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Cécile Grudet

LUND
UNIVERSITY

LICENTIATE THESIS

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Title and subtitle: Vitamin D and inflammation in Major Depressive Disorder and suicidality

Abstract

Background and aim: The aim of the licentiate was to investigate the relationship between vitamin D and inflammation in cohorts of depressed and suicidal subjects. High blood inflammatory markers have been reported in Major Depressive Disorder (MDD) subjects compared to healthy controls, and this immune activation may be even greater in suicidal subjects. Vitamin D exerts anti-inflammatory effects in the body and therefore, vitamin D deficiency may be an underlying factor of the pro-inflammatory state often seen in individuals with MDD and individuals with suicidal behavior.

Methods: Study I: Cross-sectional study with data collected at Lund University Hospital. Fifty-nine patients with a recent suicide attempt, 17 non-suicidal patients with MDD and 14 healthy controls, randomly selected from the municipal population register in Lund, were enrolled in the study. Interleukin (IL)-1β, IL-6, Tumor Necrosis Factor (TNF)-α, $25(OH)D_2$ and $25(OH)D_3$, were analyzed.

Study II: Cross-sectional study with data collected at University of California, San Francisco. Forty-eight un-medicated, somatically healthy MDD subjects without a recent suicide attempt and 54 healthy controls were enrolled in the study. IL-6, TNF-α, Neutrophil-to-Lymphocyte ratio (NLR), White Blood Cell count (WBC), $25(OH)D_2$ and $25(OH)D_3$, were analyzed.

Results: There was a significant difference in vitamin D levels between subjects with a recent suicide attempt, non-suicidal MDD subjects and healthy controls in study I, with suicidal subjects having lowest vitamin D levels. Vitamin D levels did not differ significantly between non-suicidal MDD subjects and healthy controls (study II). In both studies, vitamin D correlated significantly and negatively with inflammatory markers.in depressed and suicidal individuals, but not in controls. *Conclusion:*

Our findings suggest that low vitamin D may contribute to a pro-inflammatory state in depressed and in suicidal individuals.

Key words: Vitamin D, inflammation, Major Depressive Disorder, suicidality, IL-6, TNF-α, NLR, WBC

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Dedication

I dedicate this Licentiate Thesis to my family, who have been invaluable supporters in every aspect of my life and in all different ideas I have decided to realize. I also dedicate this Licentiate Thesis to my beloved friends, Maria Nolvi and Marianne Eriksson, who also have been long time followers and supporters over the years. Lastly, I dedicate this Licentiate Thesis to myself and to my beautiful son, Vidar, who is one of the reasons I actually started this journey.

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List of papers

The licentiate thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I: Cécile Grudet, Johan Malm, Åsa Westrin, Lena Brundin
Suicidal patients are deficient in vitamin D, associated with a pro-inflammatory status in the blood
Psychoneuroendocrinology (2014) ***50****, 210—219.*

II: Cécile Grudet, Owen M. Wolkowitz, Synthia H. Mellon, Johan Malm, Victor I. Reus, Lena Brundin, Firdaus S. Dhabhar, Åsa Westrin, Daniel Lindqvist
Vitamin D and inflammation in Major Depressive Disorder
Manuscript

Abbrevations:

MDD:	Major Depressive Disorder
SI:	mild-to-moderate Suicidal Ideation
NSI:	non-(mild-to-moderate) Suicidal Ideation
HDRS:	Hamilton Depression Rating Scale
MADRS:	Montgomery-Åsberg Depression Rating Scale
IL-1β:	Interleukine-1β
IL-6:	Interleukine-6
TNF-α:	Tumor Necrosis Factor- α
NLR:	Neutrophil-to-Lymphocyte Ratio
WBC:	White Blood Cell count
Th1:	T helper type 1 cell
Th2:	T helper type 2 cell
Vitamin D:	vitamin D3, 25(OH)D3 ()
Active vitamin D:	1,25(OH2)D3
VDR:	vitamin D receptor
UVB:	ultraviolet B radiation

Abstract

Background and aim: The aim of the licentiate was to investigate the relationship between vitamin D and inflammation in cohorts of depressed and suicidal subjects. High blood inflammatory markers have been reported in Major Depressive Disorder (MDD) subjects compared to healthy controls, and this immune activation may be even greater in suicidal subjects. Vitamin D exerts anti-inflammatory effects in the body and therefore, vitamin D deficiency may be an underlying factor of the pro-inflammatory state often seen in individuals with MDD and individuals with suicidal behavior. Few studies to date have investigated the relationship between vitamin D and inflammation in psychiatric cohorts.

Methods: Study I: Cross-sectional study with data collected at Lund University Hospital. Fifty-nine patients, with diverse psychiatric diagnoses, were enrolled in the study after a suicide attempt. Seventeen non-suicidal patients with MDD were also included. Fourteen healthy controls were randomly selected from the municipal population register in Lund. Depression symptom severity was rated with Montgomery-Åsberg Depression Rating Scale (MADRS). Interleukin (IL)-1β, IL-6, Tumor Necrosis Factor (TNF)-α and vitamin D_2 ($25(OH)D_2$), vitamin D_3 ($25(OH)D_3$) were analyzed.

Study II: Cross-sectional study with data collected at University of California, San Francisco. Forty-eight un-medicated, somatically healthy MDD subjects without a recent suicide attempt and 54 healthy controls were enrolled in the study. Depression symptom severity was rated with the 17-item Hamilton Depression Rating Scale (HDRS). IL-6, TNF-α, Neutrophil-to-Lymphocyte ratio (NLR), White Blood Cell count (WBC), vitamin D_2 and D_3 were analyzed.

Results: There was a significant difference in vitamin D levels between subjects with a recent suicide attempt, non-suicidal MDD subjects and healthy controls in study I, with suicidal subjects having lowest vitamin D levels. Vitamin D levels did not differ significantly between non-suicidal MDD subjects and healthy controls (study II). In both studies, vitamin D correlated significantly and negatively with inflammatory markers, and this correlation was generally more robust in depressed and suicidal individuals compared to controls.

Conclusion: Our findings suggest that low vitamin D may contribute to a pro-inflammatory state in depressed and in suicidal individuals.

Populärvetenskaplig sammanfattning

D-vitamin, inflammation, depression och självmordsbenägenhet

Låg energi, nedstämdhet, isolering, initiativlöshet, avsaknad av aptit, hopplöshetskänslor… Depressionen har många ansikten och några av dem liknar de upplevelser vi har när vi är sjuka i till exempel influensa, som när det pågår en inflammation i kroppen. Kan det vara så att en del deprimerade har en låggradig inflammation som bidrar till depressionssymptomen?

Enligt WHO är depression den sjukdom som orsakar störst sjukdomsbörda i världen. De befintliga behandlingarna av depression är otillräckliga då många drabbade aldrig blir helt friska och det finns ett stort behov av att hitta kompletterande behandlingsformer.

De senaste decenniernas depressionsforskning har visat att en del deprimerade och en del självmordsnära personer kan ha en ökad inflammatorisk aktivitet i kroppen jämfört med friska kontroller. Forskningen på området har dock gett olika resultat. Kanske är det så att det finns undergrupper av patienter med depression som skulle kunna klassas till att ha förhöjd inflammation.

De flesta av oss känner säkert till att D-vitamin är viktigt för skeletthälsan. D-vitamin är emellertid med och påverkar många andra viktiga funktioner i kroppen, däribland immunförsvaret i vilket D-vitamin kan sägas ha en anti-inflammatorisk effekt.

Eftersom forskning också har visat att personer med D-vitaminbrist löper större risk att drabbas av depression, har vi undersökt om D-vitamin är kopplat till inflammation hos deprimerade individer. Kan det vara så att låga D-vitaminnivåer bidrar till en ökad inflammatorisk aktivitet?

I två olika studier har vi undersökt D-vitaminets koppling till inflammation hos deprimerade individer. Båda studierna har visat att D-vitaminnivåerna är kopplade till graden av inflammation hos dessa personer, dvs ju lägre D-vitaminkoncentration i blodet, desto högre inflammatorisk aktivitet. Denna koppling är endast ett statistiskt samband och fler studier behöver göras för att kunna se om det också finns ett orsakssamband mellan D-vitamin, inflammation och depression.

Forskningen inom D-vitamin, inflammation och psykisk ohälsa är fortfarande i sin linda och det är viktigt att nämna att här har tidigare studier gett motstridiga resultat. Mycket återstår att reda ut innan det går att veta om låga D-vitaminnivåer har någon betydelse för utveckling av depression. En fråga för framtiden är om behandling med D-vitamintillskott skulle kunna påverka depressiva symptom hos individer med ökad inflammatorisk aktivitet.

Introduction

The pathophysiology and somatic manifestations of Major Depressive Disorder (MDD), a disorder causing extensive morbidity and mortality worldwide and lethal in its most severe form, are not yet fully understood. It is thought that genetic, environmental and psychological factors all contribute to the etiology of depression and suicidality. Frequently, increased blood inflammatory markers have been reported in MDD subjects compared to healthy controls (1-4), and this immune activation is suggested to be more pronounced in suicidal subjects (5). The cause of the observed low-grade inflammation is mostly unknown (6, 7). Individuals with MDD and/or suicidality have been shown to be vitamin D deficient in several studies, although not in all (8, 9). Vitamin D exerts profound immuno-modulating effects in the body and previous studies suggest an anti-inflammatory effect of active vitamin D (10, 11). There is a lack of studies investigating the relationship between vitamin D and inflammation in psychiatric cohorts. Therefore, the aim of this licentiate thesis was to add knowledge to this research field.

