *In vitro* studies have shown that by gradually adding DBP to cultures of keratinocytes, osteoblasts, monocytes, dendritic cells, and T-cells, vitamin D action was inhibited through restriction of 25(OH)D uptake (21, 49, 50).

1.2.3 The biologic importance of DBP besides vitamin D transport

DBP is responsible for actions that are independent of vitamin D, like actin scavenging and tissue neutrophil recruitment, by enhancing the chemotactic activity of complement C5α and other chemoattractants (e.g., CXCL1) (44). Moreover, DBP can become a macrophage-activating factor through deglycosylation (45, 47). A possible role for DBP in inflammation has thus been discussed. Serum DBP levels were found to positively correlate with C-reactive protein (CRP) levels in healthy individuals (51) and in a cohort of older men (52).

High levels of DBP have been reported in comorbidities associated with psoriasis (Crohn's disease, cardiovascular diseases, chronic periodontitis) (53-55). In an article by Ghaly *et al.*, high serum DBP levels could predict relapse in Crohn's disease, with a hazard ratio (HR) of 1.23 for every 50 mg/L increase in serum DBP (53). In another study by Robinson-Cohen *et al.*, high circulating serum DBP was strongly associated with increased risk of coronary heart disease (CHD) events. Participants with the highest tertile concentration of DBP were more likely to experience a CHD event (HR 1.78 per SD increment in DBP concentration, $P<0.0001$) (54).

1.2.4 Factors affecting DBP levels

The production of DBP is stimulated by estrogens, dexamethasone, and certain cytokines like the proinflammatory cytokine IL-6, and inhibited by transforming growth factor-β (TGFβ) (31, 45). Reduced DBP levels are described in primary hyperparathyroidism, liver cirrhosis, malnutrition, nephrotic syndrome, peritoneal dialysis, critical illness, and type 1 diabetes (45, 56, 57). Smoking might also down-regulate DBP levels (58).

On the contrary, pregnancy and exposure to some drugs like oral contraceptives and aspirin are associated with higher DBP levels (58).

The data regarding how obesity affects DBP levels are conflicting with some studies showing negative, positive, or no association with BMI, fat tissue or obesity (59-61).

The mean concentrations of DBP in serum are believed to be to a great extent independent of race and genotype, with the exception of the GC2 variant where the levels are slightly lower than other variants.

Vitamin D itself does not seem to influence or regulate the levels of DBP (45, 57, 62).

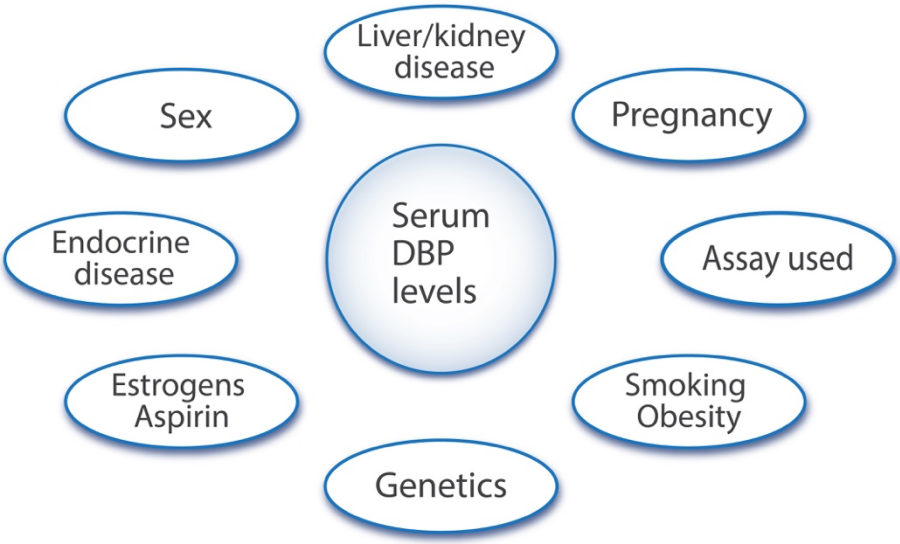


Figure 5. *Factors that might affect DBP.*

1.2.5 Measurement of DBP in serum

DBP can be measured using immunoassays (monoclonal or polyclonal) and LC-MS/MS (33). The monoclonal immunoassay for DBP has differential affinity for specific genotypes of DBP and more specifically, lower affinity for the GC1f isoform and higher affinity for GC1s (39) than the polyclonal assay. This means that GC1s is well detected while the amount of GC1f is underestimated, resulting in falsely low total DBP levels when there is an overrepresentation of the GC1f isoform, for example in individuals with African origin. This problem does not seem to be present in polyclonal immunoassays and LC-MS/MS.

To date, there is no standardized method for measurement of serum DBP. Lack of standardization makes the comparison between different studies difficult and also affects the measurement of calculated free and bioavailable vitamin concentration.

1.2.6 The free vitamin D hormone hypothesis

According to the free hormone hypothesis, it is the free (non-protein-bound) fraction that is available for diffusion through the lipid layer of the cell membrane and that is biologically more important than the total amount of the hormone (bound and free). The free hormone hypothesis is applied to thyroid, cortisol, and sex hormones as their free fraction is measured as part of everyday clinical praxis (45, 46).

In DBP-null mice or humans, calcium and bone homeostasis remain normal despite the extremely low levels of 25(OH)D and 1,25 $(OH)_2D$ (45). This is powerful evidence that the free hormone hypothesis can also be applied to vitamin D.

Applying the free hormone hypothesis, that is to measure free 25(OH)D as a marker for vitamin D status and the biologic activity of vitamin D, had not been investigated in dermatology, and more specifically in psoriasis, before this thesis work.

In clinical conditions where serum DBP levels are affected, the balance between total 25(OH)D and free 25(OH)D might alter (48), therefore the immunomodulatory effect of vitamin D might not depend exclusively on the concentration of 25(OH)D but also on the concentration of DBP in serum (21).

1.3 PSORIASIS

Psoriasis is a chronic, immune-mediated, inflammatory disease characterized by increased proliferation and decreased differentiation of keratinocytes as well as a localized and systemic inflammation (63). It affects approximately 2-3% of the Western population with equal prevalence between men and women, although severe psoriasis is more common in men. The onset of psoriasis occurs often before the third decade of life but it may appear later in life as well. There are different phenotypes of psoriasis, the most common being plaque psoriasis.

Psoriasis has a severe impact on the quality of life of patients due to the clinical appearance of the skin lesions, the itching, and not least the multiple associated comorbidities. Psoriatic arthritis is the most common comorbidity affecting approximately 30% of the patients. Patients with psoriasis more often suffer from hypertension, diabetes, being overweight, hyperlipidemia and metabolic syndrome and are thus at increased risk for cardiovascular disease (64-66). Furthermore, it has been shown that psoriasis constitutes an independent risk factor for myocardial infarction. Other comorbidities include autoimmune gastrointestinal disease like Crohn's disease and depression (67). A less commonly reported comorbidity is periodontitis (68, 69). Subjects with psoriasis tend to drink more alcohol and smoke.

The pathogenesis of psoriasis is complex and not fully elucidated but heredity constitutes a strong predisposing factor together with other environmental triggers, for example infections, trauma and medications. The inflammation in psoriasis is a result of a dysregulated cross-talk between different cells and cytokines of the innate and adaptive immune system. It is believed that there is an initial activation of the innate immune system (plasmacytoid dendritic cells, keratinocytes, natural killer T-cells, and macrophages) and production of proinflammatory cytokines (TNF-α, IFN-α, IFN-γ, interleukin (IL)-1β and IL-6). This consequently leads to an exaggerated activation of the adaptive immune system through activation of myeloid dendritic cells. Th1-, Th17-, and Th22- lymphocyte cells ascend after the activation and differentiation of naive T-cells and seem to play a central role in the inflammation. Cytokines which are produced by these activated T-cells cause the keratinocyte hyperproliferation and inflammatory infiltration in lesional skin that clinically appears as sharply demarcated, erythematous, scaly patches or plaques (63, 70-72).

1.3.1 Assessment of psoriasis disease severity

The *Psoriasis Area Severity Index (PASI),* a validated, quantitative rating score, is the most commonly used tool to assess psoriasis severity. PASI takes into consideration the area of body surface that is affected and the severity of lesions with regards to erythema, induration, and scaling. The body is divided into 4 sections (head, arms, trunk, and legs) and each area is scored separately. These variables are weighed together into a single score which ranges from 0 (no disease) to 72 (maximal disease). In this thesis, a score of up to 4 is defined as mild disease, between 5 to <10 as moderate severity, and a score equal to or higher than 10 is defined as severe disease. Lately, even PASI score 3 and 4 are considered moderate disease. PASI has showed good intra-rater and inter-rater reliability values (73).

The *Dermatology Life Quality Index (DLQI),* is a validated, standardized ten-question questionnaire, used to measure the impact of dermatological diseases on the quality of life of the patients. It is widely used in psoriasis and takes into account the intensity of the symptoms, feelings of the patient, limitations in daily life and activities because of the disease, influence of the disease on personal relationships, and treatment satisfaction. The DLQI is calculated by adding the score of each question (0 to 3, where 3 is strongest complaints), resulting in a maximum of 30 and a minimum of 0. The higher the score, the more quality of life is impaired. A score up to 5 indicates a small effect, between 6 to 9 a moderate effect, and equal or higher than 10 indicates that the patient's life is severely affected by their skin disease.

The *visual analogue scale (VAS)* is a simple method that is used to evaluate subjective symptoms. It is made up of a 100 mm horizontal line where the subject is asked to place a mark at the point that represents the intensity of the experienced symptoms. Zero means no complaints and 100 the maximum complaints. VAS has been previously used to evaluate self-rated psoriasis severity and has shown good correlation to PASI and DLQI (74).

