

Hype or hope? Vitamin D in multiple sclerosis

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Summary and general discussion

General aim of this thesis

The increase in vitamin D research during the past decades has raised hopes among many, including patients, clinicians and researchers. The main question now is whether these high expectations can be substantiated by conclusive data or that positive findings from *in vitro* studies, association studies and small supplementation studies, have hyped those hopes up. In this thesis we focussed on the clinical and immunological effects of vitamin D in multiple sclerosis (MS). We investigated the role of vitamin D in disease progression to enhance our insights in the role of vitamin D in the disease course and studied the immune regulatory effects of high dose vitamin D supplementation as a possible add on treatment in patients with MS.

Summary of results

In **chapter 1**, we first reviewed the insights in the *in vivo* effects on immunological outcomes of vitamin D in healthy controls (HC), and in patients with an auto-immune disease, at the start of the research described in this thesis. The hypothesized mechanism underlying the association between vitamin D and MS disease activity is the immune regulating capability of vitamin D. *In vitro* studies and studies in the experimental auto-immune encephalitis (EAE) model had previously shown that immune cells are functional targets of vitamin D, but *in vivo* studies in humans were heterogeneous and involved multiple immunological outcomes. Overall, there seemed to be a trend towards an anti-inflammatory role of vitamin D, but this was not supported by conclusive data. Therefore well powered studies, like RCTs, were needed before definite conclusions could be drawn.

While such trials were executed, in the first part of this thesis, we investigated the role of vitamin D levels within the physiological range in disease progression, a disability hallmark that was not investigated in great detail so far. This was a relevant question, especially for the treatment of progressive patients, since no treatments have been available to stop the progression of disease. Vitamin D could possibly fill this gap by having a direct effect in the CNS itself. In **chapter 2** we used the expanded disability status scale (EDSS) score to investigate the relationship between vitamin D levels and disability progression over a 3 year follow up. Although we could confirm the known relation of a poor vitamin D status with an increased relapse risk in younger MS patients, no statistically significant association was found with disability or disability progression in patients with either relapsing remitting or progressive MS. An alternative way to investigate disease progression is described in **chapter 3**. Here, we investigated the role of vitamin D in the (time to) transition to secondary progressive (SP)MS in patients with relapsing remitting (RR)MS. We could confirm that the patients with SPMS in our study cohort had lower serum 25(OH)D levels when compared to the patients with RRMS. Cross sectional deseasonalised serum 25(OH)D levels in patients with RRMS did not predict the 3 year-risk of conversion to SPMS, but vitamin D levels at diagnosis were significantly lower in patients with RRMS and a very rapid progression to SPMS

(median RRMS duration of 3.5 (1.0-5.7) years) when compared to matched RRMS patients with a longer RRMS duration (minimum median RRMS duration of 7.7 (6.3-10.0) years, $p < 0.01$). Long term follow up of high dose vitamin D supplementation studies is warranted, to confirm the findings in both these studies on the relation between vitamin D and disease progression.

In the second part of this thesis, the vitamin D effects on immunological outcomes are described. The most recent Th cell, that has been discovered to have strong encephalitogenic capacities in the EAE animal model, is the GM-CSF producing Th cell. Although a clinical trial in patients with MS has been initiated (NCT01517282), data on GM-CSF producing T cells in humans are limited. Therefore, we assessed the role of GM-CSF producing T cells in patients with MS and the mechanisms that might regulate them in **chapter 4**. We showed that the proportion of GM-CSF producing T cells in the circulating immune cell compartment was similar in patients with MS and HC. Furthermore, GM-CSF producing Th cells formed a significant part (39%) of the total T cell fraction in CSF of both patients with MS and non-MS controls. These results did therefore not support a specific pathogenic role of these cells in MS. *In vitro*, GM-CSF production by peripheral blood Th cells could be controlled by regulatory T cells and by vitamin D. The regulation by vitamin D was less effective in patients with MS.

The most important part of this thesis focuses on the immune regulatory effects of high dose vitamin D supplementation in a randomised placebo controlled trial in patients with RRMS. We expected vitamin D to have a function in the immune regulation, in which the cytokine IL-10 plays an important role. The detection of IL-10 producing Th cells has, however, been reported to be difficult. Appropriate detection of IL-10 is highly relevant for a correct interpretation of the vitamin D effects on immune regulation. In **chapter 5** we describe an improved method to detect IL-10 producing Th cells using flow cytometry. We showed that omitting monensin, a protein transport inhibitor, from the stimulation protocol, increased the detection of IL-10 producing Th cells, NK-T cells and monocytes, but not B cells. **Chapter 6** reveals the results of the SOLARIUM study. We showed that high dose vitamin D supplementation did not have any substantial effects on the number of circulating pathogenic and regulatory lymphocytes relevant for MS. The anti-inflammatory, IL-4 producing, Th cells decreased over time in the placebo group and were stable in the vitamin D supplemented group, but this was not reflected by a change in the IFN- γ /IL-4 ratio. Functional analyses of supernatant of PBMC upon T cell stimulation, showed an increase of cytokine production in the placebo group, most outstanding in the anti-inflammatory cytokine compartment, while the cytokine expression was stable over time in the vitamin D supplemented group. Altogether, this could point towards a role of *in vivo* high dose vitamin D₃ supplementation in the maintenance of homeostasis of the adaptive immune cell compartment during the early RRMS disease course. Importantly, lymphocytes were still reactive to *in vitro* 1,25(OH)₂D, irrespective of randomisation group. Whether this balancing role also becomes evident in the clinical outcomes will become clear in the near future.

Vitamin D research in auto-immunity and MS, is the star on the horizon still shining?