Vitamin D

Vitamin D deficiency is a global public health issue and it is estimated that 1 billion people are deficient worldwide (12). Vitamin D is most commonly known to play a central role in skeletal health. However, over the last three decades, it has become apparent that vitamin D also exerts profound effects throughout the body that goes beyond its regulation of calcium homeostasis and skeletal health. One of the most prominent extra-skeletal effects of vitamin D is its moderation of the immune system.

Vitamin D is a fat-soluble vitamin that acts as a hormone in the body in endocrine, paracrine and autocrine ways. In humans, vitamin D can be acquired through the diet, via supplements and fortified food, and is produced in the skin upon UVB-radiation exposure. The main source of vitamin D comes from sun exposure since 80-90% of the circulating inactive form of vitamin D is produced by UVB-radiation exposure. The contribution of food intake to the total vitamin D levels is therefore rather small. Both vitamin D_2 and vitamin D_3 can be obtained via food intake, but only vitamin D_3 is produced via sun exposure. Vitamin D_3 is only found in animal-

sourced foods, whereas vitamin D_2 mainly comes from plant sources and fortified foods. Research suggests that Vitamin D_2 is less effective than vitamin D_3 at raising blood levels of vitamin D. One study investigated the relative potencies of vitamin D_2 and D_3 by administrating single doses of 50 000 IU vitamin D_2 or D_3 to 20 healthy male volunteers. The two vitamin D forms gave rise to comparable initial increases in serum 25(OH)D, but 25(OH)D only continued to rise after three days in individuals treated with vitamin D_3, with a peak at 14 days after administration. In contrast, serum 25(OH)D fell rapidly in individuals treated with vitamin D_2 and returned to baseline levels at 14 days. They concluded that vitamin D_2 potency is less than one third than that of vitamin D_3 (13). However, it is still under debate whether there are any significant physiological differences in the activity between the two forms of vitamin D.

The initial form of vitamin D is synthesized when UVB-radiation penetrates the skin and converts 7-dihydrocholesterol to pre-vitamin D. This metabolite is then rapidly hydroxylated in the liver by 25-hydroxylase, to form 25(OH)D and the liver is the major source of 25(OH)D. 25(OH)D is an inactive form of vitamin D and the metabolite we are measuring while assessing the vitamin D status in the body. After hydroxylation in the liver, vitamin D is converted in the kidney by 1α-hydroxylase to its active form $1,25(OH)_2D_3$. The kidney is the main source of circulating $1,25(OH)_2D_3$, although 1α-hydroxylase is present in a number of other tissues, among them cells of the immune system such as macrophages, T and B lymphocytes and dendritic cells. Activation of 25(OH)D in the kidney is tightly regulated by parathyroid hormone (PTH) levels, calcium and phosphorus levels. The activation is also under tight regulation by a negative feedback loop, where $1,25(OH)_2D_3$ inhibits the gene expression of 1α-hydroxylase in the kidney and stimulates the enzyme metabolizing active vitamin D (24-hydroxylase) to its inactive form $1,24,25(OH)_2D_3$. However, the regulation of extra-renal 1α-hydroxylase differs from the renal regulation (13, 14). In macrophages and DCs, for example, 1α-hydroxylase is primarily regulated by interferon (IFN)-γ and lipopolysaccharides (LPS) (11).

Active vitamin D binds to nucleus vitamin D Receptors (VDRs) to exert its effects and the outcome is an altered expression of vitamin D responsive genes. These genes have multiple functions in the body and have been shown to be involved in cellular proliferation, differentiation, apoptosis, angiogenesis and immunomodulation (15). Directly or indirectly, vitamin D regulates over 200 genes (16). Most tissues and cells have vitamin D receptors (VDRs) and, as previously mentioned, many of them also possess 1α-hydroxylase, the enzyme converting the inactive, circulating inactive form of vitamin D to its active form. This suggests an autocrine/paracrine function of vitamin D, beyond the known endocrine function, i.e. active vitamin D has the capability to affect different cells and tissues locally throughout the body (14).

There is no consensus with regard to timal levels of vitamin D, but most experts define deficiency as levels <50 nmol/L. In the clinic, vitamin D levels <25 nmol/L are most often considered as severe deficiency, <50 nmol/L as deficient, 50-75 nmol/L as suboptimal/insufficient and >75 nmol/L are considered as sufficient levels (see Table 1). There is a discussion though, regarding the suboptimal vitamin D levels, whether or not these levels should be considered as a relative deficiency (16, 17). For example, the prevalence of cardiovascular diseases (CVD), has been found to significantly increase, in a dose-response manner, when serum levels drops to less than 75 nmol/L (18).

There are several causes of vitamin D deficiency; reduced skin synthesis (sunscreen use, skin pigmentation, ageing, season/latitude/time of the day), decreased bioavailability (malabsorption, obesity), increased catabolism (due to various medications) and mechanisms related to liver and kidney diseases (16) (see Fig 1). Some individuals are at higher risk of vitamin D deficiency, for example obese individuals, old individuals, people living at higher latitudes or individuals with dark skin. Toxic levels of vitamin D occur at levels above 375 nmol/L. This is a rare but serious and potentially life-threatening condition. In order to reach toxic levels, one must take vitamin D supplements at high doses for a longer period. For example, taking 60,000 international units (IU) of vitamin D per day for several months has been shown to cause toxicity in a recent study (19). Several studies of the highest 'safe' vitamin D supplementation dosage for a longer period have concluded that 10 000 IU vitamin D per day does not cause any acute toxicity or long-term adverse health outcomes, in the absence of predisposing comorbidities such as primary hyperparathyroidism and granulomatous disease (20). It is not possible to reach toxic levels of vitamin D via sun exposure. Any excess of vitamin D or pre-vitamin D formed in skin is metabolized to inactive forms and sunlight induced vitamin D intoxication has never been described, i.e. sunlight itself is the ´regulator´ of vitamin D production in the skin (21). In addition, skin melanin pigmentation, which efficiently absorb UVB photons, increases while being exposed to UVB-radiation, hence causing a decrease in vitamin D_3 production via the skin (22).

Vitamin D reference intervals

Table 1.
Most commonly used classification of vitamin D status by $25(OH)D_3$ concentration

Vitamin D concentration a)	Classification
≤ 25 nmol/L	Severe deficiency
≤ 50 nmol/L	Deficiency
51 – 74 nmol/L	Suboptimal/insufficiency
≥ 75 nmol/L	Sufficient
> 375 nmol/L	Toxic

a) To convert nmol/L to ng/mL, divide by 2,496

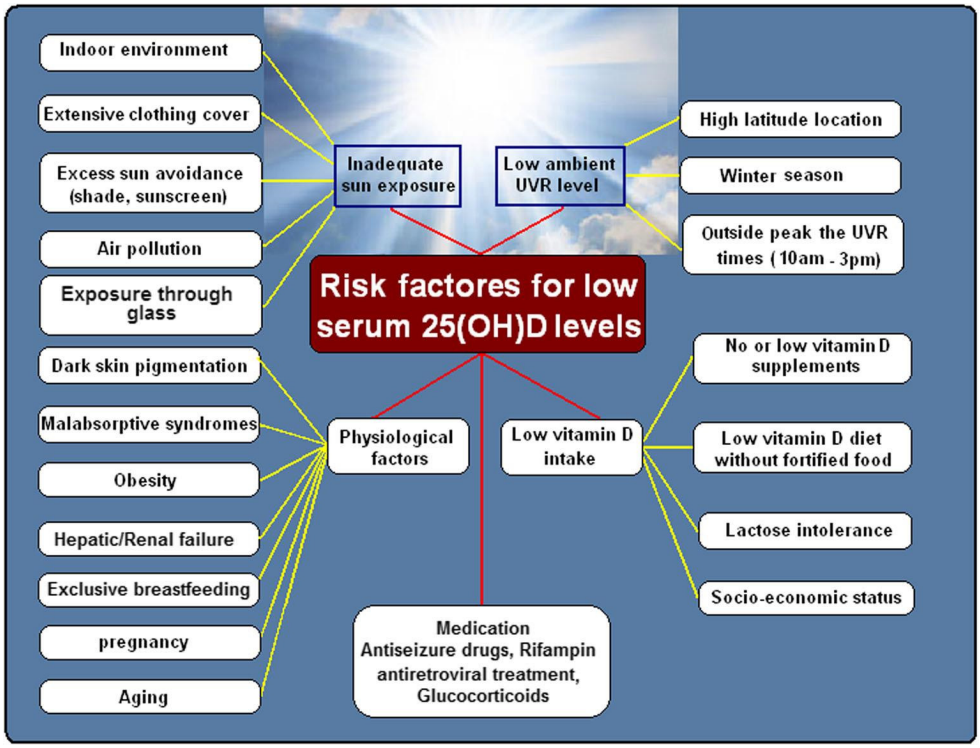


Fig 1.
Risk factors of low vitamin D status. (Holick copyright 2013, reproduced with permission)

Vitamin D and inflammation

Active vitamin D has been shown to exert strong immunomodulatory effects in the adaptive and innate immune system in experimental studies, in vitro studies, animal studies and observational studies. These immunomodulatory effects could briefly be described as promoting a shift from the pro-inflammatory T-helper 1 (Th1) cell response towards a T-helper 2 (Th2) cell response, the latter considered to mitigate the pro-inflammatory state in the body (14, 23-25). Previous studies suggest that active vitamin D increases the levels of anti-inflammatory cytokines such as interleukin-10 (IL-10), IL-4, IL-5 and transforming growth factor (TGF)- β, and decreases pro-inflammatory cytokines IL-1β, IL-2, IL-6, interferon (INF)-γ, TNF-α and IL-12 (14, 15, 23, 24). In vitro studies indicate that vitamin D has potent anti-inflammatory properties and decreases inflammation. VDRs are extensively expressed in most immune cell types; in antigen-presenting cells (APCs; monocyte/macrophage, DCs), Natural Killer cells, T cells and B cells (10). The presence of enzymes capable of synthesizing active vitamin D in immune cells

makes it possible for active vitamin D to act locally in an immunologic environment (14). Macrophages and dendritic cells, for example, express both 25-hydroxylase and 1α-hydroxylase, which enable the production of both $25(OH)D_3$ and $1,25(OH)_2D_3$ (11).

