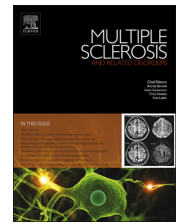




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Vitamin A is not associated with exacerbations in multiple sclerosis

T.F. Runia^{a,*}, W.C.J. Hop^b, Y.B. de Rijke^c, R.Q. Hintzen^a

^aDepartment of Neurology, MS Centre ErasMS, Erasmus MC, Rotterdam, The Netherlands

^bDepartment of Biostatistics, Erasmus MC, Rotterdam, The Netherlands

^cDepartments of Clinical Chemistry and Internal Medicine, Erasmus MC, Rotterdam, The Netherlands

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Abstract

Background: Vitamin A is a multifunctional vitamin that can inhibit the formation of Th17 cells, which are probably involved in the development of relapses in MS. Furthermore, it promotes Treg formation. Therefore, vitamin A can be hypothesized to be lower in patients than in healthy controls, and to decrease relapse risk in relapsing-remitting MS (RRMS) patients. **Objective:** To compare vitamin A levels in MS patients and controls, and to investigate whether vitamin A levels are associated with relapse risk.

Methods: In a case-control study all-trans-retinol levels were compared between 31 RRMS patients and 29 matched controls.

In a prospective longitudinal study in 73 RRMS patients, serum samples for all-trans-retinol measurements were taken every eight weeks. Associations between all-trans-retinol concentrations and relapse rates were calculated using Poisson regression with the individual serum levels as time-dependent variable. Associations between vitamin A and vitamin D were calculated.

Results: Mean vitamin A levels were lower in patients (2.16 $\mu\text{mol/l}$) than in controls (2.44 $\mu\text{mol/l}$) but with borderline significance ($p=0.05$). In the longitudinal study, during follow-up (mean 1.7 years), 58 patients experienced a total of 139 relapses. Monthly moving averages of all-trans retinol levels were categorized into tertiles: a low (<2.9 $\mu\text{mol/l}$), medium (2.9–3.7 $\mu\text{mol/l}$) and high level (>3.7 $\mu\text{mol/l}$). Relapse rates were not associated with serum all-trans retinol levels ($p>0.2$), in univariate nor in multivariate analysis.

Serum concentrations of all-trans-retinol and 25-OH-vitamin D were positively correlated, although this correlation was weak ($r=0.15$).

Conclusion: We did not find evidence for a role for vitamin A in the disease course of RRMS. We did find an association between vitamin A and D levels in the RRMS patients, possibly explained by dietary products that contain both fat-soluble vitamins.

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*Correspondence to: Erasmus MC, Kamer Ee2230, Postbus 2040, 3000 CA, Rotterdam, The Netherlands. Tel.: +31 10 7043980; fax: +31 10 7044727.

E-mail address: r.hintzen@erasmusmc.nl (T.F. Runia).

1. Introduction

Vitamin A or retinol is a fat-soluble vitamin with multiple functions, such as those in vision, growth and the normal differentiation of epithelia (Wolf, 1996; Hall et al., 2011). Over the last 2 decades, it has become clear that vitamin A also has important roles in immune functioning, both in immunological tolerance and in adaptive immune responses (Hall et al., 2011).

After absorption from food, most vitamin A is stored in the liver, from where it is added to the circulation bound to retinol-binding protein (RBP) (Blomhoff and Blomhoff, 2006; Theodosiou et al., 2010). Inside the target cells, retinol is oxidized by alcohol dehydrogenase (ADH) into retinal, which can then be oxidized into the active form retinoic acid (RA) by the more selectively expressed retinaldehyde dehydrogenase (RALDH) (Hall et al., 2011).

RA is a signaling molecule that can control gene expression, mainly through the activation of nuclear retinoid receptors. There are three subgroups of retinoid receptors: retinoic acid receptors (RAR α - γ), retinoid X receptors (RXR α - γ), and retinoic acid orphan receptors (ROR α - γ) (Hirahara et al., 2010). RXR can form heterodimers with other nuclear receptors, such as vitamin D receptor (VDR) (Theodosiou et al., 2010).

RA has been shown to inhibit the formation of Th17 cells, which are probably involved in the development of relapses in MS (Steinman, 2008), in vitro (Mucida et al., 2007) via binding to RAR α (Schambach et al., 2007; Elias et al., 2008). It can do so synergistically with 1,25-diOH-vitamin D (Ikeda et al., 2010). In EAE, a synthetic retinoid could also inhibit Th17 cell differentiation and ameliorate EAE (Klemann et al., 2009). Furthermore, RA can promote the formation of anti-inflammatory Treg cells expressing Foxp3 (Coombes et al., 2007; Mucida et al., 2007; Schambach et al., 2007). Recently, it was also found that vitamin A is possibly associated with MS risk (Salzer et al., 2013) and MRI outcomes in MS (Loken-Amsrud et al., 2012).