Vitamin D was discovered as the active anti-rachitic substance in liver oil in 1922 [322] but is mainly obtained via UVB exposure of the skin [323]. Later, in 1960, MS was related to sun exposure [324] and in 1974, the hypothesis of vitamin D being necessary for optimal formation of myelin was put forward. [101] Since then, many researchers, *i.e.* immunologists, chemists, epidemiologists, geneticists and neurologists, have tried to get a better insight in the true role of vitamin D in auto-immunity and MS. In 2008, our group at the Academic MS Centre Limburg/Maastricht University Medical Centre, jumped into this research line and started with an overview of the then available evidence of the potential immune modulating effect of vitamin D in MS. [131] Some years later, we reviewed the evidence for *in vivo* effects of vitamin D in the healthy situation and in auto-immunity and concluded that this evidence was inconclusive (**chapter 1**). We were not the only ones discussing the role of vitamin D and the potential usefulness of high dose vitamin D supplementation therapies. Vitamin D workshops, originally organized for basic aspects of vitamin D biochemistry and physiology, started to broaden their view and discussions about the pros and cons of vitamin D therapies were held. [325-328] Commercial companies started advertising campaigns to enhance the purchase of vitamin D supplementation products thereby making also layman aware of the potential beneficial role of this sunshine vitamin, beyond the scope of bone strength. However, this explosion of quantitative interest in vitamin D made the differentiation between firm conclusions drawn from solid scientific experiments and to-be-proven hypotheses drawn from exploratory scientific studies, unclear.

The clinical point of view

The association between low vitamin D levels and relapse risk in patients with MS, has been substantiated by several studies from different research groups. [71, 72, 74, 75, 103] However, more and more recent evidence suggest the strongest relation of vitamin D in the MS disease process in the early (inflammatory) phase of the disease, with the strongest association between low vitamin D levels and relapse risk in MS patients with a short disease duration [103] and in CIS patients [206, 329]. Overall, the studies on clinical outcomes in this thesis confirm this line of thoughts and increase the knowledge on vitamin D and its role in progression of disease. Disease progression is a highly relevant outcome measure as MS is a chronic disease with a major impact on an individual's quality of life and with subsequent consequences for the society. We, indeed, confirmed the relation of vitamin D with relapse rate especially in young patients with MS with a possible role of a low vitamin D at the start of RRMS disease as risk factor for early conversion to SPMS. However, later during the disease course, we did not find a relation between serum vitamin D levels and conversion to SPMS or progression of disease, neither in RRMS nor in progressive MS (**chapter 2 and 3**). Also other follow-up studies have shown that physiological vitamin D levels in (very) early RRMS and CIS patients were related to MRI outcomes [206, 234] and EDSS [206]. This was,

however, not the case in established MS where there was no relation between EDSS progression and vitamin D levels. [73, 215, 234] The dilemma in studying disease progression is that it is not a parameter easy to measure. Disability related outcome measures are most commonly evaluated by using the EDSS score (as mentioned in the general introduction of this thesis). This measure is not ideal, but arguably the best measure available. Besides, it is important that the follow-up period in studies on progression is long enough to measure an effect, keeping in mind the role of other more substantial influencing factors than vitamin D, like the immune modulating treatments.

Overall, our and other studies on the role of vitamin D in MS disease progression, suggest that the intake of (high dose) vitamin D supplementation in patients with MS later in the disease course will not affect the progression of the disease. Adequate vitamin D levels might be most relevant for disease activity and disease progression in the very beginning or even before the start of the disease when the inflammatory component is most obvious and maintenance of a (immunological) balance is still possible. This implies a narrow window of opportunity for vitamin D supplementation studies. Leray *et al.* have postulated that disability progression in MS may follow a two stage pattern: early progression till an irreversible EDSS of 3 and later progression till an irreversible EDSS of 6. They suggest the progression in the late stage to be independent of the duration and progression in the early phase, both in patients with relapsing remitting and progressive MS. Only in the early phase the number of relapses was an independent predictor of disability progression. [230] If vitamin D would be able to increase the duration of this early phase, this would mean that the onset of the second phase progression, which is probably not caused by focal inflammation, but by diffuse inflammation and neurodegeneration, could be postponed. Besides, this would have important implications for decisions on the continuation of DMTs in the SPMS phase. Currently, most treatments are especially effective in the inflammatory phase of MS, meaning that guidelines advice to stop treating patients who enter a secondary progressive phase, especially if they do not experience relapses anymore. [51, 330] However, the decision to discontinue treatment is often difficult to accept for patients and many neurologists continue to prescribe DMTs to patients with SPMS. [331] A postponement of the conversion to SPMS by adequate vitamin D levels early in the disease course could therefore supposedly also increase the window of opportunity for DMTs.

Although one supplementation study showed a trend towards a relation between vitamin D levels and EDSS in RRMS after 1 year [80], long term follow-up of high dose supplementation trials should be able to give definitive answers to the question of the role of vitamin D in MS disease progression.

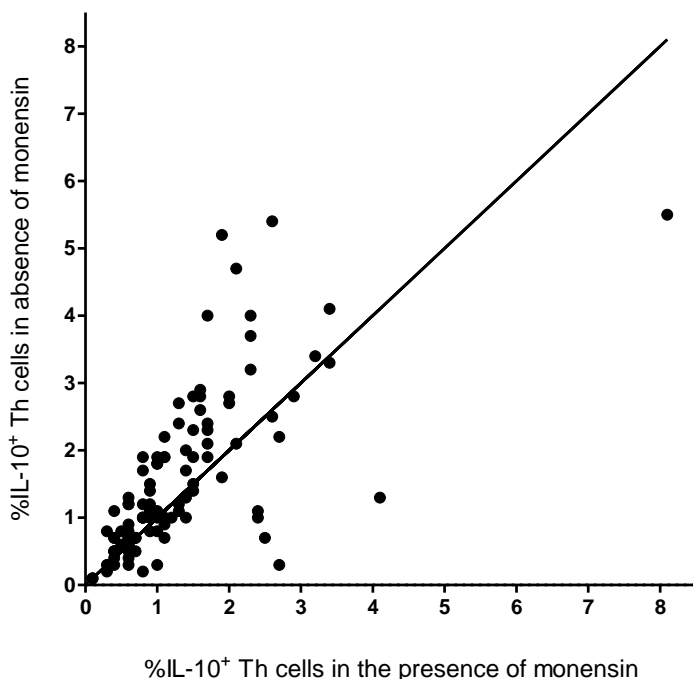


Figure 1. Proportion of IL-10⁺ Th cells detected in patients included in the SOLARIUM study at wk0 and wk48, in the presence and in the absence of monensin. The line represents the $y=x$.