Interestingly, 1α -hydroxylase is also present in neurons and glia cells in the adult brain. Microglia, one of three different glia cells, are the primary immune effector cells in the CNS and have been shown to significantly express various cytokines essential for the maintenance and protection of the neurobiological environment. In animal studies, activated microglia have been demonstrated to increase the expression of VDR and 1α-hydroxylase, thus enhancing their receptiveness to vitamin D_3. As microglia possess VDRs, 1α-hydroxylase and 24-hydroxylase, it has the ability to respond to an immunological challenge in an autocrine way. Acute activation of different immune cells during injury, infection or disease is crucial to protect and bring the CNS environment back to a healthy condition. However, a prolonged inflammatory state, such as chronic inflammation, may be very harmful to the CNS and has been shown to be associated with neurological diseases. Notably, activated microglia exposed to vitamin D_3 (both $25(OH)D_3$ and $1,25(OH)_2D_3$) had reduced expression of pro-inflammatory cytokines IL-6, IL-12, and TNF-α, and increased expression of IL-10 (26, 27). IL-10 has recently been shown to reduce pro-inflammatory cytokine expression. Activated microglia exposed to vitamin D_3 may therefore promote a switch from the pro-inflammatory Th1 cell response to the less inflammatory Th2 cell response. Thus, by altering the immune response by microglia, vitamin D is suggested to be a control mechanism for avoiding a prolonged inflammatory state in the CNS (26).

Vitamin D in psychiatric illness

Vitamin D receptors are widely expressed in the adult human brain, for example in brain areas such as pre-frontal cortex, cingulate gyrus, hippocampus, substantia nigra and hypothalamus, areas which are considered to be important in the pathophysiology of depression (28). Both $25(OH)D_3$ and $1,25(OH)_2D_3$ can cross the blood-brain barrier and, as mentioned before, there is also evidence suggesting that vitamin D can be activated and metabolized locally in the CNS (29). There are several ways by which vitamin D could affect the brain which in turn could be related to psychiatric illnesses, for example via its influences on inflammation (25), on catecholamine and serotonin biosynthesis (30, 31), as well as via its suggested neuroprotective properties (28, 32).

There is an over-representation of vitamin D deficiency among patients with psychiatric illnesses and several cross-sectional studies, as well as some longitudinal

studies, have shown an association between low vitamin D levels and an MDD diagnosis (8, 9, 33-37). Low vitamin D levels have also been shown to increase the risk for depression (35). However, there are inconsistencies across studies, which partly might be due to methodological differences and study design limitations (38) such as small sample sizes, differences in defining depression/depression symptom severity and the use of different vitamin D cut-off levels defining deficiency/sufficiency (39, 40).

The relationship between vitamin D and MDD seems to be strongest in individuals with the most severe symptoms (9, 36, 41, 42). One study on vitamin D and suicidality found that very low vitamin D levels were associated with risk for completed suicide in a sample of military service members (43). Since the relationship between vitamin D, MDD and suicidality has been addressed only in a very small number of studies, more research in the field is clearly needed.

Inflammation in psychiatric illness

There is growing evidence that depression and- suicidality are associated with inflammatory mechanisms contributing to the psychopathology of these disorders (7, 44-47). Induction of peripheral inflammation is known to result in depressive symptoms (48), which suggests a causal relationship between inflammation and depression. Evidence of causality comes both from animal studies and from studies of patients with hepatitis, in whom the risk for developing both depression and suicidality increased after treatment with interferons (INF) (49, 50). Further, healthy subjects who are given bacteria endotoxin infusions triggering the release of inflammatory cytokines, often develop depression symptoms such as anxiety, depressed mood and a decrease in memory performance (51).

The origin of the observed low-grade inflammation in individuals with depression and/or suicidal symptoms is in most cases unknown. Mechanisms that may promote inflammation in these individuals include psychosocial stressors, increased gut permeability, infectious agents, autoimmune reactions and alterations in glucocorticoid or sympathetic nervous system tone (24, 52, 53). Vitamin D deficiency is another factor that may promote an inflammatory state in depression and suicidality (7, 47, 54). The pattern of peripheral inflammation in depressed and suicidal patients involves up-regulation of common pro-inflammatory cytokines such as IL1β, IL-6 and TNF-α (4, 46).

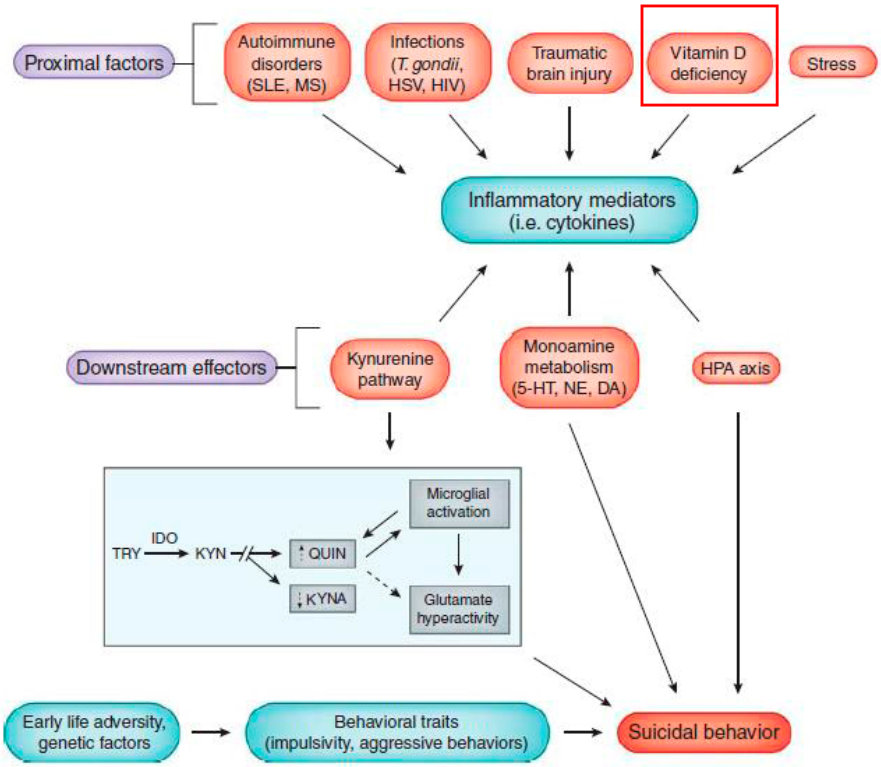


Fig 2.
Contributing factors to suicidal behavior etiology. (Bryleva et al (7), reproduced with permission.)

Vitamin D, inflammation and psychiatric illness

Over the last two decades, a negative correlation between vitamin D and inflammation has repeatedly been shown in various somatic illnesses (10, 14, 55). However, there are very few available studies on vitamin D's relationship to inflammation in psychiatric cohorts (56, 57). Though, one ongoing prospective study on the subject is investigating the relationship between low vitamin D levels, inflammation and postpartum depression (PPD) in African American women (57). They found a significant inverse correlation between vitamin D levels and PPD, which was moderated by levels of IL-6 and IL-6/IL-10, i.e. low vitamin D was associated with PPD, but only in a subgroup of women with increased inflammatory activity/higher inflammatory markers. This is an uncharted research field, ahead to be explored.

Aims

Aims of the thesis

The overall aim of this thesis is to gain a deeper understanding of how vitamin D is involved in the pathophysiology of depression and suicidality. Specifically, we wanted to investigate the relationship between vitamin D and inflammatory markers in MDD and in patients with various degrees of suicidality.

Aims, study I

1. To investigate vitamin D levels in MDD subjects with or without a recent suicide attempt and healthy controls.

2. To investigate possible associations between vitamin D and inflammation in MDD subjects with or without a recent suicide attempt and healthy controls.

Aims, study II

1. To investigate vitamin D levels in un-medicated MDD subjects without a recent suicide attempt compared to healthy controls.

2. To investigate vitamin D's relationship with inflammation in this group, and test if MDD status and suicidal symptoms moderate this relationship.

3. To investigate the relationship between vitamin D levels and depression symptom severity in this group.

Material and Methods

Study I

Study design

The study had a cross-sectional design and was approved by the Ethical Review Board for human studies, Lund/Malmö, Sweden (479/2006 and LU 82-01). All patients gave their written informed consent before enrollment. The research was performed according to the principles expressed in the declaration of Helsinki.

