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Abstract

Background: Vitamin A is important for the immune system, and might suppress inflammatory activity in multiple sclerosis (MS).

Objectives: To examine if vitamin A levels were associated with MS risk in samples collected prospectively and during gestation.

Methods: We measured Retinol Binding Protein (RBP – a surrogate marker for vitamin A) and high sensitivity C-reactive protein (hs-CRP) levels, in 1) prospectively collected biobank blood samples from MS cases and controls, and 2) gestational samples were the offspring had later developed MS, and gestational control samples. The risk of MS was calculated using matched multivariable logistic regression adjusted for confounders.

Results: In prospective samples, RBP levels within the second quintile (vs. the first) were associated with a lower MS risk (OR = 0.38, 95% CI 0.19–0.74). No effect on MS risk in the offspring by gestational RBP levels was found. In young subjects hs-CRP levels ≥ 10 mg/l in prospective samples were associated with a lower MS risk (OR = 0.36, 95% CI 0.14–0.95).

Conclusions: Our results suggest that sub-optimal vitamin A levels may be associated with MS risk. The association between hs-CRP levels and MS risk in young subjects may support the role of the hygiene hypothesis in MS aetiology.

Introduction

The causes of multiple sclerosis (MS) are unknown, although a number of genetic and environmental factors influencing the risk of the disease have been identified.¹⁻³ One of these is high vitamin D levels, that have been associated with a lower risk of MS.^{4,5} Vitamin A (retinol) has been shown to be important for the immune system,⁶ but no studies investigating the association between vitamin A and MS risk have been performed. Vitamin A derivatives have, however, been successfully used in experimental allergic encephalitis (the animal model of MS).⁷ Vitamin A deficiency is not common in the industrialised parts of the world, but in developing countries, severe vitamin A deficiency is prevalent and large-scale vitamin A supplementation programs are estimated to prevent 350,000 childhood deaths annually and many more cases of xerophthalmia and night blindness.^{6,8}

Vitamin A can be ingested as it is or as β -carotene, which is converted to vitamin A. It is essential for the making of rhodopsin, the light sensitive structure in the retina that mediates visual information to the central nervous system.⁹ Important functions of the vitamin A metabolite retinoic acid (RA) for the immune system include the survival of T-cells, the development of regulatory T-cells and inhibitory effects on Epstein-Barr virus (EBV) DNA synthesis and viral reactivation – which are all of interest in MS pathogenesis.^{2,10-13} Both vitamin A and D influence gene expression by binding to certain regions within the DNA (response elements) after forming complexes with the same nuclear receptor, retinoic X receptor (RXR).¹⁴ A functional interaction between vitamin A and D has been proposed, where the vitamins may antagonise each other.¹⁴⁻¹⁶ A season-of-birth effect has been shown in MS, and wintertime vitamin D deficiency, infections, and seasonal differences with respect to diet, possibly affecting vitamin A levels, have been proposed as possible explanations of

this.¹⁷ According to WHO, vitamin A deficiency is defined as serum levels $<0.70 \mu\text{mol/l}$, and sufficient vitamin A levels is defined as serum levels $\geq 1.05 \mu\text{mol/l}$.⁸

The primary objectives of the present study were: 1) to estimate the risk of MS by levels of vitamin A in prospectively collected blood samples in MS cases and controls, and; 2) to determine the risk of MS in the offspring by levels of vitamin A during pregnancy. The secondary objectives were to compare these estimates of MS risk for different age strata.

Methods

Procedure and participants

This study was performed at Umeå University Hospital in northern Sweden and has two study arms: 1) Risk factors of multiple sclerosis (RoMS), a nested case-control study estimating the risk of MS by levels of vitamin A in prospectively drawn blood samples and; 2) Gestational risk factors of multiple sclerosis (GRoMS), a nested case-control study estimating the risk of MS in the offspring by levels of vitamin A during pregnancy. The procedures used to find prospectively collected blood samples, and samples collected during gestation where the offspring had later developed MS, are described in detail elsewhere.⁵ Briefly, we cross-linked a database of MS cases and a database of mothers of MS cases to two local biobank cohorts, one with plasma samples collected in population based health programs (Northern Sweden Health and Disease Study cohort, NSHDS) and one with serum samples collected during early pregnancy (Northern Sweden Maternity Cohort, NSMC). We had no information on the number of freeze-thaw cycles for the samples used in this study. In RoMS, two controls for each case were selected, matched for biobank, sex, age at sampling, and date of sampling. In GRoMS, five controls for each GRoMS case mother were selected, matched for age at sampling and date of sampling.