IL: interleukin; *Th*: T-helper

The immunological point of view

In the second part of this thesis, we assessed the mechanism underlying the associations between vitamin D and MS disease activity and investigated the effect on relevant immunological outcomes. A recent addition to the panel of T-cell cytokines, suggested to be detrimental in EAE, is GM-CSF. Its role and relevance in MS has not yet been fully consolidated, but it might be a relevant marker to evaluate an immune modulating effect of vitamin D in MS. We therefore first looked at the GM-CSF producing (GM-CSF⁺) Th cells in patients with MS in a cross sectional design, and ascertained that GM-CSF⁺ Th cells are not increased in the peripheral blood of patients with RRMS in remission nor in the CSF (**chapter 4**). In succession of this finding, Noster *et al.* found that the number of GM-CSF⁺ Th cells was also not increased in patients with active MS disease, contrary to the numbers in CSF, which they found to be increased compared to non-MS controls. [39] On the opposite, Hartmann *et al.* reported a borderline significant increased fraction of these cells in the circulating immune cell compartment of patients with MS compared to patients with other neurological diseases. They also showed that DMTs could decrease the numbers of GM-CSF⁺ Th cells. [254] Up to now, it is still the question whether the pathogenicity of the GM-CSF⁺ Th cell holds for the whole group of GM-CSF⁺ Th cells or only for a subset. [39] We showed that GM-CSF⁺ Th cells

were sensitive to regulation by regulatory cells and vitamin D, but the *in vitro* inhibition by vitamin D was less pronounced in patients with MS than in HC (**chapter 4**). Possible mechanism for this may lay in the genetic differences in vitamin D metabolism or vitamin D response elements in patients with MS, or in a different composition of the gut microbiome. More details on this topic will be discussed in the next paragraph.

Next we assessed the immunological effects in our high dose vitamin D supplementation study in patients with early RRMS. Therefore, we first improved the detection of IL-10⁺ Th cells, the so called iTregs, which are important cells for immune regulation and a potential target of vitamin D therapy. The new protocol is described in **chapter 5**. We used this optimized protocol to assess iTregs in the SOLARIUM study where we could confirm our previous findings, i.e. we detected increased proportions of IL-10⁺ Th cells after omitting monensin from the stimulation protocol (median proportion of IL-10⁺ Th cell in the presence of monensin 1.0% (0.6-1.7), in the absence of monensin 1.2% (0.7-2.2); $p < 0.001$) (Figure 1, unpublished data).

Previously, we [79] and others [180, 186, 192, 302, 303, 306, 320, 332] have reported positive outcomes of vitamin D supplementation on a diverse array of immune parameters. In the SOLARIUM study, we could only detect minor changes on measures of regulatory lymphocyte subset frequencies and lymphocyte cytokine production in the circulating immune cell compartment of the RRMS patient who received placebo, while the measures in the vitamin D group were rather stable over time. The added value of the SOLARIUM study compared to previous studies was that this was a placebo controlled study with relevant power, and a high dose (14.000 IU/day) and long term (48 weeks) vitamin D₃ supplementation intervention. Results indicate that vitamin D balances the disturbed adaptive immune system during the disease course as was observed in the placebo group. In this group, the proportion of anti-inflammatory IL-4⁺ Th cells decreased while a general increase in the amounts of cytokines produced upon T cell activation was shown, most pronounced in the anti-inflammatory compartment. This might point towards a disturbed immune response with accompanying compensatory mechanisms early in the MS course. Vitamin D might hereby be able to better maintain immune homeostasis and consequently control the disease course of MS. These findings are subtle and not entirely in line with earlier cross-sectional findings on immunological outcomes, and their relevance for clinical MS outcomes still needs to be shown. Notably, an effect of vitamin D on the immune pathology of MS is not necessarily the only driver of the numerous associations between vitamin D status and evolution of disease course in MS. Possible alternative hypotheses are plentiful, but cluster around two main themes in which vitamin D either does or does not affect the pathophysiology of MS.

Food for thought - alternative hypotheses

Our studies in the context of vitamin D as an effector in the MS pathophysiology

When assuming that vitamin D does interfere with MS, particularly in the early or pre-clinical phase, our clinical studies confirm this hypothesis and the SOLARIUM study hints towards a more complex mechanistic role of vitamin D within the human body than *in vivo* animal and *in vitro* human studies have suggested. These studies showed clear inhibiting effects of vitamin D supplementation on the pro-inflammatory cells of the adaptive immune system, while regulatory lymphocytes and anti-inflammatory lymphocytes were promoted. [131] In the human body, compensatory mechanisms of the neuro-endocrine-immune system, including other vitamins and (sex) hormones, may take place. These interactions may be too complex to observe interference with a single component of this system, like vitamin D, in the immune system as a whole or on broad clinical measures. Complex multidimensional measurements, with multiple parameters at the single cell level like those obtained in the SOLARIUM study, make analyses of the right parameters a challenging one. Attempts to translate such datasets into one relevant integrated immune parameter have already been made [333], but might need further investigation.

Although we aimed to look at relevant effects of vitamin D, by taking the regulatory lymphocytes and cytokine producing Th cells as a read-out, measuring vitamin D effects will be dependent on the right location, the right timing and the investigation of the right target cells. The cells we isolated from blood represent a heterogeneous pool of lymphocytes. In contrast, *in vitro* experiments and *in vivo* models of neuroinflammation show a more homogeneous pool of (antigen-specific) activated cells, in which vitamin D may interfere. Therefore, an effect of vitamin D on pathogenic T cells in MS may be diluted and not observed in our studies. Another option is that our hypothesis, being vitamin D an immune regulator, was incorrect. Natalizumab, an effective MS drug, has for example been shown to lead to similar or even higher levels of pathogenic IL-17⁺ Th cells in the CNS, while one would expect pathogenic Th cells to decrease in number after treatment with an effective drug. [334, 335] This also illustrates that it is possibly not (only) the number of cells that counts but also the functionality of these cells. We did not include the measurement of functional regulating capacity of regulatory lymphocytes *in vitro* as an outcome measure in our RCT. Our data do not suggest a dramatic shift in markers of immune regulatory cells, but a recent trial showed an improvement of Treg function after supplementation with vitamin D in diabetes mellitus type 1 patients. [313] Other, newly discovered cells that could be relevant as detrimental cells in MS and as targets for vitamin D therapy are specific subsets of GM-CSF producing Th cells (GM-CSF⁺ IFN- γ ⁺ double producers or GM-CSF-only producers) [39], IL-2 receptor (CD25) expressing Th cells [336], CD52 Tregs [337] and GM-CSF producing B cells [338]. Furthermore, we did not investigate the direct effect of vitamin D supplementation on the immune cells in the CNS, on the CNS-cells themselves or on the blood brain barrier, which are all difficult to assess in humans. Likewise, migration of lymphocytes into the CNS would be an interesting outcome measure. A chemokine that might be relevant in this perspective

is CXCR3, involved in trafficking of potential pathogenic immune cells. This trafficking was impaired upon vitamin D supplementation in the EAE model. [339]