All subjects went through a structured interview by a specialist in psychiatry and were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders 4th edition (DSM IV) and the Structured Clinical Interview for DSM IV (SCID) I and II (American Psychiatric Association, 1994). A suicide attempt was defined as: 'Those situations in which a person has performed an actually or seemingly life threatening behavior with the intent of jeopardizing his/her life or to give the appearance of such intent, but which has not resulted in death' (58). Depression symptom severity was rated with MADRS. All study participants underwent a general physical examination, which showed no evidence of ongoing infection or other unknown somatic condition.

Subjects

Cases I: Fifty-nine patients (25 men and 34 women) were enrolled on admission to Lund University Hospital shortly after a suicide attempt during 2006-2008. The patients were diagnosed according to the DSM-IV and had a variety of different psychiatric diagnoses. Ten of the suicidal patients had a MDD diagnosis. Axis-II disorder (personality disorder), psychotropic drug treatment and/or somatic diagnosis were not exclusionary criteria (see table 2). Twenty-five of the suicidal patients had a somatic diagnosis (see table 2). Mean age of the patients was 38 years and mean Body Mass Index (BMI) 26 kg/m^2.

Cases II: Seventeen non-suicidal patients (9 men and 8 women) with MDD were recruited from the Psychiatric Clinic, Lund University Hospital during 2001-2003. The severity of the depression among the patients was a median MADRS total score

of 25 (IQR: 23-31,5), which equals moderate depression severity. The non-suicidal depressed patients were not treated with any psychotropic drugs for at least one month before sample collection. Seven of the non-suicidal depressed patients had a somatic diagnosis (see table 2). Mean age of the patients were 35 years and mean BMI 25 kg/m^2.

Control group: 14 healthy controls subjects (7 men and 7 women) without any previous or ongoing psychiatric condition were randomly selected from the municipal population register in Lund in 2001-2003, invited by letter. Exclusion criteria were pregnancy and cardiovascular disease. None of the healthy subjects was treated with psychotropic drugs. Three participants in the control group had a somatic condition (see table 2). Mean age of the healthy subjects was 33 years and mean BMI 23 kg/m^2.

Table 2. Characteristics of the study participants regarding medical treatment and somatic conditions

Variable	Suicide attempters (*n* = 59)	Non-Suicidal Depressed patients (*n* = 17)	Healthy Controls (*n* = 14)
Medication (*n*):	SNRI (11) SSRI (10) Neuroleptics + SSRI (6) Anti-epileptics (6) Anti-epileptics + other (4) Lithium (3) Other combinations (9)		
Somatic illness[a] (*n*):	[b] Endocrine- Nutritional and Metabolic Diseases (10)	[c] Endocrine- Nutritional and Metabolic Diseases (2)	
	[d] Nervous System Diseases (6)	[e] Nervous System Diseases (2)	
	Eye Diseases (1)		
	Ear Diseases (1)		
	Circulatory System Diseases (4)		
	Respiratory Tract Diseases (1)	Respiratory Tract Diseases (1)	Respiratory Tract Diseases (1)
	Digestive System Diseases (3)	Digestive System Diseases (1)	
	Skin Diseases (1)	Skin Diseases (1)	
	Musculoskeletal Diseases (6)		
	[f] Pain Conditions (3)		
	[g] Allergy (2)	[h] Allergy (1)	[i] Allergy (3)

a) 10 of the suicide attempters, 1 of the non-suicidal depressed patients and 1 of the healthy controls had two or more somatic illnesses
b) 7 of the suicide attempters had an endocrine condition; 3 had diabetes and 4 had a thyroid disease.
c) 1 of the non-suicidal depressed patients had an endocrine condition; Polycystic Ovarian Syndrome (PCO).
d) Multiple Sclerosis (1), sequelae intoxication (1), migraine (2), fibromyalgia (1), neuropathy (1)
e) Migraine (1), fibromyalgia (1)
f) Post. op. fractures after suicide attempt (1), orifice of the stomach (1), pain NOS (1)
g) Pollen allergics (1), nickel allergy (1)
h) Allergy NOS (1)
i) Cat, horse, pollen allergics, hard fruit (1), furred animal, hay fever (1), allergy NOS (1)

Blood sampling

Blood samples from all subjects were collected between 7.30 and 8.00 in the morning, after a night of fasting. The suicidal patients were psychiatric inpatients, whereas the control-groups had spent the night at home. Samples from all study participants were collected within a week from the physical and psychiatric examination. The blood was placed on ice and centrifuged (3000 r/minutes, at +4°C) within an hour. Plasma and serum were stored at -80°C until analysis. Blood samples were collected from all seasons throughout the year for the suicidal patients

and from all seasons, except for the summer, for the control groups. Blood samples from healthy controls and non-suicidal depressed patients were collected between 2001-2003 and from suicide attempters between 2006-2008.

Laboratory analyses

Vitamin D: Analyses of $25(OH)D_2$ and $25(OH)D_3$ were done by liquid chromatography mass spectrometry (LC-MS/MS) at the department of clinical chemistry, Scania University Hospital. LC-MS/MS is the most accurate analytical technique for quantification of vitamin D status (59). Stored serum samples were thawed and analyzed for $25(OH)D_2$, and $25(OH)D_3$. The samples had not been thawed prior to analyses. The time of storage, 4-12 years at -80°C, is not likely to have had an impact on the quality of the analysis due to the relatively stable vitamin D molecule (60)

Inflammatory markers: IL-6, IL-1β and TNF-α were measured in the plasma using ultra-sensitive electrochemiluminescence immunoassays according to the manufacturer's instructions (MesoScale Discovery, UK). The method and plasma concentration of the cytokines in these patients have previously been published (61). All samples were analyzed in duplicates. Samples had never been thawed prior on the cytokine analysis. The storage time of 3-10 years is not likely to impact on the quality of the analysis, since we did not find any correlation between cytokine levels and the sample storage time for over 15 years in a previous study from our research group (62).

Statistical analyses

All statistical analyses were performed using the statistical package SPSS version 20 (SPSS Inc., Illinois, US). The computed power for a two-tailed t-test showed that 87% power is achieved with n=59. Vitamin D data displayed a normal distribution and all samples were above detection limit. Data for the cytokines were transformed into normal distribution using the natural logarithms before statistical analysis. The potential effects of confounding factors were tested in linear regression models. Vitamin D was found to be associated with BMI and age of the subjects and therefore all analyses were performed correcting for these factors using linear regression. For normally distributed variables, multiple group-wise comparisons were performed using One-way ANOVAs followed by Bonferroni-Dunn's post-hoc tests. Student's T-test was used in cases when only two groups were compared. Mann-Whitney U test was used for non-normally distributed variables and groups of heterogeneous sizes. Fishers Exact Test was used for comparing proportions of patients with vitamin D deficiency between the groups. Correlations between

cytokines and vitamin D were assessed using Pearson's *r*. The alpha-level of significance was set at $p<0.05$.

Results, study I

Suicide attempters had significantly lower mean levels of vitamin D than depressed non-suicidal patients and healthy controls ($p<0.05$). The mean value of vitamin D among the suicide attempters was 47 ± 20 nmol/L whereas it was 62 ± 27 nmol/L among the depressed non-suicidal patients and 65 ± 26 nmol/L among the healthy controls. Moreover, there was a significant negative association between vitamin D and pro-inflammatory cytokines in the psychiatric patients. Low vitamin D levels were associated with higher levels of the inflammatory cytokines IL-6 and IL-1β in the blood.

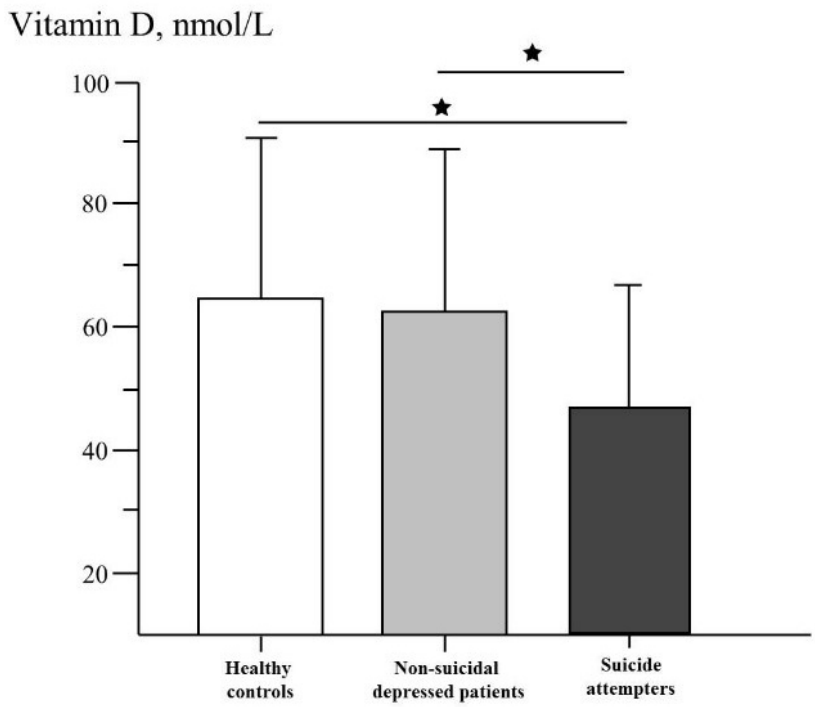


Fig 3.
Uncorrected mean vitamin D levels for the three studied groups. The significant differences in the mean vitamin D levels between the suicidal patients and the healthy controls/non-suicidal depressed patients were analyzed statistically using a variable corrected for age and BMI (*$p<0.05$).