Measurements

Direct measurement of vitamin A levels was not possible since vitamin A is light sensitive. Proper sampling procedures include protecting the samples from light immediately, which was not done in NSMC and NSHDS.⁹ We therefore measured Retinol Binding Protein (RBP), a transport molecule for vitamin A that correlates equimolar to vitamin A levels. RBP is an excellent surrogate marker for vitamin A in the absence of systemic inflammation.¹⁸ Both RBP and vitamin A levels decrease during an acute-phase response and the correlation between them becomes less pronounced.¹⁸ To assess the degree of systemic inflammation, levels of high sensitivity C-reactive protein (hs-CRP) were measured. A systemic inflammatory response was considered to be present with hs-CRP levels ≥ 10 mg/l. ELISAs (Immundiagnostik AG, Bensheim, Germany) were used to measure RBP and hs-CRP levels. Due to a limited supply of serum and plasma no hs-CRP or RBP duplicates were run. Apart from this, the manufacturer's instructions were followed. For the purpose of other reports on the same cohort, levels of 25-hydroxyvitamin D (25[OH]D),⁵ and antibody reactivity against Epstein-Barr Nuclear Antigen-1 (EBNA-1) (submitted manuscript) were measured using ELISAs, and levels of the nicotine metabolite cotinine were measured using an immunoassay (in press).

One mol RBP weighs 21,000 g, and RBP has an equimolar relationship to vitamin A in the blood. This allows for a simple equation to convert RBP levels to expected corresponding vitamin A levels: $c(\text{RBP}) \text{ (mg/dm}^3\text{)} = 21 * c(\text{vitamin A}) \text{ (}\mu\text{mol/dm}^3\text{)}$. Thus, dividing the measured RBP levels in mg/l with 21 yields the expected vitamin A level in $\mu\text{mol/l}$.

Analyses on the correlations between RBP and hs-CRP levels and sampling year were done on controls from NSMC to avoid possible MS-related bias, and to avoid problems arising

from pooling cohorts with samples collected at different ages and during different time-periods.

Sample collection routines were unchanged during the entire time period with one exception: the samples in NSMC were heat-treated during 1975–1987 to enable complement binding analyses.

Statistical methods

For statistical analyses SPSS version 19.0 was used. The chi-square test was used to compare proportions, and the Mann-Whitney U test was used to compare medians. Pearson's correlation coefficient (r) was used to assess the correlation between continuous variables. The risk of MS was estimated using cut-offs calculated as above (equation): vitamin A insufficiency ($\text{RBP} < 14.70 \text{ mg/l}$), sufficient vitamin A levels ($\text{RBP} \geq 22.05 \text{ mg/l}$), and using RBP levels stratified at median, into tertiles, quartiles, and quintiles. Matched logistic regression was used to estimate odds-ratios (OR) and 95% confidence intervals (CI) for different RBP strata. High sensitivity CRP levels stratified at 10 mg/l were included in the multivariable analyses to adjust for potential confounding, as were 25(OH)D status (< 75 vs. $\geq 75 \text{ nmol/l}$),⁵ EBNA-1 antibody reactivity tertiles and cotinine levels (< 10 vs. $\geq 10 \text{ ng/ml}$, a cut-off used to discriminate smokers from non-smokers and passive smokers). For the subgroup analyses by age at blood sampling we evaluated the heterogeneity of the stratum-specific OR estimates using the chi-square test of heterogeneity in the WinPepi statistical software, version 11.24. For the offspring of GRoMS case mothers, but not for their controls, we had access to social security numbers and were able to estimate the duration of their mothers' pregnancy at blood draw (assumed length of pregnancy 280 days). Probability is denoted by p .