Based on previous studies and our own clinical studies, described in this thesis, we included a group of patients with early RRMS who have the highest chance to benefit from high dose vitamin D supplementation. However, the IFN- β treatment they received might have obscured the vitamin D effects. Obliging patients to be treatment naïve during the vitamin D supplementation period was, obviously, unethical. Moreover, IFN- β was one of the most common MS therapies used at the start of the study. Also, associations between vitamin D status and MS outcomes have been predominantly reported in IFN- β treated MS patients [74, 206, 234, 340], suggesting this treatment not to interfere with an underlying mechanism. Furthermore, vitamin D supplementation might be effective only in those patients with very low 25(OH)D levels. Yet, the immune system of the subgroup of patients with a very low 25(OH)D status at baseline did not show more clear-cut effects of high dose vitamin D supplementation in our study (**chapter 6**). This makes it also likely that it is not the change in 25(OH)D level that counts. In line with this notion, SOLAR was certainly not a dose finding study: too high or too low doses of vitamin D may have been supplemented. In addition, we still do not know whether a certain vitamin D level is equally important for each individual. Multiple genes do affect an individual's vitamin D status. [341, 342] Black people have lower serum vitamin D levels when compared to white people, but their bone metabolism is equal. [343, 344] Furthermore, also the main risk allele for MS, HLA-DRB1*1501, is regulated by vitamin D [345] just like the risk alleles TAGAP and IL-2R in Th cells [346]. CYP27B1 and CYP24A1, involved in the metabolism of vitamin D, popped up in GWAS. [14] Therefore supplementation might be most beneficial in a certain subgroup of patients with MS, based on their genetic profile. Unfortunately, our group of included patients did not have sufficient power to perform a stratification for this. Combined results of SOLAR and other vitamin D₃ RCTs may provide more clearness on this issue.

Our studies in the context of vitamin D not affecting the MS pathophysiology

The main second theme of alternative hypotheses regarding the association between vitamin D status and MS is that findings on the effects of vitamin D in *in vitro* studies and *in vivo* animal studies do not reflect the situation in human MS. In the EAE animal models, the active component of vitamin D, 1,25(OH)₂D has been shown to be beneficial, mainly in the prevention of disease onset, but also in reducing severity of symptoms and increasing survival. [131] Substantial fewer studies have been performed on vitamin D supplementation in mice. Spach and Hayes showed that vitamin D supplementation before immunization inhibited EAE in female mice. [159] Farias *et al.* replicated this finding with a reduced EAE severity, when vitamin D supplementation was started at the moment of immunization [347] and mice from vitamin D deprived parents developed a precocious and more severe EAE [348]. Also *in vitro* studies have contributed to the hypothesis of vitamin D having an immune regulatory role by increasing regulatory cells and anti-inflammatory cells and decreasing pro-inflammatory cells. However, here as well, usually the 1,25(OH)₂D metabolite is used. In

humans, though, 25(OH)D levels are associated with clinical MS outcomes, whereas that is less clear for 1,25(OH)₂D. Besides, supplementation of 1,25(OH)₂D may lead to (unacceptable) hypercalcemia risks, while increasing the 25(OH)D levels might give the body the opportunity to regulate 1,25(OH)₂D levels, thereby diminishing the change to develop side effects. Therefore, vitamin D supplementation in humans is based on supplementation with vitamin D₂ or D₃.

Discrepancies between *in vitro* and *in vivo* human studies may also be explained by quantitative issues in the immune responses studied: the *in vitro* effects of 1,25(OH)₂D are usually assessed under quite extreme stimulating conditions, boosting the immune system to a very strong inflammatory reaction, one that will usually not arise *in vivo* in the human body. A strong aspect of the SOLARIUM is that functional characteristics of isolated PBMC (as well as their responsiveness to vitamin D) were assessed both directly *ex vivo* as *in vitro*. Furthermore, in the isolated environment of *in vitro* experiments, the dynamics of interactions in the human body are lost and other important parts of the immune system, like germinal centres cannot be easily investigated directly *ex vivo*. [349]

Another interesting point is that our GM-CSF study and another recent study [315] have suggested immune cells of patients with MS to be less sensitive to regulation by vitamin D when compared to cells of HC (**chapter 4**). This could be due to differences in the vitamin D metabolism. Previously we have shown that the gene expression of the VDR, CYP27B1 and CYP24A1, was not impaired in PBMC and Th cells of patients with MS compared to HC, suggesting a normal vitamin D response and metabolism in both groups. [350, 351] However, Bhargava *et al.* showed that MS patients have lower 25(OH)D levels after vitamin D supplementation with 5000 IU/day for 90 days than HC. [316] If it is really true that the vitamin D metabolism in patients with MS is different from that of controls, these differences may be caused by variances in gut absorption, alternations of gut microbiota or in the polymorphisms in genes related to vitamin D metabolism, which are reported in some, but not all, MS genetic studies. It might therefore be that vitamin D supplementation is most relevant for a subpopulation of patients with MS who have a more 'HC-like' microbiome or genetic profile.

In contrast to our current supplementation study, previous supplementation studies, most of them reporting much clearer effects of vitamin D on immunological and clinical outcome measures, were small and uncontrolled. Thereby introducing higher odds for chance and regression to the mean to interfere with the results. Furthermore, the impression that there is an unintended, but clear, bias of positive data selected and published, cannot be banished. Positive results of vitamin D supplementation have been selectively emphasized reporting changes of single cytokines while the more apparent negative results were just briefly mentioned. [187, 302, 306, 352] Blind analyses of all cytokines or cytokine producing T cells might have given a more general view on the effects of vitamin D on the adaptive immune system. This might have taken away the focus on changes in the pathogenic and regulatory cells in MS, and might have led to different conclusions, as has recently been suggested in Nature. [353, 354]

Ultimately, vitamin D might be a correlate of the causative factor interfering with MS. It may not be the (only) driving force in the relation between MS and sun exposure. Vitamin D is synthesized in the skin upon UVB exposure, in particular UV light with a wavelength between 280 and 310 nm. Although positive associations with vitamin D and disease severity have been described in the EAE animal model, suppression of EAE appeared to be most eminent at wavelengths of 300-315 nm and was then independent of vitamin D. [355, 356] Vitamin D levels might therefore be a surrogate marker of sun exposure. Other potential candidates of the relationship between sun exposure and MS onset and disease activity are melatonin [319], vitamin A [318] or nitric oxide [321]. A 'consumopathy' of vitamin D by the activated immune cells could also explain the low vitamin D levels in patients with MS. [317] Low levels of vitamin D are then the consequence of the inflammation rather than a cause and higher serum levels of 25(OH)D will not dampen the inflammatory component. Lastly, most MS outcomes correlate negatively with sun exposure. [73] Therefore, being less exposed to sunlight and vitamin D may be a consequence rather than a cause of having active MS.