Assessment of psoriasis disease severity is best evaluated by combining different assessment tools. The combination of PASI and DLQI is common since together both tools can give a more holistic comprehension of the severity of the disease and its impact on the individual. For example, in some cases, even if small body surface area is affected, which would result in a low PASI score, the quality of life might anyway be highly impaired if the skin lesions are located on, e.g., the face and/or the hands.

Other assessment tools include the Physician's Global Assessment (PGA), body surface area (BSA) and EuroQoL 5-Dimension Health Questionnaire (EQ-5D).

PGA is more practical and easier to use than PASI. It is based on an average assessment of all psoriatic lesion with regard to erythema, scale and induration. However it does not include an evaluation of the area affected or the localization of the lesions so its value as the only tool to assess the disease severity is limited (75). BSA is defined as the percent of BSA involvement, where the patient's palm of the hand is approximately 1% of the body area. The product of PGA X BSA, as a composite tool has been proposed in the evaluation of disease severity as it showed strong correlation with PASI (76).

EQ-5D has five dimensions of health including mobility, self-care, usual activities, pain/discomfort, and depression/anxiety. The validity and responsiveness of the EQ-5D was found to be good in people with skin diseases, especially plaque psoriasis or psoriatic arthritis (77).

1.3.2 Psoriatic arthritis

Around 30% of patients with psoriasis develop psoriatic arthritis (PsA) which is a complex inflammatory disease with heterogeneous clinical features (78). The term "psoriatic arthropathy" is sometimes used to denote the wide spectrum of this articular disease. PsA may evolve to erosive disease. There is usually no correlation between PsA and the severity of psoriasis in the skin, with patients appearing with severe PsA even with minimal skin lesions (79, 80). The negative effect on the physical function of the patient and on their quality of life is comparable to that of rheumatoid arthritis (RA) (78).

The different clinical features of PsA are:

i. peripheral mono- or oligoarthritis, characterized by joint swelling and pain, in large joints but even in the feet and the distal interphalangeal joints,
ii. enthesitis, characterized by entheseal inflammation and tenderness,
iii. axial arthritis, and
iv. dactylitis (78).

Coincident skin psoriasis is present in around 80% and nail disease in around 60% of patients with PsA (81).

The early diagnosis of PsA can be difficult to determine since there are to date no specific diagnostic criteria and no early diagnostic tests. Even CRP, which is otherwise a sensitive marker for systemic inflammation, can be normal in PsA (82, 83). PsA diagnosis is made with the help of patient history, physical examination, the usually negative serology for rheumatoid factor and anti-cyclic citrullinated peptide, inflammatory markers, radiologic imaging, and exclusion of mimics. However, in some cases, observation over time might be needed to confirm the diagnosis.

The ClASsification for Psoriatic ARthritis (CASPAR) criteria are used for classification of established PsA. Use of the CASPAR criteria as diagnostic criteria might lead to misdiagnosis due to the low prevalence of radiographic damage in early disease and consequently delayed treatment initiation and poorer prognosis (79). What further perplexes the evaluation and treatment of PsA is the reported discrepancy between the experienced symptoms and the physician's assessment of disease activity (79, 84) Thus, there is a need for the discovery of new inflammatory biomarkers for PsA.

1.3.3 Vitamin D and psoriasis

Use of locally applied vitamin D analogues is an established treatment for psoriasis (85) and a positive example of the extra skeletal, immunomodulatory effects vitamin D may exert. The calcitriol analogue, calcipotriol, when used as local treatment against psoriasis within the recommended dose limit, has the advantage of a low binding affinity to DBP which means that the majority remains in the skin and the risk for the systemic adverse effect of hypercalcemia is low. The efficacy and safety of calcitriol analogues in psoriasis are well investigated both as a monotherapy and in combination with topical glucocorticoids (85-87).

Topical calcipotriol in pharmaceutical doses has direct anti-proliferative, differentiation-inducing, and pro-apoptotic effects in the psoriatic lesions (85). Furthermore, calcitriol exerts multiple immunoregulatory effects in psoriasis as a result of its action on both the innate and adaptive immune systems. It inhibits the maturation of antigen-presenting cells that could trigger the adaptive immune system, thus promoting immune tolerance. It also directly affects T-cell proliferation by causing a switch from the overdriven Th1/Th17 skewed response observed in psoriasis towards a Th2/Treg profile (88, 89). This leads secondarily to reduced proliferation and increased differentiation of keratinocytes. The production of different proinflammatory cytokines is inhibited [e.g., IL-1β, IL-6, IFN-γ, TNF-α] and the production of the regulatory cytokine IL-10 is stimulated. Furthermore, psoriasin and koebnerisin are reduced and LL-37 and HBD2 are increased (16, 90).

Both the enzyme that is responsible for the synthesis of 1,25 $(OH)_2D$ (1α-hydroxylase) and VDR are found not only in keratinocytes but also in lymphocytes, dendritic cells, and macrophages, all of which are involved in the psoriatic inflammation.

1.3.4 Contradictory results regarding the role of vitamin D in psoriasis and possible explanatory reasons for that

Studies investigating correlations between vitamin D status and severity of psoriasis have led to conflicting results (91-93) with some studies finding a negative correlation and others not verifying this. Furthermore, the increase of serum 25(OH)D levels after UVB phototherapy does not always correlate to the degree of improvement in psoriasis severity (91, 94, 95). Multiple studies have reported an improvement in psoriasis following vitamin D supplementation but these data are predominantly from small observational or

non-randomized interventional studies (96). Some studies found psoriasis to be associated with low and others with normal 25(OH)D levels when comparing with healthy controls. Furthermore, in some studies, vitamin D status, was inversely associated with PASI scores and CRP levels, while in others, no such correlation was found (97).

One reason for these contradictions in published results might be that the amount of 25(OH)D and calcitriol which is present and active in the skin, does not reflect serum 25(OH)D or serum calcitriol levels. Therefore, it is important to find new methods that would help us investigate the *in loco* vitamin D production and metabolism and their influence on inflammation directly in the skin instead of just measuring the vitamin D metabolites in serum (87, 98).

Another reason to the contradictory results might be that these studies have been measuring an inappropriate vitamin D marker that does not accurately represent the biologic activity of vitamin D, as mentioned above (the free hormone hypothesis) (99).

1.3.5 Ultraviolet radiation B (UVB) phototherapy in psoriasis

Narrow band UVB (NBUVB) (311 and 312 nm) and broadband UVB (280–320 nm) phototherapy are commonly used as first-line treatment choices for widespread psoriasis. Both treatments lead to the production of vitamin D in the skin and consequently increased serum levels of 25(OH)D (100). In recent years, NBUVB has become the dominant phototherapy modality for psoriasis treatment. The beneficial effect of UVB phototherapy in psoriasis is believed to be partly due to the UVB-induced *in loco* production of vitamin D_3 in the skin and the subsequent synthesis of calcitriol (101-103).

UVB phototherapy reduces skin inflammation and improves PASI scores, but it has not been shown to improve circulating biomarkers of cardiovascular risk and a persistent inflammatory process has been observed after use of this treatment (104).

1.3.6 TNF-α inhibitor treatment in psoriasis

The family of TNF-α inhibitors (TNFis) was the first group of biologics used to treat psoriasis. TNFis comprise an established and effective treatment for both skin psoriasis and PsA. Furthermore, the use of TNFis has shown a beneficial effect on other comorbid conditions, e.g. it was shown that TNFis reduce the risk for major cardiovascular events (64, 105) in patients with

psoriasis. This is extremely important since it is now believed that psoriasis confers an independent risk factor for CHD and cardiovascular mortality especially in those patients with severe disease (65, 67).

Etanercept is a soluble, recombinant, TNF-α receptor protein fused with the Fc portion of IgG1 that competitively inhibits the interaction of TNF-α and its cell surface receptors (106). Etanercept was the first United States Food and Drug Administration (FDA)-approved TNFi for psoriasis treatment.

1.3.7 Anti TNF-α treatment and vitamin D

The active metabolite of vitamin D, calcitriol, seems to have an anti-TNFα effect through suppression of circulating TNF-α expression. Furthermore, it may suppress Th1-related chemokines as shown in different studies (107-109).

The synergic effect might be explained by i. an additive anti-TNFα effect of TNFis and calcitriol and/or ii. the combined effect of the two agents against Th1 cytokines, IL-17A and IL-22, as shown in some experimental studies. In an *in vitro* study in patients with RA, TNF-α inhibition as a monotherapy could not suppress IL-17A and IL-22 expression, but the combination with calcitriol resulted in control of Th17 activity and inhibited synovial inflammation (110). This synergic action between calcitriol and TNFis was also seen in other *in vitro* and *in vivo* studies in patients with inflammatory bowel disease (IBD) and RA (111, 112).

1.4 TIME-OF-FLIGHT SECONDARY ION MASS SPECTOMETRY

Time-of-flight secondary ion mass spectrometry (ToF-SIMS), a type of mass spectrometry imaging (MSI), is a very sensitive method used to analyze surface composition. Recently, it has been used in medical science to determine, map, and depict the distribution of different molecules in biological samples (113, 114). The technique can detect molecules (e.g., lipids, organic molecules, and even drug molecules) with mass-to-charge ratios (m/z) up to 1000–1500 and with a lateral resolution from 200 nm to 1–2 μm.

ToF-SIMS has many advantages: *i.* no pre-treatment of the tissue sample is required, *ii.* the imaging is very detailed and can reach the cellular and subcellular level, simultaneously delivering spatial information at high resolution, and *iii.* semi-quantitative information of the content concentration is provided (114). ToF-SIMS has been used before in analysis of skin samples (113, 115, 116). In a previous study, vitamin D and its metabolites were depicted and measured by ToF-SIMS in human adipose tissue (117).

2 AIMS

The overall aims of this thesis were:

1. To explore the possibility of mapping the distribution of vitamin D and its metabolites in skin using ToF-SIMS in psoriatic skin in order to obtain both spatial and semi-quantitative information. At the same time, to compare the skin biopsies and vitamin D production in the skin, before and after NBUVB phototherapy (**Paper I**).