Comments, study I

Study design: One limitation of the cross-sectional design is that it is not possible to determine any causality in the relationship between vitamin D and inflammatory markers. In order to investigate any causal relationship, a well-designed RCT would have been necessary, where subjects treated with vitamin D would be followed over time with vitamin D levels/cytokines measured both at baseline and at the end of the study.

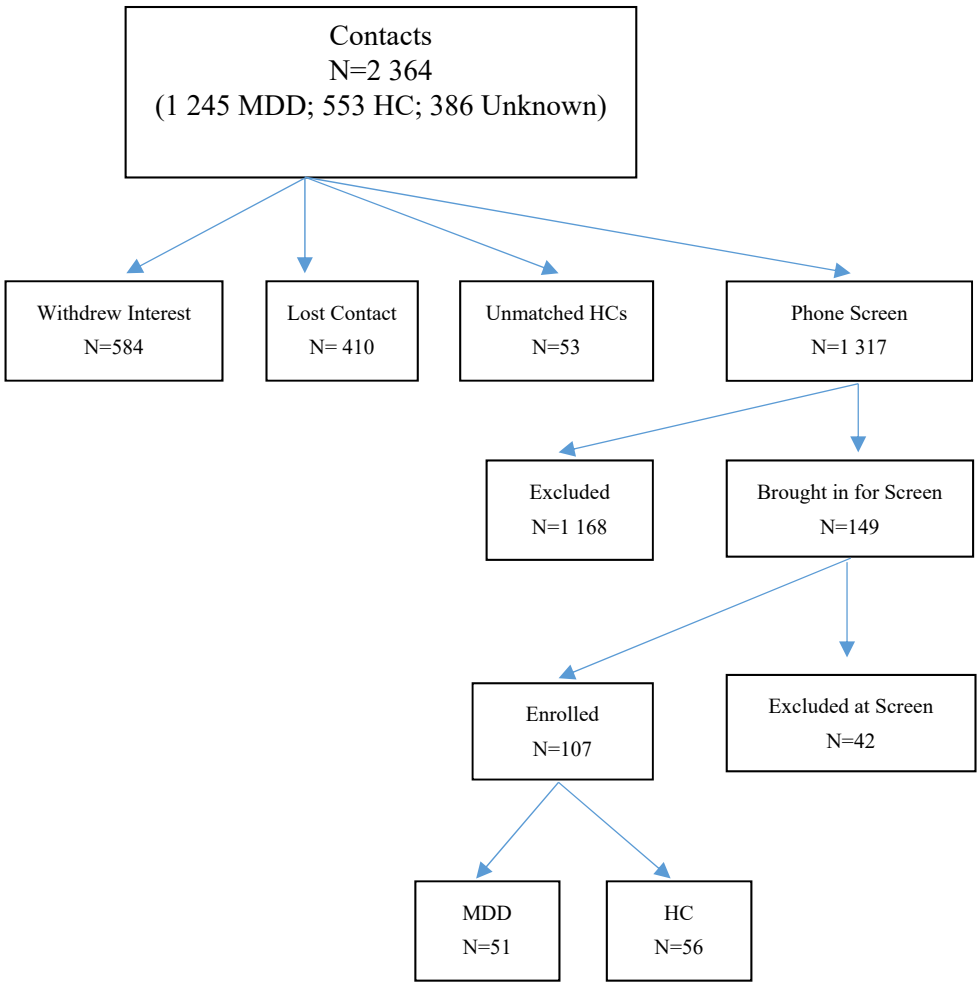
Sample size: It would have been preferable with a larger sample size in the study in order to increase the precision of the results and thus make the results more generalizable to a general population, which is the primary purpose of most research. In addition, a larger sample size would have allowed us to look at different diagnostic sub-groups separately. However, the sample had natural limits due to the use of already collected samples. For example, to subdivide and explore suicidal subjects separately in a pure MDD group, a bipolar disorder group and a psychosis related group would have been relevant considering previous literature as subjects with bipolar disorder or schizophrenia and other psychosis related illnesses have been shown to be deficient in vitamin D (63-65). In this study, ten of the suicidal subjects had an MDD diagnosis, 17 had a bipolar disorder (including both type I and II) and only 3 had psychosis related diagnoses (schizoaffective disorder and psychotic disorder NOS). A sub-group of a clinical diagnosed MDD with a recent suicide attempt would have made it possible to compare non-suicidal MDD subjects with MDD subjects with a recent suicide attempt, which would have made our results between suicidal and non-suicidal individuals more comparable. However, a cohort of suicidal subjects with mixed psychiatric diagnoses is also relevant to investigate since previous literature suggest a more profound dysregulated immune system in suicidal subjects than in MDD subjects without any suicidality (5).

Confounders: It would have been preferable if all subjects were medication free and somatically healthy to reduce the effect of potential confounders. In the present study, only MDD subjects without a recent suicide attempt were free from psychotropic drugs apart from the healthy subjects. Twenty-five of the recent suicide attempters, seven of the MDD subjects and three of the healthy subjects had one or more somatic conditions. We adjusted the analyses for known vitamin D affecting confounders such as age and BMI, however we did not have any information about ethnicity, amount of sun exposure and smoking habits. Ethnicity is not likely to be a confounder in this cohort since the large majority of the patients at Lund University Hospital are of Swedish origin with a Caucasian ethnicity. Exposure to sun is known to be related to both vitamin D levels and inflammation. In this study, seasonality did not have an impact on the group differences (or lack of differences). Smoking is known to be related to inflammation status, however,

there are controvercies in previous literature regarding a possible association between smoking and vitamin D (66, 67).

Study II

Fig 4.
Flow chart, enrollment



Study design:

The study had a cross-sectional design and was approved by the Committee on Human Research of the University of California, San Francisco (UCSF) (protocol #10-00825). All patients gave their written informed consent before participating in the study and were compensated for their participation. Subjects were recruited by flyers, bulletin board notices, Craigslist postings, newspaper ads, and clinical referrals between 2011-2015. All diagnoses were made according to the Structured Clinical Interview for DSM IV-TR Axis I Disorders (SCID) (68), and were confirmed by clinical interview with a board-certified psychiatrist. MDD subjects were excluded if they met DSM-IV criteria (which were the extant criteria in use at the time this study was conducted) for any of the following: (i) bipolar disorder, (ii) alcohol or substance abuse within the preceding 6 months, (iii) PTSD or an eating disorder within 1 month of entering the study, and (iv) for any history of psychosis outside of a major depressive episode (MDE), or the presence of any psychotic symptoms during the current MDE. Potential healthy controls were excluded for any history of DSM-IV Axis-I diagnoses. On the day of study visit, all subjects had to pass a urine toxicology screen (marijuana, cocaine, amphetamines, phencyclidine, opiates, methamphetamine, tricyclic antidepressants, and barbiturates) and a urine test for pregnancy in women of child-bearing potential. None of the subjects had had any vaccinations for at least six weeks prior to enrollment in the study, and all subjects were free of any psychotropic medications (including antidepressants), hormone supplements, steroid-containing birth control or other potentially other interfering medications for a minimum of six weeks. None of the subjects were taking vitamin D supplements above the U.S. recommended daily allowances (e.g. 15 μg/day) prior to study start and all, but one MDD participant, stopped taking vitamin D during 2 weeks before study start. Regarding acute illnesses or infections, none of the study participants had any acute or chronic inflammatory disorders, neurological disorders, or any other major medical conditions considered to be potentially confounding (e.g. cancer, HIV, diabetes, history of cardiovascular disease or stroke, (etc.).

Subjects

Cases: Forty-eight un-medicated MDD subjects with mild-to-moderate MDD were included in the study. Depressed subjects were diagnosed with current MDD, without psychotic features, and scored >17 on the 17-item Hamilton Depression Rating Scale (HDRS) (69). Exclusion criteria for MDD subjects were: a recent or any serious suicide attempt, bipolar disorder, psychotic symptoms during their current major depressive episode, history of psychosis outside of a mood disorder episode, any eating disorder or post-traumatic stress disorder within one month of entering the study, and substance abuse or dependence (including alcohol) within

six months of entering the study. Co-morbid anxiety disorders (except PTSD) were not exclusionary if MDD was considered the primary diagnosis. If needed, short-acting sedative-hypnotics were allowed in the MDD subjects, up to a maximum of three times per week, but none within one week prior to participation. Seventeen of the MDD subjects had mild-to-moderate Suicidal Ideation (SI), defined as score between 1 and 3 on HDRS suicidal ideation item. Subjects who scored 4 on the scale HDRS suicidal ideation item, i.e. subjects with a recent suicide attempt, were excluded in the study. Mean age of the MDD subjects was 39 years and mean BMI 26 kg/m^2.

Healthy controls: Fifty-four healthy control subjects were included in the study. None of them had any history of DSMIV-TR Axis I disorder, also confirmed by SCID interview. Mean age of the healthy subjects was 38 years and mean BMI 24,5 kg/m^2.