Summary

Summing up, the studies described in this thesis provide additional support for a role of vitamin D and possibly also vitamin D supplementation in the early (and maybe even pre-clinical) state of MS. Ultimately, to draw conclusions and chose directions for further research on vitamin D in MS, the results of clinical trials are extremely important. These studies will show whether modulation of vitamin D status will affect disease outcomes in MS. One of these studies is the SOLAR study. Data are not yet available, but will be announced in 2016. If modulation of the vitamin D status indeed affects disease outcomes, the question is whether these effects arise from the limited effects on the immune system or that there is a more important target. If not, other factors interfering with both vitamin D status and MS outcome may be more relevant. Altogether, as will be discussed in the next paragraphs, the star on the horizon is sufficiently shining to warrant further research on the role of vitamin D in MS.

Future prospects

This thesis describes the research done to discover some missing pieces in the story of vitamin D in MS. Although some questions have been clarified, the final answer is not there yet. As already mentioned, supporting evidence suggest the strongest role of vitamin D very early in the disease course. To enhance our knowledge on the long term effects of vitamin D, also on disease progression, it is important to follow up the patients in vitamin D supplementation studies, such as the SOLAR study. Future research will possibly also tell us whether other immune modulating drugs, currently in development, might have even more relevant synergistic effects with vitamin D than IFN- β has. Vitamin D will probably not be of help in immune cell depleting therapies or therapies blocking the entrance of the immune cells into the CNS, but a possible role in combination with immune modulation, stem cell transplantation (SCT) or remyelination therapies is not unrealistic. Preventive treatment was beyond the

scope of this thesis, but other studies on vitamin D have shown also associations and effects of vitamin D in the pre-clinical phase of MS: *i.e.* CIS [206, 329] and optic neuritis patients [357, 358]. These findings confirm our line of thoughts on a restricted window of opportunity for vitamin D supplementation (very) early in (the pre-clinical phase of) MS. One step further, vitamin D treatment could then also be useful in any pre-clinical phase, in which the disease process already started, but no actual symptoms have appeared. It might even be necessary to move our view more towards the prevention of the development of auto-immune disease in children and young adults. To obtain a healthy bone and calcium metabolism, vitamin D supplementation of 400 IU per day is already common in children and adolescents in the USA. [359] In Europe, this is advised for all children till the age of 4 or 5 and till the age of 19 for every child who is at risk of a low vitamin D level due to a dark skin or a minimal time spent outside. [360-362] The question for the future is then whether vitamin D supplementation should be advised for adolescents all over the (Western) world to prevent the development of MS and possibly other diseases. Furthermore, we will have to investigate what the desired dose of supplementation is for this target group.

To obtain more insights into the mechanism of vitamin D and its role in the MS pathogenesis, our longitudinal samples which were collected in the SOLAR and SOLARIUM study are of great value. There are still a couple of interesting questions that need an answer. To gain more insights in what happens in the vitamin D uptake, transport and metabolism in patients with MS, it might be interesting to measure the vitamin D metabolizing enzymes and associated genes in plasma or lymphocytes. Besides these mechanisms playing a role in the effectiveness of vitamin D treatment in patients with MS, also the existence of neutralizing vitamin D antibodies, which were found in serum of patients with systemic lupus erythematosus, may hamper the potency of vitamin D treatment. [363, 364] Furthermore, one could think of investigating the vitamin D binding protein (DBP). The effects of vitamin D supplementation were cancelled out in EAE mice with increased DBP expression. [365, 366] DBP levels have been reported to be increased in patients with MS [365], although our group could not confirm this [367]. Furthermore the investigation of vitamin D metabolites and the free versus bound fraction of 25(OH)D could learn us more about the actual availability of the 25(OH)D metabolite in the microenvironment of immune- and CNS cells. [368]

As mentioned before, it is possible to look at other target cells of the immune system that were not investigated so far. Furthermore, it would be compelling to know what the actual effects of vitamin D supplementation are on the target tissue, the CNS. However, human brain tissue usually originates from deceased MS patients with more advanced MS. Those samples will probably not be representative of MS manifestations at the start or during the disease course. Therefore, answers to this question will probably need an animal model, which is genetically linked to human MS, like EAE models in primates such as marmosets. Another open question is what happens in the lymph nodes of patients with MS, in reaction to vitamin D supplementation. It would be very interesting to investigate this in the (superficial) cervical lymph nodes where most probably antigen presentation takes place. Also (changes in) the gut microbiome is a hot topic in auto-immunity research. Furthermore it is

interesting to look at the relations and interactions of different environmental risk factors in MS such as the combination of vitamin D and EBV [349] and to keep our minds open for other underlying mechanism that could explain the relation between sun exposure and MS like melatonin, vitamin A and nitric oxide.