2. To compare the serum levels of DBP in patients with psoriasis with healthy controls (**Paper II**).

3. To study the effect of UVB phototherapy as well as the effect of TNF-α inhibition treatment (etanercept) on the serum levels of DBP in psoriasis (**Paper II and III,** respectively).

4. To investigate the impact of TNF-α inhibition treatment (etanercept) on vitamin D status and serum levels of DBP as well as on other variables that constitute risk factors for cardiovascular disease (**Paper III**).

5. To test the free hormone hypothesis for vitamin D in psoriasis and to investigate whether there would be a stronger correlation between directly measured serum free 25(OH)D and psoriasis disease severity compared to total serum 25(OH)D. (**Paper IV**).

6. To identify the strongest determinants for serum levels of DBP in psoriasis (**Paper IV**).

3 MATERIALS AND METHODS

3.1 SUBJECTS

3.1.1 Paper I

A 65-year-old female patient with active chronic plaque psoriasis who attended the outpatient clinic of the Department of Dermatology at Sahlgrenska University Hospital was included in this pilot case study. The patient's disease severity was classified as moderate and the patient was prescribed NBUVB phototherapy according to standard protocol. The patient did not use oral vitamin D supplements or topical corticosteroids and/or topical vitamin D treatment during the study.

3.1.2 Paper II

Sixty-eight Caucasian adult subjects (51 men and 17 women) with active plaque psoriasis who attended the outpatient clinic of the Department of Dermatology at Sahlgrenska University Hospital were recruited and treated during March to June 2006 and November 2006 to March 2007. The severity of the disease varied among the patients from mild to severe. The patients did not use oral vitamin D supplements or topical vitamin D treatment during the study. The patients had been previously treated with either NBUVB or broadband UVB but not during the last two months prior to the study. All of the patients were bio-naïve. Having kidney disease was an exclusion criterion. Information about concurrent medication and smoking was registered but no anthropometric data were collected and no information about the coexistence of PsA was registered.

Matching subjects with respect to sex and age was done from a randomly selected subsample (n=414) from a population-based study sample from the World Health Organization (WHO) MONItoring of trends and determinants for CArdiovascular disease (MONICA) project in Gothenburg, Sweden (118). The original study (WHO MONICA investigation 1995) invited a random population sample of men and women (n=2612, aged 25-64 years) from the Gothenburg city census to participate. Of the 2563 individuals who were possible to contact, 66% accepted to participate (n=1618) (119). A randomly selected subsample of those examined in 1995 were reinvited for a follow-up

examination in 2008. The participation rate was 62% and a total of 414 subjects were evaluated.

Of the total of 414 subjects from the WHO MONICA study 2008, 129 were matched with respect to age and sex to the patients with psoriasis but 24 were excluded since they had not been recruited within the same period as the patients with psoriasis. The remaining 105 individuals were used as controls.

The serum levels of DBP, free 25(OH)D index and total 25(OH)D were compared at baseline. The changes after UVB phototherapy in serum levels of DBP, 25(OH)D and calculated vitamin D metabolites (free 25(OH)D index, calculated free 25(OH)D, and bioavailable 25(OH)D) were investigated.

3.1.3 Paper III

Twenty consecutive bio-naïve patients, 18 years of age or older, with moderate to severe plaque psoriasis (defined as PASI≥10 and/or DLQI≥10), and/or where other systemic treatment was ineffective or inappropriate, were included in this study. The patients had to fulfil the criteria for biologic therapy (etanercept) according to standard clinical praxis. The patients attended the outpatient clinic of the Department of Dermatology at Sahlgrenska University Hospital.

The patients were matched to 15 healthy controls with respect to sex, age, and season of inclusion. The controls were all Caucasians, with the exception of one individual of Asian origin, and had no history of psoriasis, other skin disease or other ongoing inflammatory disorder in the skin, joints and/or bowel.

Exclusion criteria were: pregnancy/lactation or plans for pregnancy; ongoing other severe chronic or systemic disease, e.g., liver disease, kidney disease, cancer or infectious disease; treatment with oral steroids or other immunosuppressive/anti-inflammatory drugs and/or antibiotic treatment; ongoing sun-bed use or sun-bed use during the last 4 weeks and/or sun holiday during the past 4 weeks. “Sun holiday” was defined as a journey to a location where UV index was ≥3 during the period of the journey. Vitamin D supplementation as well as the use of topical vitamin D analogues were not allowed during the study.

The patients were treated with etanercept, 50 mg weekly, for 24 weeks and were followed up at baseline, after 10 weeks, and after 24 weeks. The healthy

controls did not receive any treatment but were followed up in the same way, at the same time points and with the same biochemical analyses.

3.1.4 Paper IV

Forty consecutive bio-naïve patients with mild to severe active plaque psoriasis who attended the outpatient clinic of the Department of Dermatology at Sahlgrenska University Hospital were included. Twenty out of the 40 patients were also included as subjects in Paper III. Exclusion criteria were: pregnancy/lactation; ongoing other severe chronic or systemic disease, e.g., liver disease, kidney disease, cancer or infectious disease; treatment with oral steroids or other immunosuppressive/anti-inflammatory drugs and/or antibiotic treatment; ongoing sun-bed use or sun-bed use during the last 4 weeks and/or sun holiday during the past 4 weeks.

A cross-sectional study was performed to test the free hormone hypothesis for vitamin D, to explore correlations between directly measured free 25(OH)D levels in serum and psoriasis disease severity, and to identify the strongest determinants for serum DBP levels. PASI, VAS and high sensitivity CRP (hsCRP) were used as measures for psoriasis disease severity.

Table 1. *The table below gives an overview of the four studies and the subjects included.*

Paper	I	II	III	IV
Design	Pilot study	Case-control and treatment study	Prospective observational study	Cross-sectional study
Subjects	1 bio-naïve subject with psoriasis, Caucasian. 4 biopsies	68 bio-naïve subjects with psoriasis, Caucasians 105 controls	20 bio-naïve subjects with psoriasis, Caucasians 15 controls	40 bio-naïve subjects with psoriasis, Caucasians (20 subjects from Paper III)
Matching criteria for the controls	-	Age and sex Season of inclusion was considered	Age, sex, and season of inclusion	-
Inclusion year	2014	2006-2007 2008 (controls)	2013-2017	2013-2017
Sex (M/F)	0/1	51/17	13/7	25/15
Age (mean years±SD)	65	54.4 ±15.7	51±9	47±15
Duration of psoriasis (mean years±SD)	35	29.5±15.3	28±12	24±14
Arthropathy	No	N/A	15/20	25/40

3.2 METHODS

3.2.1 Paper I

Biopsies

In total, 4 biopsies (4 mm punch biopsy) were taken, 2 biopsies before the initiation of NBUVB phototherapy and 2 biopsies after the last session. One biopsy was taken from lesional and one from perilesional skin at each occasion. The location of the biopsy was strictly defined in advance to ensure that the biopsies taken after the completion of the phototherapy would represent the same area of the body and the same psoriatic lesion as those taken before the phototherapy. The deep-frozen skin biopsies were sliced to 10-µm-thin slices at Histocenter (Gothenburg, Sweden). The samples from perilesional skin before phototherapy could not be analyzed since the surface after the sectioning was not plane enough.

Time-of-flight secondary ion mass spectrometry (ToF-SIMS)

ToF-SIMS analysis was performed using a TOF.SIMS 5 instrument (ION-TOF GmbH, Münster, Germany).

Mechanism of function

Primary ions are bombarded with the help of a cluster ion gun aimed towards a tissue surface. When the primary ions collide with the sample's surface, their energy is transported to the molecules on the surface of the examined sample and some of them acquire a high enough energy that they detach from the surface (secondary ions) (*Figure 6*). These secondary ions are then accelerated to gain the same kinetic energy and travel through the equipment towards a detector. The speed and consequently the time it takes for the ions to travel towards the detector depends on their mass (lighter molecules travel faster and heavier molecules slower). In this way, by measuring the time for travel (time-of-flight) the different molecules can be separated according to their mass-to-charge ratio (m/z). A mass spectrum (total ion signal) is obtained that can be analyzed as images, resembling those obtained from an electron microscope.

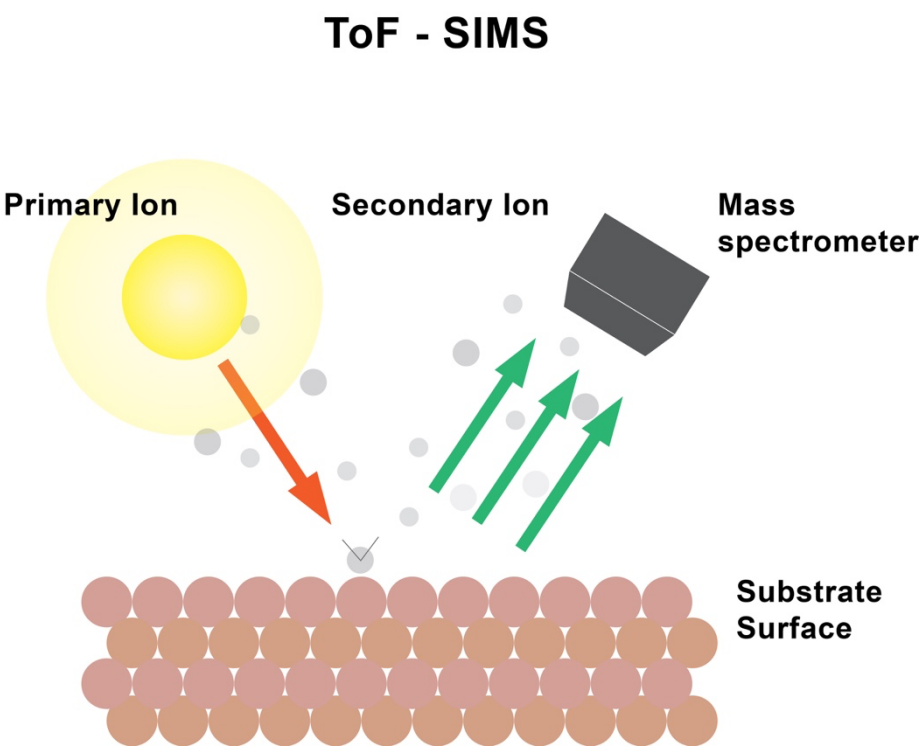


***Figure 6**. ToF SIMS, mechanism of function. Primary ions are bombarded with the help of a cluster ion gun aimed towards a tissue surface. When the primary ions collide with the sample's surface, their energy is transported to the molecules on the surface of the examined sample and some of them acquire a high enough energy that they detach from the surface (secondary ions).*