Blood sampling

Subjects were admitted as outpatients to the UCSF Clinical and Translational Science Institute between 8:00 a.m. and 11:00 a.m., having fasted (except water) since 10:00 p.m. the night before. Subjects were instructed to sit quietly and relax for 25–45 minutes before blood samples were obtained for assessment of inflammatory markers.

Laboratory analyses

Vitamin D: Stored serum samples were thawed once before the $25(OH)D_2$ (vitamin D_2) and $25(OH)D_3$ (vitamin D_3) analysis. The samples were stored in a -80ºC freezer for 4-7 years prior to assay, which is not likely to have had an impact on the quality of the analysis due to the relatively stable vitamin D molecule (60). Analyses of $25(OH)D_2$ and $25(OH)D_3$ were done by liquid-chromatography-mass-spectrometry, model Sciex API 4000 LC/MS/MS (MA, USA), which is the most accurate analytical technique for quantification of vitamin D status (59). Coefficient of variation (CV) values were as follows: for $25(OH)D_2$, 6,0% at 40 nmol/L and 5% at 120 nmol/L and for $25(OH)D_3$ 6% at 40 nmol/L and 4% at 120 nmol/L. The lowest detection limit is 6 nmol/L for both for $25(OH)D_2$ and $25(OH)D_3$. The analyses were conducted by The department of clinical chemistry at Scania University Hospital, which is accredited by SWEDAC (the Swedish Board for Accreditation and Conformity Assessment) and participates in the external assurance program of DEQAS (Vitamin D External Quality Assessment Scheme, UK). Blood sampling was performed all over the year. Sampling season was divided into 'Summertime', which equals April-September and 'Wintertime', which equals October-March.

In this study, we used a cutoff of <50 nmol/L to indicate "vitamin D deficiency" and <25 nmol/L to indicate "severe vitamin D deficiency." Levels between 50-74 nmol/L were considered "suboptimal" and levels ≥75 nmol/L as "sufficient" (16).

Inflammatory markers assays: IL-6 and TNF-α concentrations were measured in the plasma using a high sensitivity multiplexed sandwich immunoassay (Mesoscale Discovery, Gaithersburg, MD, USA). Cytokine assays were performed in the lab of Dr. Firdaus Dhabhar at Stanford University. In addition, we assessed the Neutrophil-to-Lymphocyte Ratio (NLR), as well as white blood cell Count (WBC), which are considered to be inflammatory markers of systemic and low grade inflammation. CBC and differential cell count were determined by automated differential analysis at Quest laboratories. Samples had never been thawed prior on the cytokine analysis. The storage time of 1-5 years is not likely to impact on the quality of the analysis, since we did not find any correlation between cytokine levels and the sample storage time for over 15 years in a previous study from our research group *(62)*.

Statistical analyses

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS, version 25). All tests were 2-tailed with alpha = 0.05. Skewed data were log-transformed to achieve normality. For unadjusted group-wise comparisons, we used Student's T-test and Pearson's Chi-square test. ANOVA were used, adjusting for confounders when appropriate (ANCOVA). We also used a composite inflammation score by averaging the z-sore of IL-6 and TNF-α in order to lower the number of statistical comparisons. For correlation analysis we used Pearson's correlation and we used the PROCESS tool for SPSS (Hayes 2012) in moderation analysis.

Based on their known association with vitamin D and/or inflammation we adjusted for the following variables in the analyses; age, sex, tobacco use, BMI and sample season.

Also in exploratory analysis, we subdivided MDD subjects based on the HDRS item 6.1 scores (suicidal ideation (SI), score range 0-3). The "suicidal ideation group" (SI) was defined as scoring ≥1 on this item and the "non-suicidal ideation group" (NSI) was defined as scoring 0 this item. Subjects scoring 4 on HDRS item 6.1 were excluded in the study; i.e. subjects with a recent suicide attempt (70).

Results, study II

There were no significant differences in plasma vitamin D levels between MDD and controls (p=0.48), nor between MDD without SI and MDD with SI (p=0.41). Vitamin D was negatively correlated with all inflammatory markers and these correlations were generally stronger in the MDD group compared to controls as well as in the SI group compared the non-SI group (see Fig 5-10). In multivariate analyses, the MDD group and SI group significantly moderated the relationship between vitamin D and NLR and between vitamin D and WBC (all p<0.05). Adjusting for age, sex, BMI, sampling season, and smoking did not change any of the results.

Fig 5-7. Correlations vitamin D and inflammatory markers, MDD vs controls.

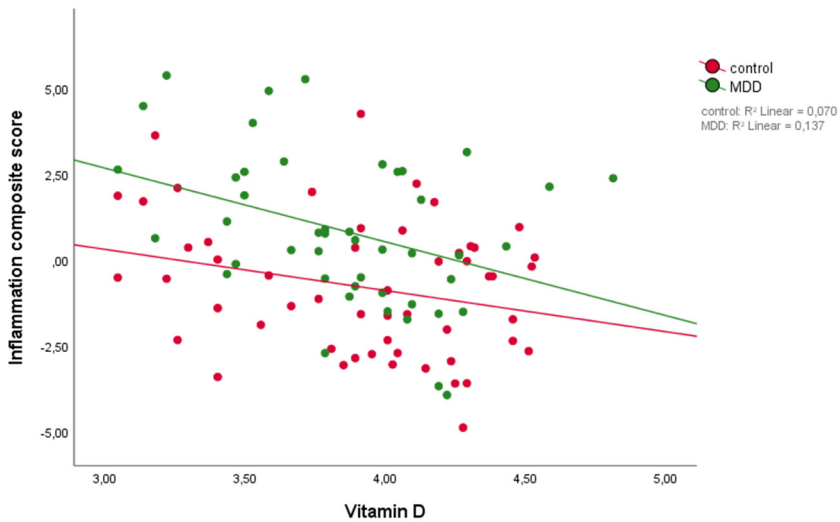


Fig 5.
Significant correlation between Vitamin D and inflammatory composite score in MDD subjects (r=-0.37; p=<0.05). Close to significant correlation between Vitamin D and inflammatory composite score in healthy controls (r=-0.26; p=0.056). No significant interaction effect.

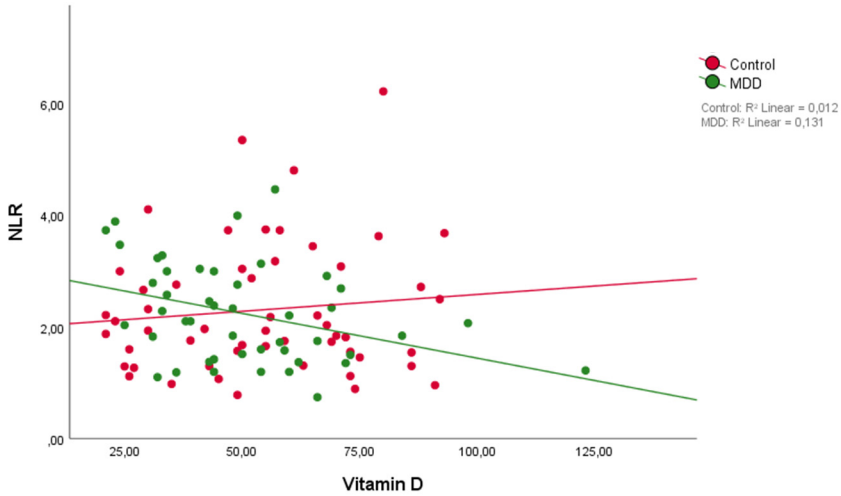


Fig 6.
Significant correlation between Vitamin D and Neutrophil-to-Lymphocyte ratio (NLR) in MDD subjects (r=-0.39; p<0.01). No significant correlation between Vitamin D and NLR in healthy controls (r=0.09; p=0.57). Significant interaction effect (p<0.05).

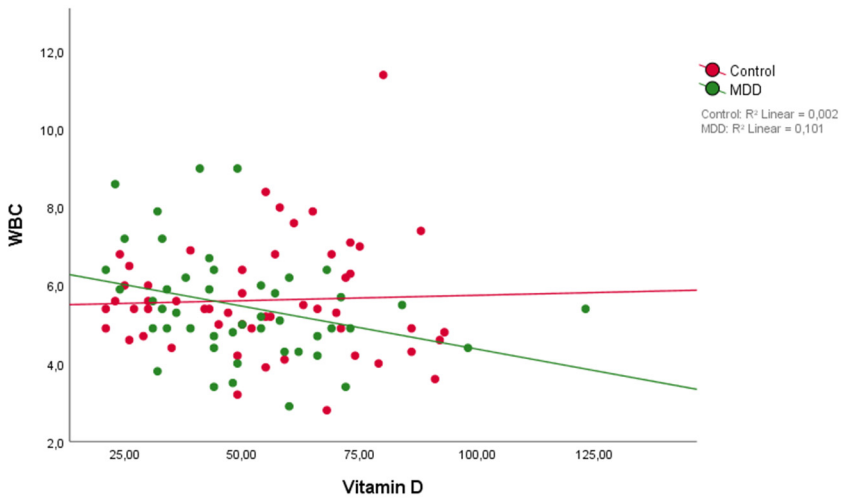


Fig 7.
Significant correlation between Vitamin D and White Blood Cell Count (WBC) in MDD subjects (r=-0.36; p<0.05). No significant correlation between Vitamin D and WBC in healthy controls (r=-0.02; p=0.90). Significant interaction effect (p<0.05).

Fig 8-10. Correlations vitamin D and inflammatory markers, MDD NSI vs SI.