Intriguing is the difference between *in vitro* and *in vivo* vitamin D supplementation studies. Instead of thinking about explanations, we could also turn this phenomenon to our advantage. By *in vitro* treatment of autologous immune cells, for example T cells, and replacement of those cells in the human body, we could circumvent the *in vivo* (compensatory) effects. [347] Research on this topic is already ongoing in the group of B. Roep at the University Medical Centre in Leiden (the Netherlands). They are investigating the possibilities of *in vitro* vitamin D treatment of dendritic cells (DC) of diabetes mellitus patients. Once placed back in the body these DC could induce the development of Tregs. [369, 370]

More in general, to enhance the progress in MS and environmental factor research, especially vitamin D, it is important that researchers in all fields of auto-immunity are open to each other and work together to disentangle the common denominators in the pathogenesis of auto-immune diseases. It is important that people involved continue to look beyond the own niche, traditionally based on the target organ that is affected. Initiatives to facilitate this have already been undertaken by the Dutch Society of Immunology (Balie debat, 20 Nov 2014). [371, 372] Another hot topic in the scientific community is the question how to obtain objective analysis and reproducibility of data, which are translatable to the clinic. [353, 354, 373, 374] To this end, it is important that everybody involved in scientific research, i.e. tutors, researchers, journal editors and reviewers is aware of this challenge and continues to do their best to prevent the realisation and publication of low quality research. Recently, it has been shown that the use of positive and negative words in research papers has increased substantially in the last decades, while the use of neutral words decreased. It might be that scientific findings are sometimes overstated or exaggerated in order to be published. [375] Also the high rate of discontinuation and non-publication of RCTs [376] might lead to waste of research sources, ethical concerns, and affect research and patient care. Therefore, it is important to make the publication of negative results not inferior to the publication of positive results. This will overcome false hopes and hypes and will make it possible to focus research sources on the really relevant topics.

Vitamin D in MS: hype or hope?

The role of vitamin D in MS has been proposed to be twofold. First of all it is presented as one of the identified environmental risk factors in the development of MS. Second, vitamin D has been related to MS disease activity. The research in this thesis focused on the latter topic. Vitamin D research and papers published on this topic have increased significantly in the last decades. In the previous 5 years the increase in papers on vitamin D appears to be stronger than the increase in the total amount of papers published, confirming the gained interest in vitamin D in the scientific community (Figure 2).

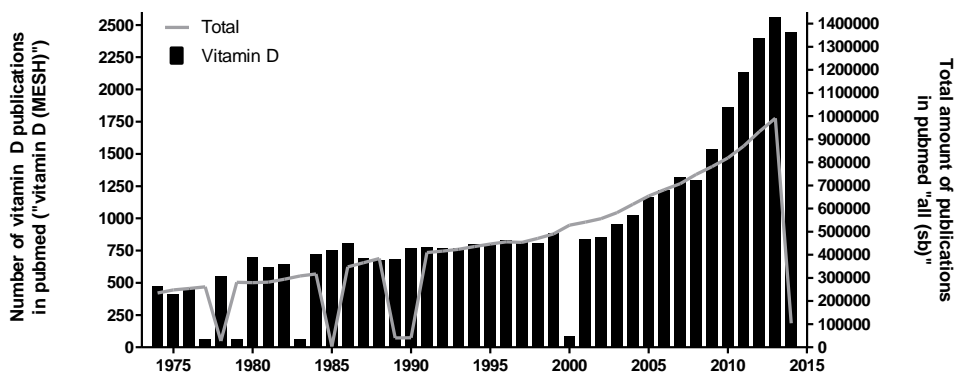


Figure 2. Quantification of publications cited in Pubmed on vitamin D (MESH) in black bars and total publications (all (sb)) in grey, chronologically ordered.

The proposed role of vitamin D in relation to MS and multiple other auto-immune diseases, but also non auto-immune diseases, like cardiovascular diseases, infectious diseases and cancer, has provided the ideal background for vitamin D to become a hype. Some clinicians already prescribe high doses of vitamin D to their patients and some countries included the advice to supplement patients with MS with 2000-4000 IU per day in their protocols. [377] However, hypes can induce false hopes and make it a challenge to disentangle the truth. We presume that the work in this thesis contributes to a better understanding and an increased nuance in the role of vitamin D in MS. Of course we will have to wait for the clinical outcomes of high dose vitamin D supplementation studies first, but findings so far suggest a window of opportunity for vitamin D treatment in pre-clinical and early MS. By extrapolating these results, we can speculate about the relevance of vitamin D supplementation even before the onset of disease. The role of vitamin D later in the disease seems negligible, but long term follow-up of randomised controlled trials have to confirm our findings. One of the mechanisms involved in this might be the maintenance of immunological homeostasis. Immune cells of patients with MS do react differently to vitamin D than those of HC. Our immunological analyses of the blood of patients with MS in a high dose vitamin D₃ supplementation study showed that vitamin D is possibly able to maintain the immunological balance in the circulating immune cell compartment during the disease course. What the effect of vitamin D is on the immune cells in lymph nodes and in the CNS or on CNS cells themselves, and whether other (environmental) factors in addition to vitamin D play an essential causative role in reported associations, remain open questions.

Nederlandse samenvatting (Dutch summary)

Introductie

In dit proefschrift hebben we een bijdrage willen leveren aan de kennis met betrekking tot de rol van vitamine D in multiple sclerose (MS). We hebben ons hierbij gericht op de rol van vitamine D spiegels op de ziekteprogressie en beschrijven de effecten van hoge dosis vitamine D suppletie op het immuunsysteem van patiënten met MS.

MS is een inflammatoire ziekte van het centrale zenuwstelsel (CZS), bestaande uit het ruggenmerg en de hersenen. In Nederland hebben ongeveer 16.000 mensen MS, op de wereld ongeveer 1,3 miljoen. Het is een ziekte die zich voornamelijk bij jonge vrouwen manifesteert, tussen de 20 en 40 jaar. De precieze oorzaak is niet bekend; het is hoogstwaarschijnlijk een combinatie van genetische factoren en omgevingsfactoren die een rol speelt. De belangrijkste omgevingsfactoren zijn een lage vitamine D status in het bloed, roken en infecties met bijvoorbeeld het Epstein Barr virus (EBV). Het meest voorkomende type MS is de zogenaamde relapsing remitting MS (RRMS), waarbij periodes van neurologische symptomen (relapsen), afgewisseld worden door periodes van herstel (remissie). De ziekte kan zich met verscheidenheid aan symptomen uiten en deze kunnen bestaan uit gevoelsverlies, tintelingen, vermoeidheid, spierspasmes, zwakte, problemen met lopen, pijn, problemen met het zien, een gestoorde verstandelijke functie, depressie en het niet goed functioneren van blaas en darmen. Als de symptomen niet volledig herstellen en er ook tussen de relapsen verergering van klachten optreedt, spreken we van progressieve MS (secundair progressieve MS (SPMS) of primair progressieve MS (PPMS)).