Because of the roles described for vitamin A on Th17 and Treg cells, we hypothesized that (1) vitamin A would be lower in MS patients than in healthy controls, and (2) MS patients with higher vitamin A levels would have a lower relapse risk. To investigate this, we performed two studies: a case-control study of vitamin A levels, and a prospective longitudinal study of vitamin A levels in relapsing-remitting MS (RRMS) patients to investigate the association between vitamin A and relapse risk. Because of possible synergistic effects, we also looked at the associations of vitamin A and vitamin D in the longitudinal study.

2. Patients and methods

2.1. Case control study on vitamin A and MS

2.1.1. Patients and controls

We randomly selected 31 patients with RRMS from our pool of MS patients, subsequently selecting 29 controls matched for age and sex. All controls had signed for informed consent, and all patients were aware that serum would be stored for later use. The Medical Ethical Committee of the

Erasmus Medical Center University Hospital approved the use of these materials.

2.1.2. Measurement of vitamin A

All-trans retinol was measured because this is the main form of retinol in the circulation (Royal et al., 2002; Theodosiou et al., 2010; Tanumihardjo 2012), and also the best measure of vitamin A status. For analysis of retinol levels, the samples were extracted with hexane and, after evaporation, dissolved in methanol. Retinol levels were measured through reverse-phase high-performance liquid chromatography (HPLC) (column: 150 \times 4.8 mm, Polaris C18A, Waters Alliance HT2795; detector: Waters 2475 [Waters, Milford, MA]), with excitation at 328 nm and detection of emission at 468 nm. The intra-assay variability of retinol measurements was 3.9% and the inter-assay variability was 5.1%.

2.1.3. Statistical analysis

Student's *T*-test was used to compare mean all-trans retinol levels between RRMS patients and healthy controls. ANOVA was used to adjust for age. $p=0.05$ (two-sided) was considered the limit of significance in all analyses. All calculations were done using SPSS 20.0 for Windows.

2.2. Longitudinal study on vitamin A and exacerbations

2.2.1. Patients

For the longitudinal study, data and samples were collected in the Rotterdam Study on Exacerbations in MS, a prospective study in patients with relapsing-remitting MS (Buljevac et al., 2002). Patients aged 18-55 years could be included in the study if they had clinically definite MS with a relapsing-remitting disease course and at least two exacerbations in the previous 2 years. Patients were excluded from participation if they suffered from another serious disease. All patients signed for informed consent. The study protocol was approved by the Medical Ethical Committee of the Erasmus Medical Center University Hospital.

2.2.2. Definitions

Exacerbation was defined as a worsening of existing symptoms or the appearance of new symptoms lasting for more than 24 h after a period of more than 30 days of improvement or stability, if confirmed by neurologic examination (Schumacker et al., 1965). A temporary neurological deterioration associated with fever was not considered to be an exacerbation.

Because infection is a known risk factor for exacerbations in multiple sclerosis, the 'at risk period' around infection was used as a covariate in this study, as described previously (Buljevac et al., 2002).

2.2.3. Visits, samples and measurement of exacerbations

All patients visited the outpatient clinic of the Erasmus Medical Centre University Hospital regularly every 8 weeks. At every visit, blood samples were taken and disability was measured using the Kurtzke Expanded Disability Status Scale (EDSS) (Kurtzke, 1983). In the event of a suspected exacerbation or infection, additional visits were arranged within

Table 1 Characteristics of patients and controls of the case-control study.

Characteristic	Patients	SD	Controls	SD
Sex (f/m)	22/7		24/7	
Age mean (range)	35.4 (19-56)	10.3	35.9 (19-56)	10.4
Ethnicity (%)				
White caucasian	77.4		89.7	
Mediterranean	16.1		10.3	
Black	6.5			
Disease duration (years) mean (range)	4.5 (0-29)	5.4		

3 days. Serum samples were stored at -80°C until serum vitamin A measurement.