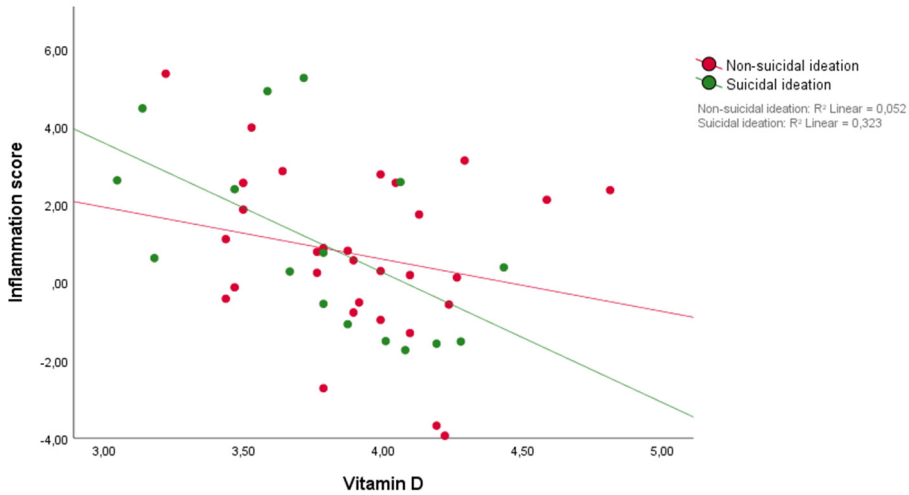


Fig 8.
Significant correlation between Vitamin D and inflammatory composite score in Suicidal Ideation subjects (r=-0.57; p<0.05). No significant correlation between Vitamin D and inflammatory composite score in non-Suicidal Ideation subjects (r=-0.21; p=0.23). No significant interaction effect.

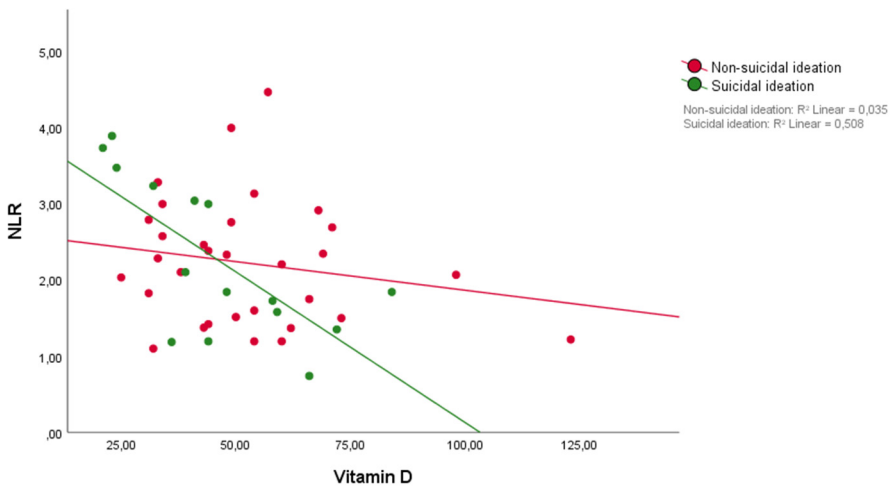


Fig 9.
Significant correlation between Vitamin D and NLR in Suicidal Ideation subjects (r=-0.71; p<0.01).
No significant correlation between Vitamin D and NLR in non-Suicidal Ideation subjects (r=-0.48; p=0.33). Significant interaction effect (p<0.05).

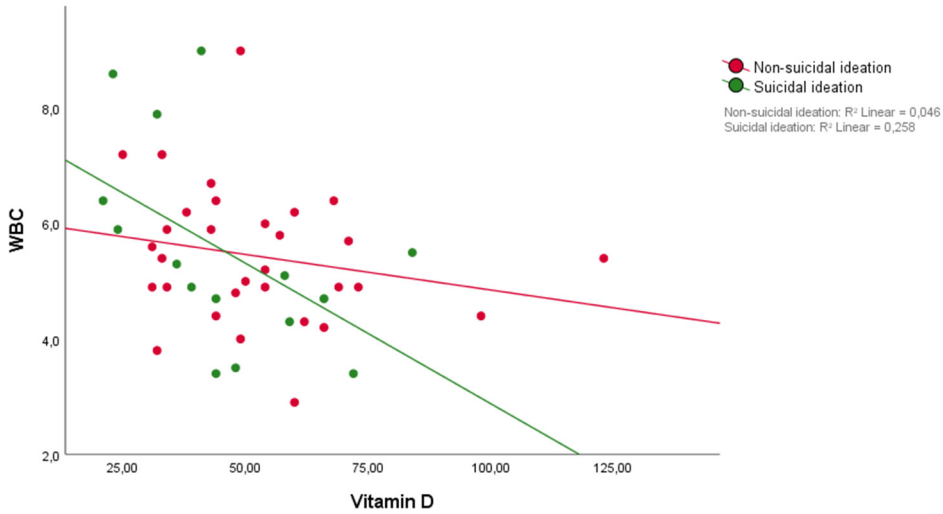


Fig 10.
Significant correlation between Vitamin D and WBC in Suicidal Ideation subjects (r=-0.56; p<0.05).
No significant correlation between Vitamin D and WBC in non-Suicidal Ideation subjects (r=-0.23 p=0.21). Significant interaction effect (p<0.05).

Comments, study II

Study design: As already mentioned in 'comments', study I, a limitation of the study design is the impossibility to determine any causality in the relationship between vitamin D and inflammatory markers. In order to do that, a well-designed RCT would be needed to conduct.

Sample size: As mentioned before in study I, it would have been preferable to have a larger sample size in the study in order to increase the precision of the results and thus make the results more generalizable to a general population. Also, a larger sample size would have given us a wider vitamin D range to work with, and thus, comparisons between subjects within different vitamin D level groups (severely deficient, deficient, sub-optimal, sufficient) could have been performed. It would have been relevant to compare subjects with vitamin D levels < 75 nmol/L to subjects >75 nmol/L. The relevance of this division will be reflected on in the discussion part later on. However, due to the modest variety in vitamin D levels in the material, we did not even have the power to compare subjects < or > 50 nmol/L. Also, our study was underpowered in the subgroups MDD analysis, which makes our exploratory results more of an indication of a possible more pronounced relationship between vitamin D and inflammatory markers in SI subjects.

Subjects: All participants in study II were free from potentially interfering medications, well phenotyped and somatically healthy, which is a rare setting in similar studies and a major strength of the study. This setting provides more reliable outcomes in the inflammatory analyses. Additionally, it would have been desirable with inclusion of MDD subjects, or subjects with mixed psychiatric diagnoses, with a recent suicide attempt. That would have made it possible for us to compare subjects with different degrees of depression symptom severity; MDD without mild-to moderate SI, MDD with mild-to-moderate SI and subjects with severe depression symptom severity, i.e. subjects with a recent suicide attempt. It would also have given us results with greater comparability in relation to study I. However, as in study I, the study material had already been collected before study start and we did not have the possibility to influence the study design.

Confounders: We adjusted our results to several relevant confounding factors; age, sex, BMI, sampling season and smoking. In addition, as mentioned before, all subjects were medication-free, well phenotyped and somatically healthy, which lessen the amount of confounding factors in the study. The main confounder, however, that we did not have the possibility to adjust for was the amount of sun exposure. Although, we adjusted all analyses to seasonality and this adjustment did not change any of the results. In this study, we had data about ethnicity and a Chi-2 test did not show any significant differences in ethnicities between MDD and healthy controls.

General discussion

In summary, this thesis generated three main results:

1. Suicidal subjects had significantly lower vitamin D levels than MDD subjects without a recent suicide attempt and healthy controls (study 1).

2. There were no significant differences in vitamin D levels between MDD subjects without a recent suicide attempt and healthy controls (study II).

3. In both studies, we found a significant negative correlation between vitamin D and inflammation that was more robust in in subjects with psychiatric diagnoses compared to healthy controls.

Our results suggest that individuals with suicidal behavior are deficient in vitamin D and have significantly lower vitamin D levels than non-suicidal MDD subjects with mild-to-moderate depression severity. In contrast to previous literature, we did not find any significant differences in vitamin D levels between non-suicidal MDD subjects and healthy controls (9, 36, 37, 41, 42, 71, 72).

In our studies, we have adjusted for, and examined, several relevant confounders. However, both studies had limitations with regard to additional relevant confounding factors, especially study I. In study I, we lacked data about ethnicity and smoking habits, which are important factors to consider; ethnicity, due to different skin pigmentations and thereby different ability to produce vitamin D upon sun exposure and smoking habits, because its' known influence on inflammation status and possible influence on vitamin D levels. There are divergent results in previous literature regarding the possible effect of smoking on vitamin D levels and the issue has not yet been thoroughly investigated. Several studies have found exposure to smoking to be related to low vitamin D levels. A recent study found exposure to smoking to be an independent predictor of low vitamin D levels among US children (73). In another study, smoking was a significant determinant of low vitamin D levels in young and middle-aged males (74). However, there are studies that have failed to find such relationship (67). As mentioned before in the Methods part, ethnicity is not likely to be a confounder in study I since the majority of subjects most certainly were of Caucasian ethnicity. Regarding smoking, we can not be certain that a possible difference in smoking habits between subjects with a recent suicide attempt, non-suicidal MDD subjects and healthy controls, could be a

confounder to our results of significantly lower vitamin D levels among suicidal subjects compared to non-suicidal MDD and healthy controls.