Er wordt over het algemeen gedacht dat MS een auto-immuunziekte is. Dit geldt in het bijzonder voor het RRMS subtype. Auto-immuunziekten ontstaan door een fout in het immuunsysteem waardoor dit systeem niet goed meer in staat is eigen weefsel van niet-eigen weefsel te onderscheiden. In MS betekent dit dat lichaamseigen weefsel van het CNS door het immuunsysteem wordt aangevallen. Hierdoor raken zenuwcellen (neuronen) en myeline, het vetachtige zenuwmerg dat normaal voor een snelle en vloeiende overdracht van prikkels zorgt, beschadigd. Op de lange termijn zijn deze beschadigingen niet meer te herstellen en treedt verergering van de klachten op.

Vitamine D is een belangrijk vitamine dat wij voornamelijk binnenkrijgen onder invloed van UVB straling in zonlicht. Een goede vitamine D spiegel in het bloed, gemeten als 25(OH)D, is van belang voor een goede calciumregulatie en bothuishouding. Maar lage vitamine D spiegels worden ook in verband gebracht met het risico op het ontwikkelen van auto-immuunziekten als MS. Daarnaast lijkt het erop dat lage vitamine D spiegels in patiënten met MS de kans op het ontwikkelen van relapsen kan vergroten. Een belangrijke open vraag was of vitamine D spiegels ook in verband kunnen worden gebracht met de mate van MS gerelateerde beperkingen en de achteruitgang daarin, later in het ziekteproces. Dit is een belangrijke uitkomstmaat omdat deze achteruitgang van ziekte voornamelijk speelt bij patiënten met progressieve MS, voor wie op dit moment alleen behandeling bestaat die symptomen kan verminderen, maar niet de ziekte kan remmen of genezen.

Voor patiënten met RRMS zijn er wel al verschillende ziekte remmende medicijnen op de markt en vele meer in ontwikkeling. Gebaseerd op de eerdere verbanden die gelegd zijn tussen lage vitamine D spiegels en de kans op het ontwikkelen van een relapse, ontstond de gedachte dat vitamine D suppletie een goede aanvulling zou kunnen zijn op de al bestaande therapieën voor patiënten met RRMS. Eerdere kleine studies hebben uitgewezen dat dit waarschijnlijk veilig is en resultaten waren hoopgevend. Het onderliggende mechanisme van vitamine D is waarschijnlijk een immuunregulatorisch effect, waardoor het immuunsysteem minder snel ontspoord.

In dit proefschrift hebben we allereerst de rol van vitamine D in de achteruitgang van de ziekte onderzocht. Daarnaast hebben we in een grootschalige gerandomiseerde en gecontroleerde studie gekeken naar de effecten van vitamine D suppletie op immunologische uitkomstmaten (de SOLARIUM studie).

Samenvatting van het beschreven onderzoek

Om te beginnen, hebben we in **hoofdstuk 1** de *in vivo* effecten van vitamine D op het immuunsysteem van gezonde controles en van patiënten met een auto-immuunziekte beschreven. Hierin wordt een overzicht gemaakt van de kennis op het moment van de start van het onderzoek dat in dit proefschrift is beschreven. Het mechanisme dat het verband tussen vitamine D en de ziekteactiviteit van patiënten met MS zou kunnen verklaren, is gebaseerd op de gedachte dat vitamine D immuun regulerende eigenschappen heeft. *In vitro* studies (studies buiten het lichaam van een levend organisme) en studies in het diermodel van MS, experimentele auto-immuun encefalitis (EAE, ontsteking van het encephalon/ brein), hadden eerder aangetoond dat immuun cellen functionele doelwitten zijn van vitamine D. In dit overzichtsartikel bleek echter dat de resultaten die tot dan toe bij mensen beschreven waren, heterogeen waren. Bovendien was er sprake van een grote verscheidenheid aan immuun parameters die bestudeerd werd. Over het algemeen leek het erop dat er een trend zichtbaar was richting een anti-inflammatoire rol van vitamine D, maar dit kon niet bevestigd worden door overtuigend bewijs. Daarom waren er krachtige studies, zoals gerandomiseerde gecontroleerde onderzoeken nodig, voordat er definitieve conclusies met betrekking tot dit onderwerp getrokken konden worden.

Terwijl dit soort gerandomiseerd en gecontroleerd, grootschalig onderzoek van start ging, hebben wij de rol van fysiologische niveaus vitamine D in het bloed, op progressie van de ziekte onderzocht. Progressie van ziekte is een belangrijke uitkomstmaat, in het bijzonder voor progressieve MS patiënten. Op dit moment zijn er geen medicijnen beschikbaar die ook daadwerkelijk de ziekteprogressie stoppen. Vitamine D zou hierin mogelijk wel een rol kunnen spelen, door een direct positief effect op de cellen van het brein. In **hoofdstuk 2** hebben we gebruik gemaakt van de EDSS score om in een retrospectieve follow-up studie van 3 jaar, de relatie tussen vitamine D status in het bloed en progressie van de invaliditeit te onderzoeken. Hoewel we de relatie tussen een lage vitamine D status en een toegenomen

kans op het ontwikkelen van relapsen in jonge patiënten konden bevestigen, konden we geen statistisch significant verband aantonen tussen de mate van invaliditeit of de progressie van invaliditeit in patiënten met RRMS of patiënten met progressieve MS. Een andere manier om ziekte progressie te onderzoeken is beschreven in **hoofdstuk 3**. Hier hebben we de rol van vitamine D bekeken in de overgang naar een secundair progressieve MS in patiënten met RRMS. We konden bevestigen dat patiënten met SPMS lagere 25(OH)D spiegels hadden dan patiënten met RRMS. Cross-sectioneel gemeten en voor het seizoen gecorrigeerde 25(OH)D spiegels van deze RRMS patiënten voorspelden niet of zij in de daaropvolgende 3 jaar overgingen naar een SPMS. Vitamine D spiegels op het moment van diagnose waren echter wel significant lager in RRMS patiënten met een snelle progressie naar SPMS (mediane RRMS duur van 3.5 (1.0-5.7) jaar) vergeleken met vergelijkbare RRMS patiënten met een langere RRMS duur (minimum mediane RRMS duur 7.7 (6.3-10.0) jaar). Langdurige follow-up van hoge dosis vitamine D suppletie studies zullen de bevindingen in deze beide klinische studies m.b.t. de relatie tussen vitamine D en ziekteprogressie moeten bevestigen. In het tweede gedeelte van dit proefschrift, zijn de effecten van vitamine D op immunologische uitkomstmaten beschreven. De meest recent ontdekte T-helper cel, die in het EAE diermodel, sterk encephalitogeen (dat wil zeggen die een ontsteking in de hersenen kan veroorzaken) is, was de GM-CSF producerende T helper cel. Hoewel er een klinische trial in MS patiënten is geïnitieerd (NCT01517282), zijn data m.b.t. deze cellen in mensen schaars. Daarom, hebben wij deze cellen en de mechanismen die deze cellen reguleren bekeken in **hoofdstuk 4**. Wij hebben laten zien dat het aantal GM-CSF producerende T cellen in het circulerende immuun cel compartiment niet verschilt tussen patiënten met MS en gezonde controles. Daarnaast zagen we dat GM-CSF producerende cellen 39% van de T cellen in de hersenvloeistof van zowel patiënten met MS als van controle patiënten uitmaakten. Deze resultaten konden zodoende niet een specifieke pathogene rol van deze cellen in MS bevestigen. *In vitro* konden regulatoire T cellen uit het bloed en vitamine D de hoeveelheid GM-CSF, geproduceerd door T cellen, beïnvloeden. Deze regulatie door vitamine D was minder effectief in MS patiënten.