2.2.4. Measurement of vitamin A and vitamin D

Measurements of all-trans retinol were as described above. To investigate the association between vitamin A and D, we used a RIA method (DiaSorin, USA) using an extraction method, to measure 25-OH-vitamin D levels. The inter-assay variation coefficient at a concentration of 62 nmol/L was 11.6%; at 109 nmol/L it was 10.3%. The respective intra-assay variation coefficients at these levels were 5.7 and 6.6%. Only serum samples taken at the regular eight-weekly visits were used for the measurement of all-trans retinol and 25-OH-vitamin D; samples taken during exacerbation visits were not evaluated.

2.2.5. Statistical analysis

To assess the association between individual serum all-trans retinol concentrations and the incidence rate of exacerbations, we split the follow-up time for each patient, which covered a maximum period of 2.3 years, into intervals of one week each. The number of exacerbations was determined for each of these intervals. The individual exacerbation rate was assumed to depend on the mean serum all-trans retinol concentration over the previous 4 weeks. To obtain this mean level, the weekly levels between measurements were determined per individual using interpolated values. These interpolated values were subsequently averaged. During the period of 4 weeks that followed an exacerbation, an individual was not considered to be at risk for another exacerbation. It was decided a priori to categorize the mean serum levels of the 4 preceding weeks into tertiles: low ($<2.9\ \mu\text{mol/l}$), medium ($2.9\text{--}3.7\ \mu\text{mol/l}$) and high ($>3.7\ \mu\text{mol/l}$). The relationship between serum all-trans retinol concentrations and the incidence rate of exacerbations was assessed using the Poisson regression models with the mean individual serum levels as a time-dependent variable. In the calculations we used generalized estimating equations with an exchangeable covariance matrix for the subsequent study weeks. The effect of other factors, including gender, age, EDSS, number of exacerbations before study entry and use of interferon- β during the study, was also estimated using a multivariable generalized linear model with a log-link function. Associations between measured vitamin A and vitamin D concentrations, the latter log-transformed to get an approximate normal distribution, were calculated using mixed model regression

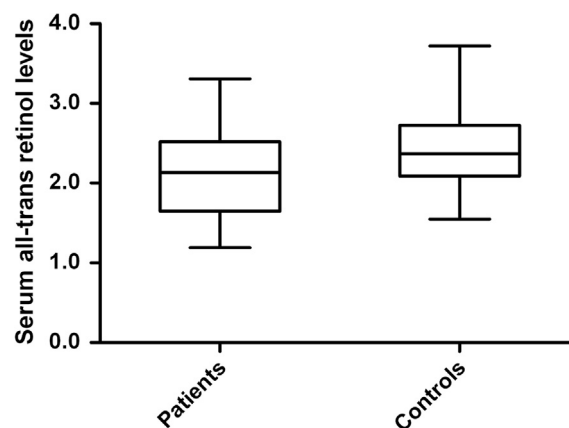


Fig. 1 Serum all-trans retinol concentrations of relapsing-remitting MS patients and healthy controls.

analysis for repeated measurements. $p=0.05$ (two-sided) was considered the limit of significance in all analyses. All calculations were done using SPSS 20.0 for Windows.

3. Results

3.1. Case control study on vitamin A concentrations and MS

3.1.1. Patient characteristics

The baseline characteristics of the 31 patients and 29 controls included are shown in Table 1. We had no information on the use of vitamin supplements of patients and controls. None of the patients were using interferon β at the time of blood sampling, because sampling was done during a clinical workup for therapy advice within our MS center.

3.1.2. Serum vitamin A concentrations

For most patients and controls, serum all-trans retinol concentrations fell within the normal range. None of the patients and none of the controls were vitamin-A deficient. As Fig. 1 shows, all-trans retinol concentrations were somewhat lower in patients than in controls (mean $2.16 \pm 0.55\ \mu\text{mol/l}$ vs. $2.44 \pm 0.52\ \mu\text{mol/l}$), but this difference was only borderline significant ($p=0.050$). Vitamin A depended on age in the case-control study, but not in the longitudinal study. It did not depend on sex. When adjusting

Table 2 Characteristics of patients of the longitudinal study at baseline.

Variable (n=73)	Mean (range)	Std. deviation
Age, years	39.4 (19-55)	9.1
Disease duration, years	5.2 (0-25)	4.1
Disability (EDSS) ^a	2.5 (0-6.0)	1.6
Exacerbations in previous 2 years	2.2 (1-8)	1.3
Variable (n=73)	Proportion (%)	
Gender, F/M	77/23	
IFN use, N/Y	82/18	

^aEDSS is a method for quantifying disability in MS, ranging from 0.0 (normal neurological exam) to 10.0 (death due to MS).

for age, there was no significant difference between patients and controls ($p=0.070$).