All subjects consented to donate blood samples for biobank storage. All subjects received written information about the study with the option to opt out. All aspects of the study were approved by the local ethics committee in Umeå (Dnr 08-135M).

Results

A total of n=192 MS cases with prospectively drawn plasma and serum samples in RoMS, and n=37 mothers of MS cases with serum samples drawn during pregnancy in GRoMS, were identified, and cases and controls in both RoMS and GRoMS were well matched.⁵ Most RoMS cases (92%) were female since NSMC contains only women, and the samples were drawn median 9 years (range 2 months–32 years) before disease onset. Median age at sample collection was 26 years (range 16–60 years). The GRoMS offspring with MS were at data collection young and most had a relapsing-remitting disease.⁵ The majority (78%) of the GRoMS case samples were drawn during the first trimester.

RBP and hs-CRP levels in prospective samples, RoMS

Most cases (86.5%) and controls (90.9%) had RBP levels ≥ 22.05 mg/l indicating sufficient vitamin A levels (≥ 1.05 $\mu\text{mol/l}$), $p = 0.1$. RBP levels ≥ 22.05 mg/l (adjusted for hs-CRP stratified at 10 mg/l) were associated with a non-significant decreased risk of MS (OR = 0.56, 95% CI 0.31–1.02). RBP levels in the second quintile (corresponding to vitamin A levels between 1.23 and 1.47 $\mu\text{mol/l}$) were associated with a lower risk of MS than in the first quintile (OR = 0.45, 95% CI 0.24–0.85), and the ORs for quintiles three–five increased gradually towards 1.0 (Table 1). These findings did not change when adjusted for potential confounders in a multivariable analysis (Table 1). None of the other pre-defined RBP categories yielded significant findings (data not shown).

In those below median age at sampling (<26.4 years), the odds-ratio pattern for RBP quintiles was similar to that for the entire cohort (Table 2). In this subgroup of young individuals, hs-CRP levels ≥ 10 (vs. <10) mg/l were associated with a lower MS risk, both in the bivariable (OR = 0.39, 95% CI 0.16–0.93) and the multivariable analyses (Table 2). When analysing only older subjects (≥ 26.4 years at sampling), hs-CRP levels ≥ 10 were not associated with MS risk (OR = 1.3, 95% CI 0.68–2.7) (P for heterogeneity = 0.04).

RBP and hs-CRP levels in gestational samples, GRoMS

Most case (83.8%) and control mothers (85.4%) had RBP levels ≥ 22.05 mg/l indicating sufficient vitamin A levels (≥ 1.05 $\mu\text{mol/l}$), $p = 0.8$. None of the pre-defined RBP strata yielded significant results regarding MS risk. Gestational hs-CRP ≥ 10 mg/l was not associated with MS risk in the offspring.

Correlation analyses and trends over time

In the 515 NSMC controls, the RBP levels did not correlate to hs-CRP levels ($r = -0.08$; $p = 0.06$), or to year of sampling ($r = -0.04$; $p = 0.3$). The hs-CRP levels did correlate to year of sampling ($r = 0.27$; $p < 0.001$). The median (range) hs-CRP levels in heat treated samples 1.6 (0–79) mg/l was slightly lower than non-heat treated samples 1.9 (0–79) mg/l, although this difference was non-significant ($p = 0.06$). The median (range) RBP levels did not differ between heat treated samples 35 (0–173) mg/l and non-heat treated samples 36 (0–173) mg/l ($p = 0.7$).

Discussion

In this study we used RBP as a surrogate marker for vitamin A status, and demonstrated an association between RBP levels and MS with a non-significant decreased risk of MS with RBP levels ≥ 22.05 mg/ml. When analysing the risk of MS over quintiles of RBP, we found a

55% decreased risk of MS with RBP levels in the second vs. the first quintile. The ORs for quintiles three–five increased gradually towards 1.0 in a U-shaped manner. These findings remained when adjusted for potential confounders in a multivariable analysis.