Het belangrijkste onderdeel van dit proefschrift richt zich op de immunologische effecten van hoge dosis vitamine D suppletie in een gerandomiseerde studie bij patiënten met RRMS. Wij verwachtten dat vitamine D een rol heeft in de immuun regulatie en hierin speelt het cytokine IL-10 een belangrijke rol. Het detecteren van T helper cellen die IL-10 produceren is echter lastig, maar wel belangrijk om de effecten van vitamine D op het immuunsysteem juist in te schatten. Daarom beschrijven we in **hoofdstuk 5** een verbeterde methode om deze cellen te detecteren m.b.v. flow cytometrie. Door het weglaten van de eiwit transport remmer monensine uit het protocol, neemt de detectie van het aantal IL-10 producerende T helper, NK- en NK-T cellen toe, maar niet het aantal B-cellen. In **hoofdstuk 6**, worden de resultaten van de SOLARIUM studie beschreven. We laten zien dat hoge dosis vitamine D₃ suppletie geen duidelijke effecten heeft op het aantal, voor MS relevante, pathogene en regulatoire lymfocyten. De anti-inflammatoire IL-4 producerende T helper cellen namen wel af over de follow-up tijd in de placebo groep en dit aantal was stabiel in de vitamine D₃

gesuppleerde groep, terwijl dit niet terug te zien was in een verschil van de IFN- γ /IL-4 ratio. Functionele analyse van het supernatant van PBMC na T cel stimulatie liet een toename van cytokines zien in de placebo groep. Deze was het meest uitgesproken voor de anti-inflammatoire cytokines. De cytokine expressie in de vitamine D₃ gesuppleerde groep veranderde niet. Dit zou kunnen wijzen op een rol van *in vivo* vitamine D₃ suppletie in het behoud van homeostase van het adaptieve immuunsysteem vroeg in het beloop van MS. Belangrijk om te vermelden is daarnaast, dat lymfocyten van beide groepen nog steeds reageerden op *in vitro* 1,25(OH)D toevoeging. Of het behoud van deze balans ook tot uiting komt in klinische uitkomstmaten zal in de nabije toekomst duidelijk worden.

Vitamine D in MS, hype of hoop?

De rol van vitamine D in MS zou tweeledig kunnen zijn. Enerzijds is het een van de omgevingsfactoren die geassocieerd is met het risico op het ontwikkelen van MS. Anderzijds, is een lage vitamine D status in het bloed gerelateerd aan de ziekteactiviteit van MS patiënten. Het onderzoek in dit proefschrift heeft met name dit laatste onderwerp verder uitgediept. Het onderzoek naar vitamine D en het aantal artikelen dat hierover gepubliceerd is, is de laatste tientallen jaren enorm toegenomen. Dit feit bevestigt de toegenomen aandacht voor vitamine D in de medisch wetenschappelijke wereld. Daarnaast heeft de mogelijke rol van vitamine D in relatie tot MS en verscheidene andere auto-immuunziekten, maar ook in bijvoorbeeld cardiovasculaire ziekten, infectieuze ziekten en kanker, de ideale voedingsbodem gegeven om uit te groeien tot een hype. Sommige artsen schrijven zelfs al hoge dosis vitamine D voor aan hun patiënten. Ook wordt er in sommige landen al geadviseerd aan patiënten met MS om 2000-4000 internationale eenheden vitamine D per dag te gebruiken. Hypes kunnen er echter voor zorgen dat er valse hoop gecreëerd wordt en het wordt dan een uitdaging om de waarheid te achterhalen. Wij zijn van mening dat het werk in dit proefschrift bijdraagt aan een beter begrip en een verfijndere blik op de rol van vitamine D in MS. Dit onderzoek zal bijdragen tot het beter onderscheiden wanneer en voor welke patiënt met MS, vitamine D suppletie van nut kan zijn. Natuurlijk moeten we wachten op de klinische uitkomsten van hoge dosis vitamine D suppletie studies, maar het onderzoek tot nu toe wijst op de beste kansen voor vitamine D behandeling in vroege, en misschien wel preklinische, MS. Als we deze resultaten extrapoleren naar preventie, is het de vraag of vitamine D suppletie niet zelfs het meest relevant zou kunnen zijn vóór de start van de ziekte. De rol van vitamine D in de latere fase van de ziekte lijkt verwaarloosbaar, maar langdurige follow-up van gerandomiseerde en gecontroleerde suppletie studies zal dit moeten bevestigen. Eén van de onderliggende mechanismen kan de invloed van vitamine D op het behoud van de immunologische balans zijn. Immunocellen van patiënten met MS reageren anders op vitamine D dan cellen van gezonde controles. Onze immunologische analyses van het immuunsysteem van patiënten met MS in een hoge dosis vitamine D₃ suppletie studie lieten zien dat vitamine D mogelijk in staat is de immunologische balans van het immuunsysteem, vroeg in de ziekte, te bewaren. Wat er echter gebeurt met immunocellen in lymfeklieren en in het brein, of met

de cellen van het brein zelf, en of ook andere (omgevings) factoren naast vitamine D een essentiële rol spelen in de beschreven verbanden, blijven open vragen.