3.2. Longitudinal study on vitamin A and exacerbations

3.2.1. Patient characteristics

73 patients were included in this study. The mean follow-up time of all patients was 1.7 years (range 0.4-2.3). Nine patients had dropped out of the study before the intended completion date, one due to participation in another study, the other eight for unknown reasons. All patients were Dutch Caucasians; their baseline characteristics are shown in Table 2. In addition to the 13 patients who used interferon- β at study entry, 15 started to use interferon- β during follow-up; these 28 patients used interferon- β at some point during an average of 56 weeks. Vitamin supplements were not widely used among the patients: five used vitamin B complex (without vitamin A) and two used multi-vitamin pills, one of which contained 6 mg of beta-carotene.

58 patients experienced a total of 139 exacerbations during this study. Median time from inclusion to first exacerbation was 20 weeks. Thirty-three patients had more than one exacerbation; the average exacerbation rate was 1.2 per year (range 0-6.2 per year). Three patients experienced a sixth exacerbation during follow-up.

3.2.2. Serum vitamin A concentrations

Serum all-trans retinol concentrations fluctuated considerably, without a seasonal pattern or other clear pattern. Mean serum all-trans retinol concentration was 3.31 ± 1.14 $\mu\text{mol/l}$. Mean levels varied substantially between patients (Anova: $P<0.001$). Within patients there was also a considerable variation between measurement occasions. Of the total variation in levels 40% was due to differences between patients while 60% was due to differences within patients.

3.2.3. Association between serum vitamin A concentrations and exacerbation risk

Univariate analysis did not show exacerbation rates to be associated with serum all-trans retinol levels ($p>0.2$).

As shown previously (Runia et al., 2012), infections were associated with the risk of an exacerbation, the exacerbation rate within an "at risk period" being 2.1-fold higher (95% CI 1.6 to 2.8, $P<0.001$). Also, vitamin D (25-OH-D) serum levels were found to be associated with exacerbation risk. In the multivariate model including 25-OH-D and infections, infections and 25-OH-D were both still related to the exacerbation rate, whereas all-trans retinol was not (Table 3). Adding gender or the use of interferon- β to the model did not alter these results. Also age, EDSS and the number of exacerbations in the 2 year period before entry into the study were not significantly associated with the exacerbation rates (all $p>0.18$).

3.2.4. Association between vitamin A and vitamin D

Serum concentrations of all-trans retinol and 25-OH-D had a significant linear correlation. Mixed model regression analysis showed that for every doubling of serum 25-OH-D concentration the mean all-trans retinol level increased by 0.59 $\mu\text{mol/l}$ ($P<0.001$). The correlation however was weak ($r=0.15$).

4. Discussion

This case-control study shows that vitamin A concentrations are not significantly lower in MS patients than in healthy controls. Our longitudinal study also shows that vitamin A is not associated with relapse risk in MS patients. We also found that vitamin A and vitamin D were associated in a linear manner.

There are several reasons to hypothesize that vitamin A is involved in MS. In the past, it has been hypothesized that the susceptibility period in multiple sclerosis lies in early childhood (Pugliatti et al., 2006) and that the element responsible was (a deficiency in) vitamin A (Warren, 1982). This hypothesis was based mainly on epidemiological evidence, and the mechanisms through which vitamin A deficiency was thought to cause MS were effects on normal CNS myelination and effects on skull and bone growth affecting normal CNS growth. Since knowledge of the functions of vitamin A in immunity has grown in the last decades (Hall et al., 2011) (inhibiting Th17 cell formation (Mucida et al., 2007) and promoting Treg formation (Coombes et al., 2007; Mucida et al., 2007; Schambach et al., 2007)), the hypothesis that vitamin A might be involved in the development and disease course of MS has become stronger. Recently, it was also found that RXR agonists can stimulate remyelination (Huang et al., 2011). Vitamin A has even been suggested as a (supplementary) treatment option in MS (Royal et al., 2002; Klemann et al., 2009). However, in the present case-control and longitudinal studies we cannot provide any evidence that vitamin A is really involved in MS.

In the case-control study, we found somewhat lower retinol concentrations in patients than in controls, but this was not significant. Other studies that addressed this topic had conflicting results. One study also did not find lower

Table 3 Association between exacerbation rate and categorized serum all-trans retinol concentrations according to the multivariable analysis including infection and 25-OH-vitamin D concentrations. ARP infection=at risk period for infection.