Vitamin A plays a role in several aspects of immune function, including both the innate and adaptive immune systems, and may be linked to the development of regulatory T-cells.^{10, 11} In a recent study, repeated estimates of vitamin A levels and MRI activity were performed in 88 MS cases, and increased vitamin A levels were associated with decreased inflammatory activity on MRI.¹⁹ One study on the other hand, designed to assess the association between vitamin D intake and MS risk, found no association between vitamin A intake, mainly from multivitamins, and MS risk.²⁰ The ORs of MS for RBP quintiles in the present study described a U-shaped pattern, which is not easily interpreted. As discussed above, there are several mechanisms that may be responsible for the protective effect associated with vitamin A above a certain level. It is possible that too high vitamin A levels inhibit the protective effect of 25(OH)D through competitive RXR interaction,¹⁴⁻¹⁶ an explanation that may also apply to the finding of a U-shaped risk curve over vitamin A quintiles regarding hip fracture risk.²¹

Since no duplicates were run in this study, the precision of the RBP measurements is probably low, and the cut-points found should be seen as an approximation. Also, an unknown number of previous freeze-thaw cycles of the samples might have effected sample quality. These weaknesses of the study increases the risk for false negative findings (type II errors) due to reduced precision, and should therefore not challenge the validity of the main results. The possibility of residual confounding by other factors than those included in the matching and multivariable analysis cannot be ruled out, and the findings need to be replicated in other prospective MS cohorts. Because of the exploratory nature of the study, correction for multiple comparisons was not performed.

Levels of hs-CRP ≥ 10 (vs. < 10) mg/l were associated with a 61% decreased risk of MS in those below median age at sampling (< 26.4 years). This was an unexpected finding as our rationale for hs-CRP analysis was to enable estimation of vitamin A levels through RBP levels,¹⁸ as discussed above. The association was found in a subgroup post-hoc analysis, and the finding should therefore be taken cautiously, although adjustment for possible confounding by several well-known MS risk factors did not change the OR or CI (Table 2). Interestingly, when analysing only older subjects (above median age at sampling), this association was annulled, suggesting a specific time-window within which this risk factor is active. If elevated CRP levels in young subjects are indeed associated with a decreased risk of MS, this may support the hygiene hypothesis, which says that less hygienic surroundings – more frequent infections – early in life are associated with a decreased risk of MS and other autoimmune disorders.^{22, 23} Support for this also comes from recent observations that cytomegalovirus (CMV) infection, which may lead to higher CRP levels,²⁴ although the results are conflicting,²⁵ was associated with a decreased risk for both paediatric and adult onset MS.^{26, 27} Alternatively, the low hs-CRP levels in young MS cases may reflect the EBV-latency pattern that is typical for MS, with less frequent EBV reactivation,²⁸ or an impaired oxidative burst in phagocytic cells that has been implicated in the development of autoimmune diseases such as MS.²⁹

No association between first trimester gestational RBP or hs-CRP levels and the risk of MS in the offspring was found in the very limited GRoMS material. The matching for date of sampling removed the possibility to study season-of-birth related effects such as gestational CRP levels as a marker for maternal infectious load.¹⁷ The cohort is small and the results should therefore be interpreted with caution. An expanded study of gestational samples from mothers of MS cases would be needed to clearly refute that vitamin A and CRP levels during pregnancy influence MS risk in the offspring.

The hs-CRP levels increased with year of sampling in the NSMC controls. Since CRP correlates with body mass index (BMI), the higher hs-CRP levels in later samples might reflect the increase in BMI seen over time in northern Sweden.^{30, 31} Unfortunately, we had no data on BMI for the subjects in the present study. Another explanation could be that samples from later years were drawn later in pregnancy, as CRP increases during pregnancy,³² but there was no correlation between sampling year and days between estimated conception and sampling in cases (data not shown). Also, no changes in sampling and sample handling routines have been made over the years except for the termination of heat-treatment of sera in NSMC in 1988, and that seems to have had no relevant effect on hs-CRP and RBP levels as presented in the results section. Furthermore, it cannot be excluded that the storage time may have influenced the hs-CRP levels, although the opposite pattern was seen for 25(OH)D levels.⁵