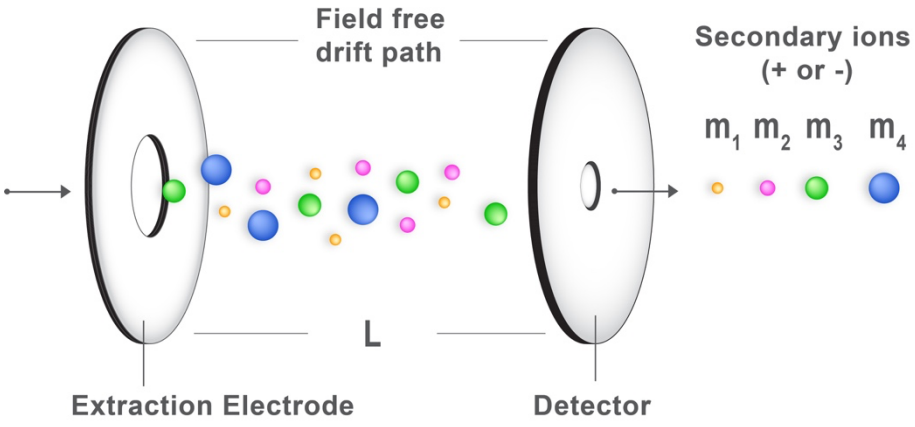


Figure 7. *ToF SIMS, mechanism of function. The secondary ions are accelerated to gain the same kinetic energy and travel through the equipment towards a detector. The speed and consequently the time it takes for the ions to travel towards the detector depends on their mass. In this way, by measuring the time for travel (time-of-flight) the different molecules can be separated according to their mass-to-charge ratio (m/z).*

3.2.2 Papers II-IV

Season of inclusion

We categorized the subjects into those recruited during the summer and those recruited in the winter. Summer was defined as the period from April to September when the UV index in Gothenburg is ≥3 and vitamin D production in the skin is possible. Winter was defined as the period from October to March, when the UV index in Gothenburg is <3 and production of vitamin D through the skin is not possible. Season of inclusion was taken into consideration when matching was done between patients and controls. In Paper II, the chosen controls were recruited during the same months as the patients with psoriasis (March-June and November-March). In Paper III, the healthy controls were matched to patients with respect to season.

Questionnaires

Paper II

Information on psoriasis debut, sun habits, sun holidays, concomitant medication, and smoking was obtained for the patients with psoriasis. Unfortunately, information on sun habits and sun holidays was not available for the healthy controls from the WHO MONICA project in Gothenburg, Sweden, and therefore this information was not presented for the patients with psoriasis either.

Paper III and IV

Information on psoriasis debut, presence of arthropathy, sun habits, sun holidays, intake of fish meals per week, detailed medical history, and concomitant medication was obtained for both patients and controls.

Anthropometry

Body weight was measured on a digital scale without shoes and with light clothing. Height was measured on a height scale that was wall-mounted, also without shoes. BMI was calculated as weight in kilograms divided by height in meters squared.

Vitamin D analyses

Paper II

Total 25(OH)D and 1,25$(OH)_2$D levels in serum samples were measured using ^{125}I RIA (radioimmunoassay, Diasorin, Stillwater, MN, USA).

Papers III and IV

Total 25(OH)D serum levels were measured with the electrochemiluminescence immunoassay (ECLIA), Elecsys Vitamin D Total II assay, a certified method according to United States Centers for Disease Control and Prevention Vitamin D Standardization-Certification Program (CDC VDSCP).
Free vitamin D in serum was directly measured with a two-step ELISA developed by Future Diagnostics Solutions, based on monoclonal antibodies from DIAsource ImmunoAssays. In the first step, free 25(OH)D found in the serum binds to an anti-vitamin D monoclonal antibody which is found on a microtiter plate. After washing away excess serum, biotin-labeled 25(OH)D in a known amount is added in order to react with the remaining unoccupied binding sites on the microtiter plate. After a second washing step, streptavidin-peroxidase conjugate is added and the amount of the bound enzyme is

measured through spectrophotometry. The level of free 25(OH)D is inversely proportional to the intensity of the signal.

$1,25(OH)_2D$ was analyzed with an automated CLIA.

Measurement of DBP

Papers II-IV

DBP serum levels were measured using the same method in all studies, a monoclonal ELISA (R&D systems).

Other biochemistry

Serum intact PTH was measured using an immunochemical luminescence method in Paper II while in Papers III and IV it was done using ECLIA. Remaining biochemistry analyses were performed with standardized laboratory techniques in an accredited laboratory (the Biochemistry Laboratory at Sahlgrenska University Hospital, Gothenburg, Sweden).

UVB phototherapy

Paper I

The patient was treated with whole body NBUVB according to a standardized protocol. Successively increasing doses from 0.3 j/cm^2 to 3 j/cm^2 were administered over a total of 24 sessions.

Paper II

The patients with psoriasis were treated with either whole body NBUVB or broadband UVB according to their personal preference, which was based on their previous personal experience with the respective modality. A standardized protocol with successively increasing doses adjusted according to the patient's skin type was also applied in this study.

Treatment with etanercept

Paper III

The 20 patients with psoriasis who fulfilled the criteria for biologic treatment and were previously not exposed to biologics were treated with etanercept via subcutaneous injection, 50 mg weekly for 24 weeks.

Assessment of psoriasis disease severity

Papers I and II

PASI was used to assess the severity and later any improvement of the disease after phototherapy.

Paper III

PASI, VAS, and DLQI were used as measures for disease severity and its impact on the patients' quality of life.

Paper IV

PASI and VAS were used.

Blood pressure

Paper III

Each patient's blood pressure was taken after a minimum of 10-minutes rest and the test was repeated at least once before the end of the visit.

Calculation of free, bioavailable 25(OH)D and free 25(OH)D index

The following formulas were used according to Bikle (120):

$$\text{Calculated free 25(OH)D} = \frac{\text{Total 25(OH)D}}{1+(6\text{x}10^{5}\text{x[Albumin]}+(7\text{x}10^{8}\text{x[DBP]})}$$

$$\text{Bioavailable 25(OH)D} = (6\text{x}10^{5}\text{x[Albumin]}+1) + \text{calculated free 25(OH)D}$$

$$\text{Free 25(OH)D index} = \frac{\text{Total 25(OH)D}}{\text{DBP}}$$

$$\text{Percentage of free 25(OH)D} = \frac{\text{free 25(OH)D}}{\text{Total 25(OH)D}}$$

3.3 ETHICAL CONSIDERATIONS

Each patient was given detailed oral and written information about the study. Written informed consent was obtained from all participants. The ethical principles as defined by the Declaration of Helsinki were followed.

All of the included studies were approved by the Ethics Committee at the University of Gothenburg and the Swedish National Data Inspection Board.

Papers I, III and IV: Reference number (dnr) 089-12, approved 2012-05-12. Complementary applications T763-12, approved 2012-10-29, and T663-14, approved 2014-09-09.

Paper II: Dnr 025-06, approved 2006-02-20, for the psoriasis patients receiving phototherapy. Dnr 088-06 approved 2006-05-22 and dnr T282-11, approved 2011-03-22, for WHO MONICA**.**

3.4 STATISTICS

Papers II-IV

All data were analyzed using R version 3.5.3 (The R Foundation for Statistical Computing, Vienna, Austria).

Simple descriptive statistics were performed. Non-parametric tests were used to avoid making any assumptions about the distribution of the data. All tests were two-sided and a *P*-value below 0.05 was considered statistically significant. Spearman's correlation was used to measure the degree of association between two variables. Fisher's exact test and Wilcoxon's rank sum test were used for two sample tests and Wilcoxon's signed rank test was used for paired tests.

Paper III

In order to test for a change over time and at the same time to control for different baselines for different patients, Spearman's test stratifying with respect to patient was used, yielding one *P*-value.

4 RESULTS

4.1 PAPER I

4.1.1 Visualization of vitamin D metabolites

Vitamin D_3, $25(OH)D_3$ and $1,25(OH)_2D_3$ could be distinguished and were visualized in both psoriatic lesional and perilesional skin. Vitamin D_3 could not be distinguished from 7-DHC but could be distinguished from cholesterol, which is the precursor of 7-DHC. $1,25(OH)_2D_3$ could not be distinguished from its major catabolite, $1,24,25(OH)_3D_3$, (*Figure 8)*.

4.1.2 Distribution and appreciation of the quantity of vitamin D metabolites in the skin

The distribution of vitamin D metabolites was quite homogenous in the epidermis and dermis but a higher amount was appreciated at the basal part of the epidermis compared to the upper part of the epidermis and in perilesional skin. The highest amount of vitamin D metabolites was noted in perilesional skin after NBUVB phototherapy.

4.1.3 Morphology of the skin and information on a subcellular level

Information about the morphology of the skin was obtained and a decrease in the thickness of the epidermis was noted after NBUVB phototherapy. High lateral resolution could be reached in the sample from perilesional skin after NBUVB phototherapy and important information was received regarding the location of vitamin D_3 at subcellular level. Vitamin D_3 was localized on the cell membrane and in the cytoplasm of the keratinocytes, (*Figure 9)*.