		Relative exacerbation rate	95% CI	P-value
All-trans retinol concentrations	Low <2.9 $\mu\text{mol/l}$	1.2	0.8-1.8	0.329 ^a
	Medium 2.9-3.7 $\mu\text{mol/l}$	1.3	0.8-1.9	0.254 ^a
	High >3.7 $\mu\text{mol/l}$	1 (reference)	-	-
ARP infection	Yes	1 (reference)	-	-
	No	0.4	0.3-0.6	<0.001
25-OH-D concentrations	Low <50 nmol/l	2.0	1.2-3.4	0.012 ^b
	Medium 50-100 nmol/l	1.3	0.8-2.2	0.281 ^b
	High >100 nmol/l	1 (reference)	-	-

^aOverall p-value: 0.489.

^bp-value for trend: 0.010.

levels than in controls (de Bustos et al., 2000). Another study found lower levels in MS patients than in controls (Besler et al., 2002). Differences with our study were that all patients had a secondary progressive disease course, and that a different technique was used for the measurements (Neeld-Pearson with trifluoroacetic acid instead of HPLC). A third study compared retinol levels in patients and controls, finding significant differences only between subgroups (Royal et al., 2002). It should be noted that in that study retinol levels were slightly higher in interferon- β treated patients.

The borderline significance in our results suggests that our groups might have been too small. On the other hand, as Fig. 1 shows, the mean levels of vitamin A in patients and controls are not far apart, and confidence intervals are wide, suggesting substantial variation within the patient and control group. In our longitudinal study, we also found substantial within-patient variation of retinol levels. We found that vitamin A was also dependent on age, which has been described before by Looker et al. (1988) but was not found by Hallfrisch et al. (1994). Because our control group was age- and sex matched, this has not influenced our conclusions.

In the longitudinal study we found no association between vitamin A and exacerbation rate. One recent study using the same technique for retinol assessment, found an inverse association between new lesion formation on MRI and vitamin A levels (Loken-Amsrud et al., 2012). We could not confirm an association between vitamin A and clinical disease activity in MS in our study. But although our longitudinal study with its frequent serum sampling provides a robust way of studying this topic, the fact that we found no association does not totally exclude a role for vitamin A in MS relapses. There might be local function or production of RA in the CNS associated with relapses, that cannot be measured systemically, for example by tissue-specific expression of retinoid receptors or retinaldehyde dehydrogenase (RALDH) (Hall et al., 2011).

A limitation of our study is that none of the patients in the case-control study used interferon- β . In the longitudinal study, the use of interferon- β did not influence our results. In studies by others on this topic conflicting results have

been found: some found a synergistic effect of RA with interferon- β on T suppressor cell augmentation (Qu et al., 1998), others found the association between vitamin A and MRI outcomes to be non-significant during interferon- β use (Loken-Amsrud et al., 2012). We were unable to confirm any of this here.

We found an association between serum levels of vitamin A and D. Vitamin A can be absorbed from food, but its levels can also be increased via a rise in hepatic production as a result of exposure to light (Pang et al., 2008; Mehta, 2010). Because vitamin D is also a fat-soluble vitamin that can be absorbed from food and can also be synthesized under the influence of sun light, it is not surprising that the concentrations of both vitamins increase and decrease simultaneously. However, we did not find synergistic action in this study: whereas a higher vitamin D level was associated with a lower relapse risk, vitamin A was not.

For our analyses of retinol levels, we used random serum samples and not fasting samples. This is justified because retinol is derived from hepatic and other body stores and is a good measure of vitamin-A status, unlike retinyl-esters, which are absorbed after a vitamin-A rich meal (Royal et al., 2002).

In recent years, several studies on the relation between vitamin A and MS have been performed, using different techniques and markers, sometimes with conflicting results (de Bustos et al., 2000; Besler et al., 2002; Royal et al., 2002; Munger et al., 2004; Loken-Amsrud et al., 2012; Salzer et al., 2013). Routine measurement of vitamin A and carotenoids is now generally done using high performance liquid chromatography (HPLC). Lately, the use of plasma RBP as a surrogate marker for retinol is increasing. It should be realized that this is only valid in the absence of infection or when adjusted for CRP levels (2012; Salzer et al., 2013).

In conclusion, there are several reasons to hypothesize that vitamin A has a role in the development and disease course of MS. Here we did not observe differences in retinol levels between patients and controls. In addition, in the prospective study on exacerbations, we found no association with disease activity. These results do not feed the perception that vitamin A could be a useful treatment for MS patients (Royal et al., 2002; Klemann et al., 2009).

Conflicts of interest

The authors declare no conflicts of interest.

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