The most relevant confounder in both studies, of which we lacked data and hence could not control for, was the potential differences in sun exposure among the study participants. As the major source of vitamin D production is in the skin, vitamin D status can be considered a surrogate marker of sunlight exposure. Furthermore, exposure to UV-radiation is also known to induce an inflammatory response in the body. In study I, there were no significant differences in vitamin D levels in samples collected during winter, fall, spring or summer. In order to control for any potential effects of sampling season in study I, group comparisons were re-run excluding samples from suicide attempters collected during summertime, and this did not have any impact on the results. In study II, there were no group differences in seasonality and adjusting for sample season did not affect the results. However, in view of the major impact of sun exposure to vitamin D levels, adjusting only for seasonality may not have been sufficient. Thus, adjusting for actual amount of UVB-radiation exposure would have been preferable.

There is an ongoing discussion regarding causal mechanisms underlying the negative relationship between vitamin D and inflammation. The question to be answered is if it is low vitamin D levels that cause increased inflammation or if it is the inflammation *per se* that results in lower vitamin D concentrations (75, 76). To date, it is generally thought that low vitamin D levels may promote higher inflammation grade as a result of its profound regulation of the immune system. However, it has also been proposed that the circulating, inactive form of vitamin D decreases in response to an immunological challenge, i.e. the vitamin D that we measure is being metabolized, which in turn causes the negative association between vitamin D and inflammation (75). This question clearly needs further investigations. Regardless of the direction of causality, both ways may be possible explanations behind our results of a negative correlation between vitamin D and inflammatory markers in psychiatric cohorts. Previous studies have shown increased inflammation in depressed individuals compared to healthy controls, and the inflammation grade seem to be even higher in suicidal individuals (3, 7, 46, 47, 52, 77, 78). If the theory of high inflammation leading to low vitamin D levels is correct, it may be a possible explanation to our finding that suicidal individuals have significantly lower vitamin D levels than MDD without suicidality and healthy controls, considering the before mentioned suggestion of a more pronounced biological disturbance in suicidal individuals (3, 7, 46, 47, 52, 77, 78).

In addition to pro-inflammatory cytokines, we investigated two other inflammatory markers in study II, NLR and WBC, both considered as inflammatory markers of systemic and low-grade inflammation, which recently also have been related to vitamin D (79-82). We have found few studies about NLR and WBC in MDD

subjects and existing studies have yielded conflicting results (83-85). To our knowledge, there are no previous studies on vitamin D's relationship with NLR, WBC in a psychiatric cohorts to date. Interestingly, in a study conducted by Ekinci et al (2017), the authors suggest NLR to be a possible trait marker of suicidal vulnerability. The study showed that suicidal depressive patients had significantly higher NLR than both the non-suicidal patients and the healthy controls. A logistic regression analysis showed that NLR was a significant predictor of a recent suicide attempt. In our study, the relationship between vitamin D and NLR, WBC was more pronounced in MDD with SI compared to MDD without SI. Unfortunately though, we were not able to investigate the relationship between vitamin D and NLR, WBC in subjects with a recent suicide attempt.

Conclusion

In summary, we found a significant negative relationship between vitamin D and inflammatory markers in a cohort of MDD subjects and in subjects with mixed psychiatric diagnoses whom all had a recent suicide attempt. Our findings suggest that low vitamin D is associated with a pro-inflammatory state, frequently observed in depressed and suicidal individuals, and that this relationship between vitamin D and inflammation may be differentially regulated in MDD subjects with or without SI, suicidal individuals and healthy controls. The cross-sectional design in both studies included in the Licentiate Thesis does not allow any conclusions regarding possible causality between vitamin D and inflammation in MDD and/or suicidal behaviour. Therefore, more studies, preferably RCTs, in this field are warranted.

Future outlooks

Several cross-sectional, longitudinal and meta-analytic studies have identified associations between depression and low vitamin D levels, but studies have failed to clarify whether vitamin D deficiency is a cause, correlate or consequence of depression (42). The few RCTs on the subject so far have yielded inconsistent results. It seems to be hard to confirm the promising results from cross-sectional and longitudinal studies in RCTs, i.e. there is a discrepancy between observational studies and RCTs. Why is that so?

There are methodological issues in all vitamin D research on depression provided that different vitamin D cut-off levels are used to defined 'deficiency'/'sufficiency' as well as different inclusion criteria of depressed subjects, which make research in the field hard to compare. These issues are especially prominent in RCTs. Gowda et al found, in a meta-analysis on vitamin D supplementation to reduce depression, that vitamin D supplementation did not significantly reduce depression symptoms. However, individuals included in most of the studies had low levels of depression and sufficient serum vitamin D at baseline. Gowda et al defined these issues as 'biological flaws' (86). According to Gowda et al, in order to achieve positive effects of vitamin D supplementation in different illnesses, supplemented subject shall be low in vitamin D levels at baseline, have severe-to-very severe illness symptoms and be substituted up to at least sufficient vitamin D levels (>75 nmol/L). Otherwise, the study should be regarded as having 'biological flaws', which is the case in most RCTs in the field to date. These 'biological flaws' in treatment studies may possibly be a reason to the existing discrepancies between observational studies and RCTs.

As for future cross-sectional studies on vitamin D, inflammation and MDD, my suggestion is firstly to have a large enough sample size to ensure a wide range in vitamin D levels within the studied groups, so that it is possible to study differences between subjects with deficient/sufficient vitamin D levels. In the two studies included in the Licentiate Thesis, few subjects had sufficient vitamin D levels, therefore separate analyses were not possible to conduct. Secondly, included MDD subjects should be diagnosed clinically and analyses be done in MDD subjects with mild-to-moderate and severe-to-very sever depressions symptoms separately.

It is tempting to suggest more well-designed RCTs, without 'biological flaws', in the future, in order to resolve some of the questions about vitamin D's possible

association to psychiatric illness. However, I do not believe this is the most optimal way to assess the underlying mechanisms and influences of vitamin D with regard to different illnesses. Both depression and suicidal behaviour are very heterogeneous and complex conditions, some of which might be related to a dysregulated immune response or other dysregulated biological pathways. A difficulty in psychiatric research today is to create pure study cohorts considering the diversity among, for example subjects with depression or suicidal behaviour. There are very likely sub-groups within subjects diagnosed with depression who may have different biological traits and this may possibly blur associations and contribute to the inconsistency between studies in the field. My suggestion to future studies is therefore to attack this problem via a different angle, namely to study the effects of vitamin D in the plasma proteome, before and after treatment with vitamin D in different psychiatric cohorts. I believe this is necessary to do prior to initiating a large number of RCTs on the subject. By only one single drop of blood, it is possible to identify 10-12000 different proteins in the blood via mass spectrometry (MS). A very interesting study to conduct, exemplified in an ideal form, would be to investigate MDD subjects without SI, MDD subjects with mild-to-moderate SI, MDD subjects with a recent suicide attempt and healthy controls, where all included subjects were severely deficient in vitamin D. If unlimited resources, make sub-groups within the different study groups and conduct a double-blinded RCT, where one sub-group receives vitamin D supplements and the other group placebo. Take a baseline blood sample, analyze the protein pattern at study start and look at possible differences between the groups. Then treat the subjects with vitamin D, according to the suggested principles to avoid biological flaws, with significant doses of vitamin D. i.e. 5000 IU/day for 2-3 months and take a new blood sample at study end. Identify which proteins that have changed the most by the increase in vitamin D levels, for example a 50-100% increase or decrease. After that, design a multiplex MS-based assay of the 25 most relevant proteins within each group. The results of the multiplex assay would then be a mark of the vitamin D response and a 'picture' of the biological effects in the different study groups. Lastly, try to relate these disclosed biological pathways to different psychiatric traits and after that, design RCTs to evaluate the effects of vitamin D treatment in the, by protein patterns, differentiated psychiatric cohorts. A hypothetic finding could be that it is inflammatory-related proteins and signal pathways that have been affected by vitamin D treatment the most within the MDD group with a recent suicide attempt, and serotonin-related proteins within the MDD group with mild-to-moderate SI. Perhaps there were no significant changes in proteins previously suggested to be related with psychiatric illness in MDD without mild-to-moderate SI and healthy controls. Relevant RCTs to conduct would then be to study the effects of vitamin D treatment on depression symptom severity in MDD with mild-to-moderate SI with, our without, additional SSRI treatment and MDD with suicidal behaviour with, or without, additional anti-inflammatory treatment.

Licentiate contributions

This Licentiate Thesis has added knowledge in the sparse field of vitamin D's relationship with MDD and suicidal behavior. When study I was published, it was the first time an association between vitamin D and inflammation was shown in a psychiatric cohort. In study II, we replicated these findings and added two inflammatory markers, NLR and WBC, which, to our knowledge, never have been studied in relation to vitamin D in a psychiatric setting before. The Licentiate has mostly been working independently in both studies in regard with hypotheses, data management, administration of vitamin D analyses, writing manuscript I and II and submission of manuscript I. The Licentiate also initiated the explorations of vitamin D in relation to inflammation in psychiatric cohorts, as well as formulated the research questions. Considering the extensive contribution by the Licentiate to both studies, the Licentiate is first author on the article and the manuscript included in this Licentiate Thesis.

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