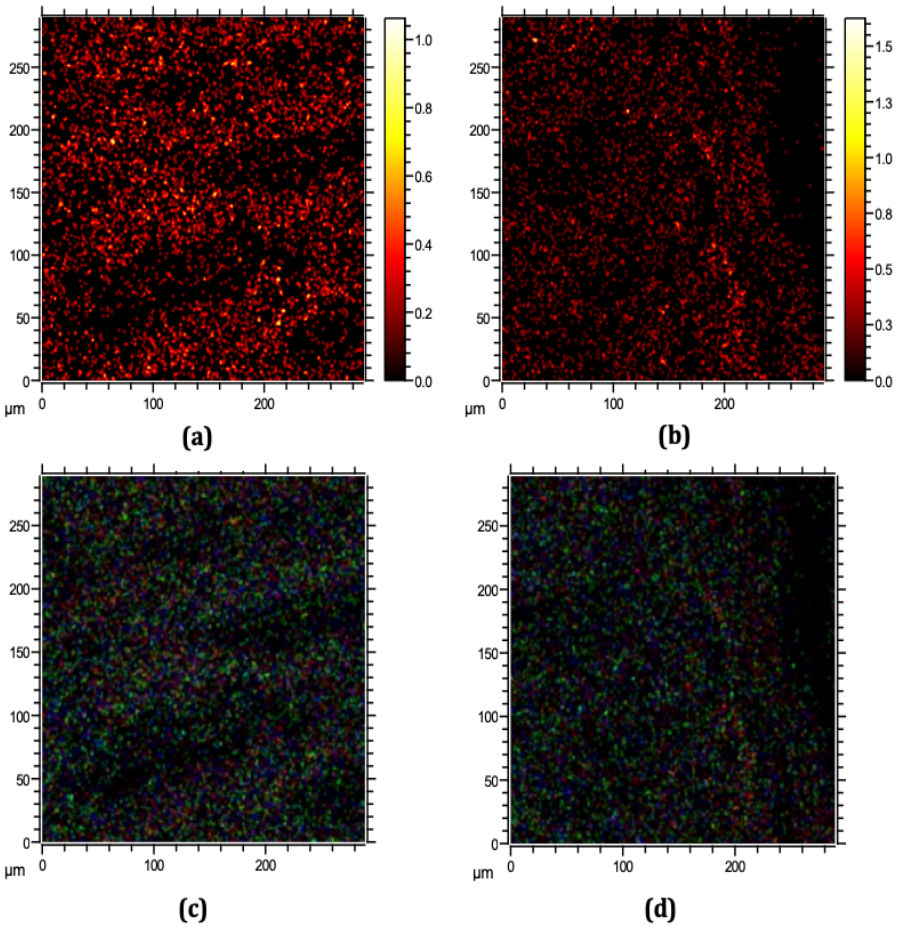


Figure 8. *High lateral resolution ToF-SIMS images with a field of view of 289x289 µm showing distribution of vitamin D_3 [M-OH]+ at m/z 367.3 in (a) the basal part of epidermis and (b) the superficial part of epidermis in lesional psoriatic skin (LS) before NBUVB, (c) and (d) overlay images showing vitamin D_3 [M-OH]+ at m/z 367.3 in red, 25OHD_3 [M-OH]+ at m/z 383.3 in green and 1,25(OH)2D_3 M-OH]+ at m/z 399.3 in blue for (a) and (b) respectively. In these images one can appreciate a tendency to higher levels of vitamin D_3 in the basal part of epidermis. Reproduced with permission, Vandikas MS et al, Imaging of vitamin D in psoriatic skin using time-of-flight secondary ion mass spectrometry (ToF-SIMS): A pilot case study. The Journal of Steroid Biochemistry and Molecular Biology. 2019;189:154-60.*

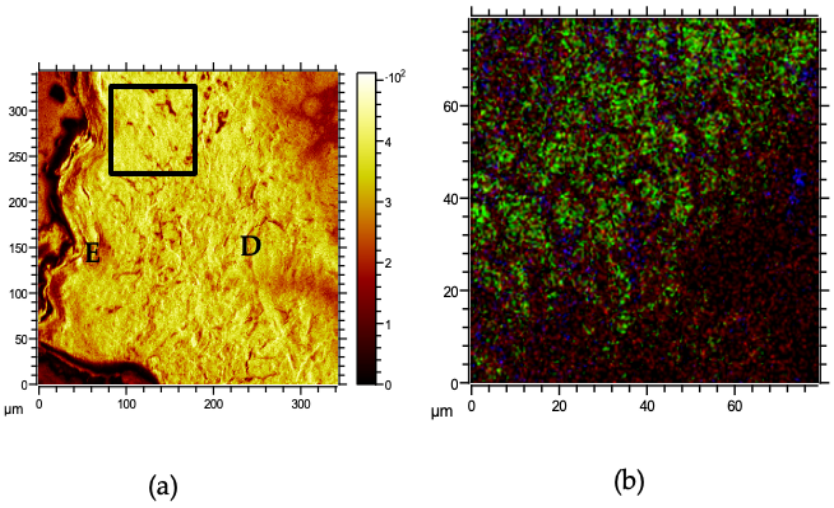


Figure 9. *ToF-SIMS images from perilesional, clinically unaffected skin (PS) after NBUVB) (a) Total ion picture (b) 80x80 µm² field of view overlay image obtained using the Burst align mode in the TOFSIMS of the area indicated with a black box in (a) showing the distribution of vitamin D_3 ($[M\text{-}OH]^+$ at m/z 367.3) in red, cholesterol ($[M\text{-}OH]^+$ at m/z 369.3) in green and phosphatidylcholine(PC) headgroup at m/z 184 in blue. PC headgroup is a fragment of phospholipids in the cell membrane and thus a good indicator of cell boundaries. In figure 9b, vitamin D_3 seems to be partly co-localized with the PC-headgroup but also fill out the spaces framed by the PC-headgroup which in a ToF-SIMS experiment generally indicate the intracellular space. One can therefore draw the conclusion that vitamin D_3 seems to be present both in the membrane part and the cytoplasmic or nuclear part of the keratinocyte. Reproduced with permission, Vandikas MS et al, Imaging of vitamin D in psoriatic skin using time-of-flight secondary ion mass spectrometry (ToF-SIMS): A pilot case study. The Journal of Steroid Biochemistry and Molecular Biology. 2019;189:154-60.*

4.2 COMPARISON BETWEEN THE STUDIES, PAPERS II-IV

Table 2. *The table below gives an overview of the results for the patients with psoriasis regarding the major variables for Papers II-IV.*

Variable	*Paper II*	*Paper III*	*Paper IV*
Serum DBP (µg/mL)	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
Baseline	350 ± 181	234 ± 54	226 ± 52
After treatment	338 ± 164	202 ± 39	-
Serum 25(OH)D (nmol/L)	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
Baseline	90 ± 35*	74 ± 31	69 ± 26
< 50	5 / 68 (7%)	3 / 20 (15%)	9 / 40 (23%)
≥ 50	63 / 68 (93%)	17 / 20 (85%)	31 / 40 (78%)
≥ 75	41 / 68 (60%)	9 / 20 (45%)	15 / 40 (38%)
	Mean ± SD	**Mean ± SD**	**Mean ± SD**
After treatment	151 ± 49*	76 ± 23	-
< 50	0 / 68 (0%)	2 / 17 (10%)	-
≥ 50	68 / 68 (100%)	15 / 17 (75%)	-
≥ 75	66 / 68 (97%)	9 / 17 (45%)	-
Directly measured free 25(OH)D (pmol/L)	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
Baseline	-	12 ± 4.0	10 ± 3.4
After treatment	-	12 ± 2.8	-
Calculated free 25(OH)D (pmol/L)	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
Baseline	23 ± 14	24 ± 11	23 ± 9.0
After treatment	40 ± 20	27 ± 8.6	-
Serum iPTH (pmol/L)	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
Baseline	3.5 ± 1.8**	3.8 ± 1.7	3.7 ± 1.3
After treatment	2.9 ± 1.5**	4.5 ± 2.8	-
PASI	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
Baseline	9.0 ± 4.7	13 ± 5.4	11 ± 4.9
After treatment	2.6 ± 1.6	2.8 ± 2.7	-

*values originally measured in ng/ml were converted to nmol/L for comparison, using the converting factor 2.496.
**values originally measured in ng/L were converted to pmol/L.
SD, standard deviation. iPTH, intact parathyroid hormone. PASI, Psoriasis Area Severity Index.

4.2.1 Serum DBP levels

The levels of DBP were significantly higher in the psoriasis cohort included in Paper II compared to the ones in Paper III and Paper IV (P=0.006 and P= 0.0001 respectively). This difference could not be explained by sex and age. However, the mean duration of psoriasis was significantly higher in Paper II versus Paper IV (P=0.032) but not Paper II versus paper III (P=0.30). Furthermore, there were no data for arthropathy in the psoriasis cohort in Paper II.

Compared to baseline, DBP serum levels remained unaltered after the UVB phototherapy (Paper II) but reduced significantly after 24 weeks of etanercept treatment (Paper III).

4.2.2 Serum total 25(OH)D levels

The serum levels of total 25(OH)D were higher in the psoriasis cohort in Paper II compared to the ones in Paper III and Paper IV. Compared to baseline, 25(OH)D levels increased significantly after UVB phototherapy (Paper II) while they remained unaltered during 24 weeks of etanercept treatment (Paper III).

4.2.3 Serum iPTH

Compared to baseline, the levels of intact iPTH decreased in the patients with psoriasis in Paper II after UVB phototherapy, while they increased after 24 weeks of etanercept treatment.

4.3 PAPER II

Major results

4.3.1 Comparison between the patients with psoriasis and the population-based controls at baseline

DBP, total 25(OH)D, and free 25(OH)D index were higher in the subjects with psoriasis compared to healthy controls (P=0.004, P=<0.0001 and P=0.045 respectively).