In conclusion, by using prospective biobank samples, we have shown an association between the risk of MS and vitamin A levels, as measured by the surrogate marker RBP, with a 55% lower risk of MS with RBP levels in the second quintile compared to the first, and gradually increasing ORs for the subsequent quintiles. We have also shown an association between hs-CRP levels and the risk of MS in young individuals with a 61% decreased risk of MS with hs-CRP levels ≥ 10 (vs. < 10) mg/l. These findings need replication in other prospective materials with samples collected at a young age. Our findings are suggestive of a novel risk factor of MS – suboptimal (too low *and* too high) vitamin A levels – and emphasise the need for further research on pre-symptomatic systemic inflammation. This involves infectious agents, including the hygiene hypothesis, and interaction studies on genetic and environmental factors involved in autoimmune processes.²²

Conflict of interest statement

Dr. Salzer has received financial support for this study from Biogen Idec, Merck Serono, Sanofi Aventis and The Swedish Association of Neurologically Disabled, received honoraria from Merck Serono for a lecture, and received support to travel to scientific meetings from Biogen Idec and Merck Serono.

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Dr. Hallmans reports no disclosures.

Dr. Wadell reports no disclosures.

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Table 1 Odds ratios of Multiple Sclerosis for retinol binding protein quintiles and high sensitivity C-reactive protein levels in prospective serum and plasma samples from n=192 cases and n=384 matched controls.

Variables	Categories	Number of		Bivariable analysis		Multivariable analysis ^a	
		Cases	Controls	OR	95% CI	OR	95% CI
RBP quintiles (mg/l)	≤25.7	50	77	1.0	(referent)	1.0	(referent)
	25.8–30.9	26	77	0.45	0.24–0.85	0.38	0.19–0.74
	31.0–38.2	34	77	0.64	0.36–1.1	0.60	0.33–1.1
	38.3–51.9	40	77	0.75	0.41–1.4	0.61	0.32–1.2
	>51.9	42	76	0.83	0.45–1.5	0.76	0.39–1.5
hs-CRP levels (mg/l)	<10.0	168	325	1.0	(referent)	1.0	(referent)
	≥10.0	24	59	0.77	0.46–1.3	0.68	0.38–1.2

Matched logistic regression was used to calculate ORs and 95% CIs.

^aAdjusted for the variables included in this table, and 25(OH)D status (<75 nmol/l vs. ≥75 nmol/l), EBNA-1 antibody reactivity tertiles, and cotinine levels (<10 vs. ≥10 ng/ml).

OR = odds ratio; CI = confidence interval; RBP = retinol binding protein; hs-CRP = high sensitivity C-reactive protein; 25(OH)D 25-hydroxyvitamin D; EBNA-1 = Epstein-Barr Nuclear Antigen-1.

Table 2 Odds ratios of Multiple Sclerosis for retinol binding protein quintiles, and high sensitivity C-reactive protein levels in prospective serum and plasma samples from subjects younger than 26.4 years (below median age at sampling), n=96 cases and n=192 matched controls.

Variables	Categories	Number of		Bivariable analysis		Multivariable analysis ^a	
		Cases	Controls	OR	95% CI	OR	95% CI
RBP quintiles (mg/l)	≤26.2	22	38	1.0	(referent)	1.0	(referent)
	26.3–30.7	14	39	0.47	0.18–1.2	0.42	0.16–1.1
	30.8–35.9	17	38	0.77	0.34–1.7	0.74	0.30–1.8
	36.0–47.5	19	39	0.84	0.36–1.9	0.63	0.25–1.6
	>47.5	24	38	1.2	0.54–2.8	0.87	0.33–2.3
hs-CRP levels (mg/l)	<10.0	88	157	1.0	(referent)	1.0	(referent)
	≥10.0	8	35	0.39	0.16–0.93	0.36	0.14–0.95

Matched logistic regression was used to calculate ORs and 95% CIs.

^aAdjusted for the variables included in this table, and 25(OH)D status (<75 nmol/l vs. ≥75 nmol/l), EBNA-1 antibody reactivity tertiles, and cotinine levels (<10 vs. ≥10 ng/ml)

OR = odds ratio; CI = confidence interval; RBP = retinol binding protein; hs-CRP = high sensitivity C-reactive protein; 25(OH)D 25-hydroxyvitamin D; EBNA-1 = Epstein-Barr Nuclear Antigen-1.