DBP levels were similar between men and women and no seasonal variation was noted (summer versus winter). No correlation was found between DBP and total 25(OH)D serum levels nor between DBP serum levels and PASI scores.

Confounding factors regarding the serum levels of DBP like medication (hormonal contraceptives and aspirin use), smoking or other comorbidities did not differ between the patients and the controls.

4.3.2 Changes in the different variables before and after UVB phototherapy in patients with psoriasis

DBP serum levels remained unaltered (P=0.21) while total 25(OH)D and all of the other calculated vitamin D metabolites increased compared to baseline. PASI scores improved and iPTH decreased compared to before UVB phototherapy. Baseline levels of DBP and 25(OH)D in serum did not affect the improvement in PASI.

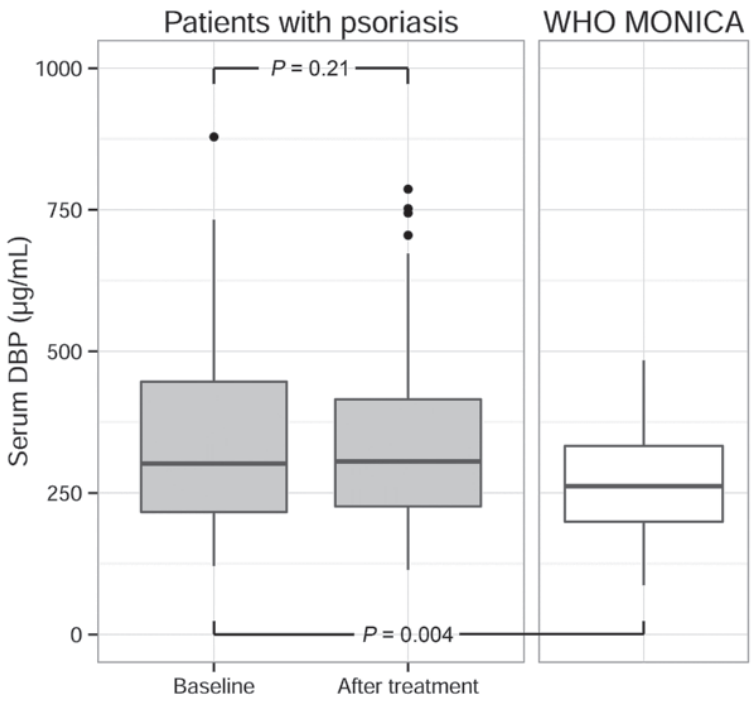


Figure 10. *Vitamin D-binding Protein (DBP) in the patients with psoriasis before and after phototherapy treatment and in comparison to levels in healthy individuals from the WHO MONICA cohort. Reproduced with permission, Vandikas MS et al, High levels of serum vitamin D-binding protein in patients with psoriasis: A case-control study and effects of ultraviolet B phototherapy. The Journal of Steroid Biochemistry and Molecular Biology. 2021;211:105895.*

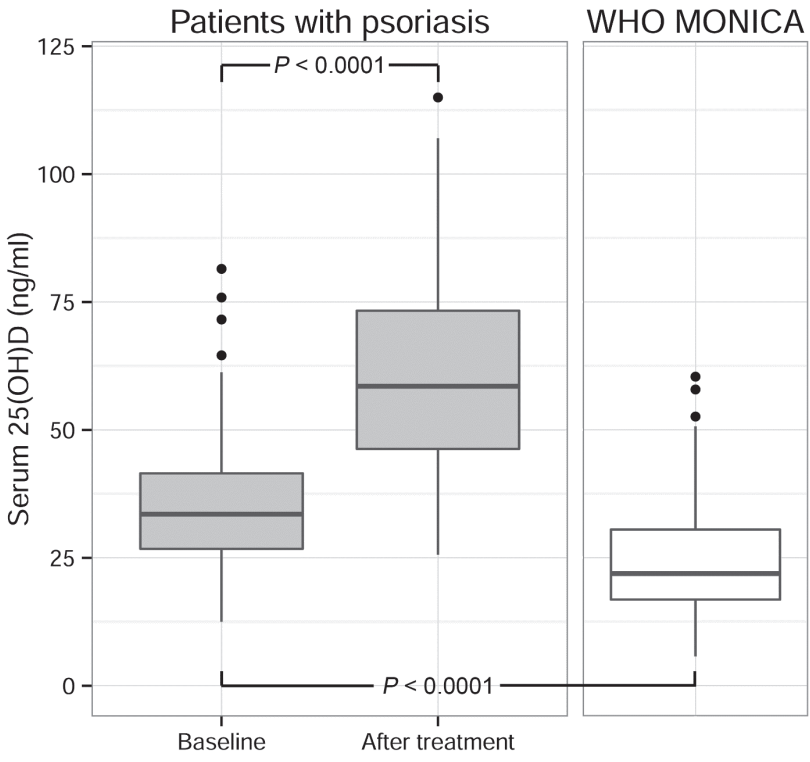


Figure 11. *Serum total 25(OH)D levels in patients with psoriasis before and after phototherapy treatment and in comparison to levels in healthy subjects from the WHO MONICA cohort. Reproduced with permission, Vandikas MS et al, High levels of serum vitamin D-binding protein in patients with psoriasis: A case-control study and effects of ultraviolet B phototherapy. The Journal of Steroid Biochemistry and Molecular Biology. 2021;211:105895.*

4.4 PAPER III

Major results

4.4.1 Comparison between patients with psoriasis and healthy controls at baseline

The patients with psoriasis had higher levels of both total and directly measured 25(OH)D compared to healthy controls. The levels of DBP were similar between the two groups but in a subsample analysis, it was found that serum DBP levels were higher in the patients with psoriasis reporting arthropathy compared to those who did not report arthropathy. HsCRP was higher in the serum of patients with psoriasis compared to healthy controls.

No correlation between hsCRP and DBP serum levels or hsCRP and 25(OH)D serum levels was found in either group.

4.4.2 Changes in the patients with psoriasis on etanercept treatment and the healthy controls during the 24-week follow-up

Psoriasis disease severity measured with PASI, DLQI, and VAS improved already after 10 weeks of treatment with etanercept (P<0.001) and the effect remained at week 24 (*Figure 12*). HsCRP in serum decreased following treatment. However, hsCRP and DBP levels remained unaltered in healthy controls (P = 0.55 and P = 0.083, respectively).

DBP serum levels decreased after 24 weeks of etanercept treatment (P=0.003) (*Figure 13*). In a subsample analysis, it was observed that DBP levels decreased significantly only in the group of patients with self-reported arthropathy (P = 0.009 vs. P = 0.11 for those without self-reported arthropathy) (*Figure 14*).

Both total and free 25(OH)D serum levels increased (P = 0.029 and P = 0.028, respectively) in healthy controls but not in the patient group.

All serum levels of vitamin D metabolites with the exception of calculated free 25(OH)D remained unaltered on etanercept treatment but iPTH levels increased.

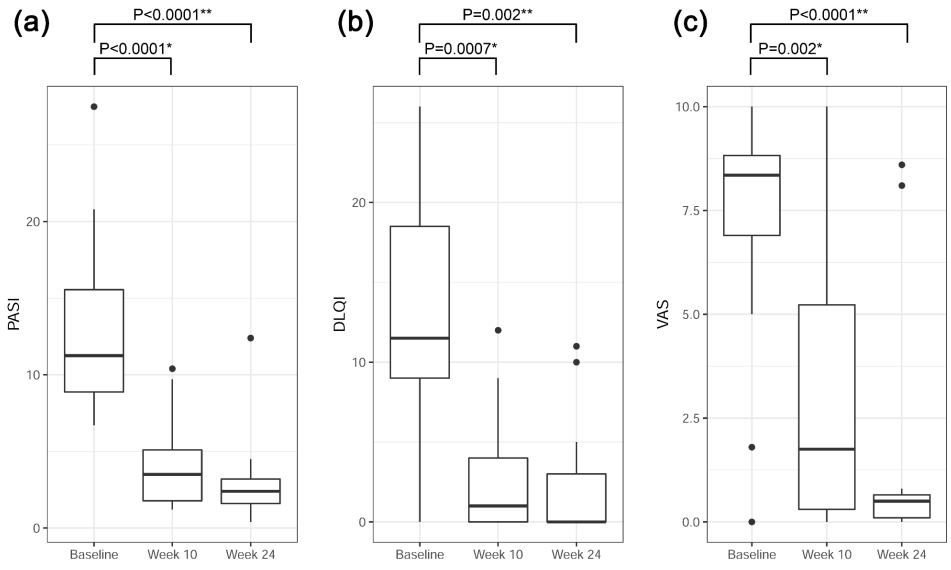
Regarding cardiovascular risk factors, total cholesterol and triglycerides remained unaltered in both groups but BMI increased (P = 0.026) during etanercept treatment. Both systolic and diastolic blood pressure decreased in the healthy controls but only systolic blood pressure decreased during etanercept treatment.

4.4.3 Sun habits during the etanercept study

Total days of sun holiday per individual per group were similar between the two groups (P = 0.39).

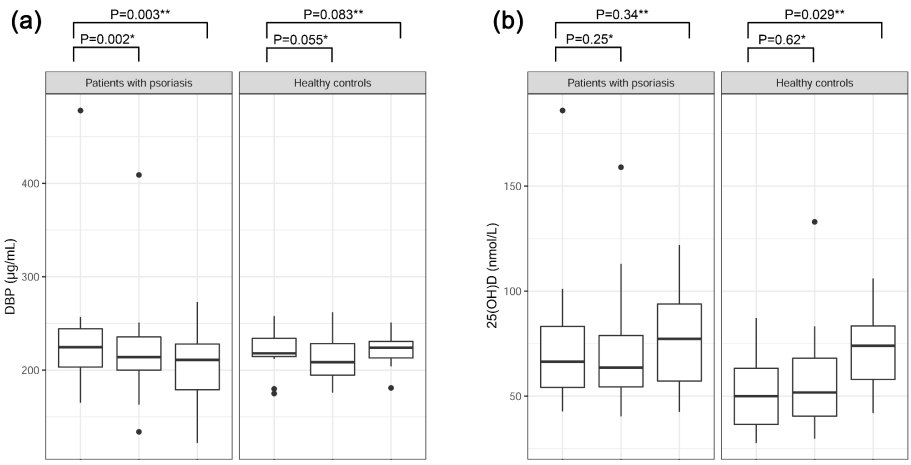
4.4.4 The significance of serum 25(OH)D levels at baseline as a predictor of the treatment

The group of patients with sufficient 25(OH)D levels at baseline, defined as ≥ 75 nmol/L, had a greater reduction on the VAS (mean reduction 9.1 versus 5.5, P = 0.023) compared to those with 25(OH)D levels <75 nmol/L and showed a trend (P = 0.092) towards achieving PASI75 (a 75% reduction or more of PASI) at week 10 of the etanercept treatment. Furthermore, the 25(OH)D sufficient group showed a greater improvement on the DLQI (mean reduction 19.4 *versus* 16.9, P = 0.045) compared to the 25(OH)D-insufficient group.



**P value using Wilcoxon's signed rank test for changes from baseline to week 10.*
***P value using Spearman's test, stratifying with respect to patient, for comparing the changes within each group.*

***Figure 12**. Boxplots showing (a) Psoriasis Area Severity Index (PASI), (b) Dermatology Life Quality Index (DLQI) and (c) Visual Analogue Scale (VAS) in patients with psoriasis treated with etanercept, at baseline, after 10 weeks, and after 24 weeks.*



*P value using Wilcoxon's signed rank test for changes from baseline to week 10.
**P value using Spearman's test, stratifying with respect to patient, for comparison of changes within each group.

Figure 13. *Boxplots comparing the levels of (a) vitamin D-binding protein (DBP) and (b) total 25(OH)D between patients with psoriasis treated with etanercept and healthy controls. Data are shown for baseline, after 10 weeks and after 24 weeks. Reproduced with permission, Vandikas MS et al. Impact of etanercept on vitamin D status and vitamin D-binding protein in bio-naïve patients with psoriasis, Acta Dermato-Venereologica, in press 2021.*

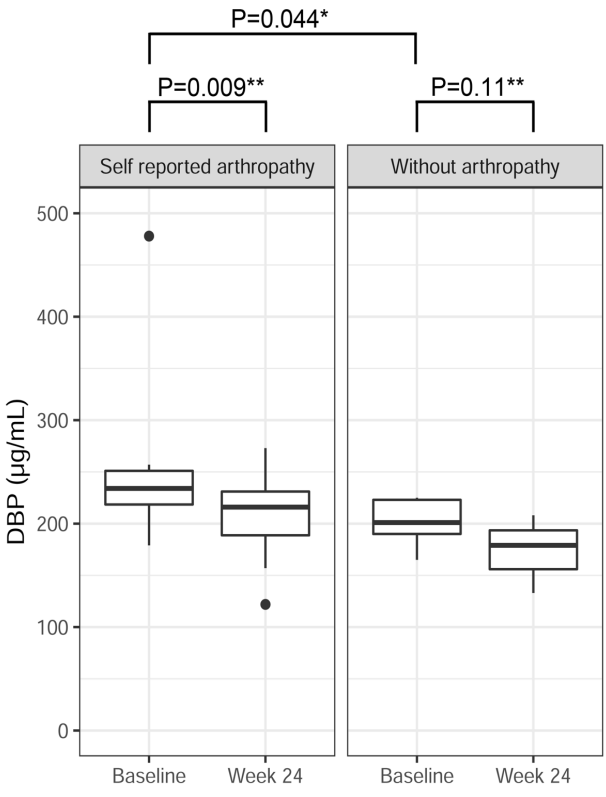


Figure 14. *Boxplots comparing the levels of vitamin D-binding protein (DBP) in the subsamples of patients with psoriasis, with self-reported arthropathy, and without arthropathy. Data are shown for baseline and after 24 weeks of etanercept treatment. *P value using Wilcoxon's signed rank test for comparing DBP levels at baseline between the two subgroups. **P value using Spearman's test, stratifying with respect to patient, for comparison of changes within each group over time. Reproduced with permission, Vandikas MS et al. Impact of etanercept on vitamin D status and vitamin D-binding protein in bio-naïve patients with psoriasis, Acta Dermato-Venereologica, in press 2021.*

4.5 PAPER IV

Major results

4.5.1 The free hormone hypothesis for vitamin D

Total 25(OH)D serum level was a reliable measure for vitamin D status in this psoriasis cohort as total and directly measured free 25(OH)D levels correlated well (ρ=0.77, P<0.0001), and both were negatively correlated to iPTH levels (ρ= –0.33, P=0.038 and ρ= –0.40, P=0.010 respectively).

The levels of calculated free 25(OH)D were higher than the levels of directly measured free 25(OH)D.

4.5.2 Vitamin D metabolites and psoriasis disease severity

No correlations were found between free and/or total 25(OH)D serum levels and hsCRP, PASI, and VAS. There was no difference in disease severity between vitamin D-sufficient [25(OH)D≥75 nmol/L] and vitamin D-insufficient subjects.

A negative association was found between total 25(OH)D levels and the percentage of free 25(OH)D (ρ=-0.66, P<0.001) in serum.

HsCRP serum levels correlated positively with BMI (ρ=0.52, P<0.001).

4.5.3 DBP determinants

Higher DBP levels were found in patients with self-reported arthropathy compared to those without (mean value 239 µg/mL versus 201 µg/mL, P=0.002).

DBP levels were independent of season of inclusion, age, sex, duration of disease, disease severity (hsCRP, PASI, VAS), BMI, and smoking status.

5 DISCUSSION

5.1 MAIN FINDINGS AND CLINICAL IMPLICATIONS

5.1.1 Imaging of vitamin D and its metabolites in psoriatic skin using ToF-SIMS

The controversies around the role and importance of vitamin D and the complexity of its endocrine and metabolic pathway advocate for the use of new methods to investigate in depth the *in loco* synthesis, metabolism, and catabolism of vitamin D. Measurements of vitamin D and its metabolites in the blood most probably do not reflect their presence and activity in the different tissues where they exert important extra-skeletal effects in an autocrine and paracrine manner.

The skin constitutes an excellent example of tissue that can be used for this purpose since it is directly and easily available for sample taking and it is the only organ that autonomously performs all the steps of the vitamin D pathway.

We contribute with a method to study the whole vitamin D pathway in the psoriatic skin and in combination with other techniques like immunohistochemistry, this work opens the way for a complete investigation of vitamin D metabolism and the inflammatory process in psoriasis. Furthermore, ToF-SIMS is a great tool to use to study the local effects of phototherapy and other topical treatments.

Through better understanding of the mechanisms of action of phototherapy and vitamin D in psoriatic skin, we will be able to gain insight on the pathogenesis of psoriasis.

5.1.2 Vitamin D-binding protein as a new potential inflammatory biomarker in psoriasis

In a cohort of 68 subjects with psoriasis (Paper II), DBP serum levels at baseline were higher compared to population-based controls. Further on in this thesis work, as found in Papers III and IV, high DBP levels were associated with the presence of arthropathy and finally, 24 weeks of etanercept treatment reduced the levels of DBP only in those patients with reported arthropathy

(Paper III). Meanwhile, UVB phototherapy, which is a less potent treatment regarding the systemic inflammation in psoriasis, did not affect the measured serum DBP levels (Paper II). Possible confounding factors for DBP that were investigated were otherwise similar between the patients and the controls and between the patients with self-reported arthropathy and those without. Furthermore, there were no correlations between DBP and season of inclusion or PASI.

These results have led to the conclusion that DBP might be a new inflammatory marker in psoriasis, especially in PsA, and a possible marker of treatment response. Given the need for the discovery of new inflammatory biomarkers for the early detection of PsA, this finding warrants further investigation.

A limitation of this study is that there were no clinical data about the self-reported arthropathy. The question the patients were asked to answer was whether they experienced any problems from the joints. This could include both arthralgia and/or morning stiffness. The presence of PsA was not verified by a rheumatologist. Therefore, the self-reported arthropathy might reflect other kinds of joint problems besides PsA. Unfortunately, there were not any follow up data either, in order to study the effect of etanercept on the self-reported arthropathy.

5.1.3 A possible synergic effect between vitamin D and etanercept (Paper III)

TNFis constitute established and effective treatment choices in psoriasis even if many new and disease specific treatments have been developed. There are individuals who do not respond adequately or who show a relapse despite an initial response. In the etanercept study (Paper III), it was shown that patients with adequate pre-treatment 25(OH)D levels defined as ≥75 nmol/L had a partly faster and greater improvement in psoriasis compared to those with 25(OH)D levels <75 nmol/L. Similar results indicating a synergic effect between vitamin D and TNFis were shown in different *in vitro* and *in vivo* studies in patients with IBD and RA regarding different aspects of this synergy: *i.* positive correlation of vitamin D concentration and trough levels of TNFis (infliximab and adalimumab) (121), *ii.* positive association between vitamin D levels and a greater odds of remission with TNFis (112), *iii.* vitamin D insufficiency (serum levels <75 nmol/L) was associated with earlier cessation of TNFi treatment and loss of response (122), and *iv.* enhancement of the anti-inflammatory effect (110, 111).

The above results urge the need for larger clinical trials with vitamin D supplementation before and during TNFi treatment.

5.1.4 Vitamin D status in psoriasis (Papers II, III and IV) and the optimal level for sufficiency

In Papers II and III, patients with psoriasis had significantly higher levels of total 25(OH)D compared to controls, which is in contrast to previously published studies in which either no difference or lower levels were found (96). This is difficult to explain but there are several possible confounding factors that might be implicated. The most important confounding factor is the method used for 25(OH)D measurement, as comparison across studies when the gold standard method for 25(OH)D measurement (LC-MS/MS) was not used is confounded by the variability of the accuracy of different immunoassays.

Other confounding factors might be the frequency of previous UVB phototherapies and sun habits in the studied populations. This kind of information is not always given and especially regarding sun habits and sun exposure, it can be very difficult to quantify and standardize for comparison between studies.

The duration of the disease might also indirectly affect vitamin D status. The realization of the beneficial effect of heliotherapy on psoriatic skin lesions might cause a change in the sun habits of the individual over time and after being diagnosed with psoriasis. This is something that needs to be further investigated.

Furthermore, a possible confounding factor for this difference is how the definition of sufficiency and insufficiency was made. In the current studies, when defining vitamin D sufficiency as total 25(OH)D >20 ng/ml or 50 nmol/L, the vast majority of patients will be characterized as vitamin D sufficient (93% Paper I, 85% Paper II and 78% Paper III), which is in contrast to other studies that have shown high prevalence of vitamin D insufficiency in patients with psoriasis (92, 123). When defining vitamin D sufficiency as total 25(OH)D ≥ 30 ng/ml or ≥75 nmol/L, then the prevalence of sufficiency drops to 60% in Paper II, 45% in Paper III and only 38% in Paper IV.

What the optimal vitamin D status is in psoriasis is insufficiently studied and defined. As shown in a recently published review article by McCullough *et al.* (42), many pre-treatment serum 25(OH)D levels in patients with psoriasis fell

within the current normal range of sufficiency, while many post-treatment concentrations fell outside the upper limit of this normal (>100 ng/ml). Nevertheless, patients with psoriasis showed significant clinical improvement without any adverse events of these high levels and treatments. This leads to the conclusion that the target serum concentration of 25(OH)D for a beneficial effect of vitamin D in psoriasis needs to be further examined and might lie much higher than the currently recommended levels of vitamin D sufficiency.

Another interesting study was recently published by Fu *et al.* showing, for the first time, that serum 25(OH)D concentration was significantly inversely associated with all-cause mortality among patients with psoriasis (124). This also warrants consideration and further investigation.

5.1.5 The assessment of calculated free 25(OH)D

Attempts to calculate free and bioavailable vitamin D may result in inaccurate results for different reasons. Firstly, the formulas used are dependent on the correct measurement of the concentration of total 25(OH)D, DBP, and albumin. The risk of introducing bias will be high if standardized methods for measurement of these compounds are not used.

Furthermore, the *in vivo* interaction between DBP and 25(OH)D is most probably different than that observed *in vitro* and the binding affinity constants reported might not be representative of the variation occurring *in vivo*.

Therefore, directly measured free 25(OH)D and even free 1,25(OH)2D may be superior for investigating vitamin D physiology in psoriasis than calculated free and bioavailable vitamin D. However, in Papers III and IV, even though calculated free was much higher than directly measured 25(OH)D, the two variables correlated well. This might depend on the very selective population studied, without other serious comorbidities and relatively low mean age. These relationships need to be investigated in large scale studies.

5.1.6 The relative importance of total 25(OH)D versus free 25(OH)D (Paper IV) and the optimal measure for vitamin D status

When testing the free hormone hypothesis for vitamin D in psoriasis (Paper IV), it was found that levels of total 25(OH)D correlated well with the levels of free 25(OH)D and both were inversely correlated to iPTH, an expected

physiological phenomenon. None of the two metabolites were correlated to psoriasis disease severity. This led to the conclusion that total 25(OH)D level seems to be a reliable marker for vitamin D status in psoriasis and not a confounding factor for the controversy regarding the results of the association of vitamin D levels and psoriasis disease severity.

However, some concern was expressed as the studied cohort had a relatively low mean age, did not have an overrepresentation of different comorbidities, and had no ongoing systemic treatment that could affect the levels of DBP nor the vitamin D metabolism and catabolism. Since there are data showing that DBP levels might be high in some cases in patients with psoriasis (125) and the levels of both total 25(OH)D (126) and free 25(OH)D (127, 128) can be affected by biologic treatment, it is wise to recommend the investigation of free 25(OH)D in future large scale studies in psoriasis.

The vitamin D metabolic pathway and vitamin D physiology are very complicated. Free 25(OH)D levels might be more relevant in situations with total 25(OH)D insufficiency. Adjusting mechanisms seem to exist especially in the extreme cases of insufficiency, as shown in Paper IV where the percentage of free 25(OH)D increased in a negative correlation to the levels of 25(OH)D.

Measuring a single metabolite in order to estimate the functionality of the complex vitamin D endocrine/autocrine/paracrine system and to define hypovitaminosis D is most probably not pragmatic. There is a need to simultaneously investigate different vitamin D metabolites and compounds involved in vitamin D physiology, e.g., the vitamin D metabolite ratio [total 25(OH)D divided by 24,25$(OH)_2$D] (129), the activity and expression of the different metabolizing enzymes, e.g., 24-hydroxylase, and even in some cases, the expression of VDR (85) that could help distinguish “responders” from “non-responders” to vitamin D. Lastly, regarding specific tissues like the skin, correlation of the findings in serum with direct visualization and quantification of the metabolites in the skin, using for example ToF-SIMS, could be the only way to find answers regarding the real role of vitamin D in different skin diseases.

5.2 METHODOLOGICAL CONSIDERATIONS AND LIMITATIONS

5.2.1 The methods used for measurement of vitamin D metabolites

The gold standard method for measurement of total 25(OH)D was not used. Instead ^{125}I RIA (radioimmunoassay, Diasorin, Stillwater, MN, USA) was used in Paper II. This assay is not a certified method according to the CDC VDSCP, which makes comparison between the studies difficult.

In Paper III and IV, total 25(OH)D was measured with ECLIA using the Elecsys Vitamin D Total II assay, which is a certified method but with high coefficient of variance, 12% at 66 nmol/L and 17% at 26 nmol/L.

The method used for the measurement of direct free 25(OH)D (a two-step ELISA, Future Diagnostics Solutions) needs to be further standardized and validated in different pathological conditions and with altered amounts of DBP. The decreased affinity for 25(OH)D_2 might have resulted in an underestimation of the true concentration of the 25(OH)D_2 and thus total amount of free 25(OH)D (130).

5.2.2 The method used for DBP measurement

The monoclonal ELISA (R&D systems) that was used to measure DBP might lead to falsely low DBP levels when the GC1f polymorphism is overrepresented. The polyclonal ELISA is more accurate. We believe, however, that this might have not affected our results since there were no participants of African ancestry in whom the GC1f variant is more common.

5.2.3 Sample sizes and sex distribution

Use of hypertensives and anti-depressants was higher in the population-based control group (Paper II) compared to the patients with psoriasis, which is not in line with the overrepresentations of the metabolic syndrome and of depression that are reported in psoriasis. This is an example of how the small sample sizes may have affected the results.

Men were overrepresented in the psoriasis cohorts in our studies, which is common in studies within psoriasis.

5.2.4 Confounding factors for vitamin D

BMI is a known confounding factor for 25(OH)D levels and might eventually affect DBP levels (though this is still controversial), and this important data was missing in Paper II.

Furthermore, there was no information about other confounding factors like physical activity, alcohol intake, genetic polymorphisms in the metabolic enzymes, and VDR.

5.2.5 Information about health-related quality of life

Information about the impact of psoriasis on the quality of life of patients is an important parameter when examining the effect of a treatment and should be included in the assessment of disease severity. This information was missing in Papers II and IV.

5.2.6 Clinical data about the coincidence of PsA

As mentioned before, there were no clinical data about the self-reported arthropathy. Under this symptom, both PsA but also unspecific arthralgia could be included and the probability of patients unknowingly suffering from other diseases, like osteoarthritis and gout rather than PsA, cannot be excluded. There were not any follow up data either, so any treatment effect of etanercept could not be evaluated.

6 CONCLUSIONS

1. ToF-SIMS is a potentially powerful tool to use for the investigation of the vitamin D pathway in psoriatic skin and its autocrine and paracrine effects in the inflammatory process.
2. DBP might be a new inflammatory biomarker in psoriasis.
3. Patients with psoriasis do not seem to suffer from vitamin D insufficiency, as defined today with regard to the reference ranges recommended mainly for skeletal health. The optimal vitamin D status for psoriasis is not yet defined.
4. There might be a synergic effect between TNFi treatment (etanercept) and vitamin D, augmenting the treatment effect, that needs to be further investigated.
5. Measurement of total 25(OH)D in serum seemed to be a reliable marker for vitamin D status in psoriasis. There were no associations between vitamin D metabolites (total and free) and disease severity.
6. Measurement of free 25(OH)D in serum should be considered in patients with ongoing biologic treatment and with conditions that might affect DBP serum levels.

7 FUTURE PERSPECTIVES

1. It would be very interesting to study the vitamin D metabolic pathway and inflammatory response in psoriatic skin by combining ToF-SIMS with other methods, e.g. immunohistochemistry, and to use ToF-SIMS to investigate the local effect of different treatments.

2. The possible inflammatory role of DBP in psoriasis and PsA needs to be investigated in large-scale prospective studies, measuring not only serum DBP concentration but also defining the different genotypes and their possible relation to psoriasis.

3. Randomized controlled vitamin D supplementation studies including only patients with vitamin D insufficiency, preferably <50 nmol/L, and the measurement of a panel of vitamin D metabolites, the vitamin D metabolizing ratio, and the activity of vitamin-metabolizing enzymes should be considered. Furthermore, VDR polymorphisms as well as polymorphisms on the enzymes implicated in the vitamin D pathway should also be investigated.

4. Randomized controlled vitamin D supplementation studies during TNFi treatment should be conducted to further investigate any possible synergic effects.

5. To study the levels of vitamin D in new-onset psoriasis and to prospectively examine how vitamin D status and sun habits change during the course of the disease.

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