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Keynotes:

- Vitamin A (retinol) is central in tolerance development and regulation of mucosal immunity.
- Previous studies show contradictory results on the relevance of vitamin A status in clinical allergy
- We found no clear association between serum levels of vitamin A and atopic rhinoconjunctivitis in Swedish teenagers.

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Research Article

Serum levels of Vitamin A and Atopic Rhinoconjunctivitis in Swedish adolescents

Abstract

Aim: Vitamin A plays a role in mucosal immunity and tolerance, but the association between vitamin A status and allergy is still unclear. The aim of the study was to analyze the levels of vitamin A in serum from adolescents with or without atopic rhinoconjunctivitis.

Method: Thirteen-year-old children with atopic rhinoconjunctivitis (n = 53) and non-allergic, non-sensitized controls (n = 52) were randomly selected from a population based prospective birth cohort comprising 1228 children in Northern Sweden born in 1996-1997. Vitamin A (retinol) concentrations in serum were measured with high performance liquid chromatography mass spectrometry. Multiple logistic regression was used to evaluate the association between allergy prevalence and serum vitamin A levels.

Results: Multiple logistic regression analysis showed no association between serum vitamin A levels and atopic rhinoconjunctivitis prevalence; OR = 1.00 (95% confidence interval 1.00-1.00), p = 0.81. Stratification for gender revealed a trend for a higher risk for having atopic rhinoconjunctivitis with higher concentrations of vitamin A in serum for females, OR = 1.02 (1.00-1.05), p = 0.07. No such associations were found in male subjects OR = 0.99 (0.97-1.01), p = 0.15. A dose-response relationship between allergy and vitamin A concentrations were also calculated but no such relationships were found, neither for all subjects nor for male and females separately.

Conclusions: Serum levels of vitamin A could neither be positively nor negatively associated with atopic rhinoconjunctivitis in Swedish teenagers.

Introduction

Allergy is the most prevalent chronic disease in affluent countries with a Western lifestyle, and its prevalence has increased globally. Decreased exposure to environmental microbes [1,2], and changes in dietary patterns, including an increased intake of margarine and oils [3], are different factors that may explain the rise in the prevalence of allergy.

Vitamin A (retinol) is a fat-soluble vitamin that is important for vision, gene expression and tissue differentiation. Vitamin A deficiency has profound effects on the immune system [4], and is associated with increased mortality from infectious disease in developing countries [5]. Animal studies show that vitamin A-deficient animals have reduced mucosal immune responses [6], and a reduced capacity to develop oral tolerance [7]. A particular subset of antigen-presenting dendritic cells (DC), the CD103+DC subset, possesses the vitamin A converting

enzyme RALDH [8], and is central in mucosal immunity and tolerance [7]. These cells traffic the intestinal mucosa, where they pick up antigens, migrate to the mesenteric lymph nodes and present the antigens to naïve T cells trafficking the lymph nodes. Retinoic acid (a metabolite of vitamin A) produced by the CD103+DCs is needed to imprint a gut-migrating phenotype on the naïve T cells [8], and to convert naïve T cells to regulatory T cells [9]. Retinoic acid has been shown both to promote Th2 cell differentiation by increasing the ratio of Th2 cytokines relative to Th1 cytokines [10], and to downregulate Th2 immune responses by inhibiting IL-6 driven induction of Th17 cells and increasing the proliferation of T-regulatory cells [11,12].

The importance of retinoic acid for mucosal immunity and oral tolerance and the effect on T-regulatory cells implies that the availability of vitamin A could have an impact on allergy development. A systematic review from 2009 on dietary intake and serum levels of vitamin A metabolites and asthma found serum levels of retinol to be inversely associated with asthma

in children [13]. Similar results were reported in another meta-analysis from 2010, which revealed lower serum levels of vitamin A in children with asthma compared with controls [14].

The aim of the present study was to analyze the association between vitamin A in serum and allergy in Swedish adolescents selected from a prospective and population based mother and child birth cohort. Allergy has many diagnoses and nuances with different, and sometimes transient, age influenced symptoms in combination with environmental changes, and is best observed in longitudinally prospective cohorts. In the present study, we had the opportunity to select non-allergic children with no sensitization or reported allergic symptoms neither at 1, 4 nor 13 years of age. For a well-defined group of children with allergy at 13 years of age (time point for retinol analyses) we chose to study allergic rhinoconjunctivitis, defined as coherently reported symptoms and sensitization to airborne allergens.

Methods

Study subjects

Cases with atopic rhinoconjunctivitis ($n = 53$) and non-sensitized non-allergic controls ($n = 52$) were selected at 13 years of age from a population based birth cohort consisting of 1,228 children born during one year between February 1996 and January 1997 at the Östersund Hospital in Northern Sweden [15]. The children were followed with skin prick tests and questionnaires regarding allergic symptoms at 1, 4 and 13 years of age. At 13 years of age, 789 adolescents responded to the questionnaire (together with a parent) and participated in sensitization test. The subjects were skin prick tested with standardized extracts for ten allergens, including milk, egg, fish, wheat, soy, cat, dog, horse, timothy grass, and birch (ALK, Hørsholm, Denmark), as earlier described [16,17].

Allergy diagnosis

Atopic rhinoconjunctivitis was defined as a positive skin prick test to one or more inhalant allergens (cat, dog, horse, birch or timothy), in combination with a positive answer to the question "Have you had any allergic symptoms from eyes and/or nose in contact with pollen or furred animals during the last 12 months?". Hence, all subjects in the allergic group had symptoms in contact with pollen or furred animals in combination with a positive skin prick test against an airborne allergen. In total, 174 of the subjects in the cohort fulfilled the criteria for atopic rhinoconjunctivitis. Non-allergic, non-sensitized controls were defined as having neither allergic symptoms nor any positive reaction in the skin prick test at 13 years of age or in any of the previous follow-ups ($n = 331$).

Selection of subjects

Fifty-three subjects who had atopic rhinoconjunctivitis were chosen randomly; of these, 19 subjects also had asthma, 4 subjects also had food allergy and 30 subjects had only rhinoconjunctivitis. As controls we selected a similar number of subjects that had been non-allergic and non-sensitized in all follow-ups at 1, 4, and 13 years of age.

Collection of serum

Venous blood (10 mL) was drawn, allowed to clot and centrifuged. Serum was separated, aliquoted and frozen within three hours. The serum samples were stored for a maximum of five years at -80°C before analyses.

Analysis of vitamin A in serum

Serum vitamin A concentrations were measured using liquid chromatography mass spectrometry (LC-MS) simultaneously as 25-hydroxy vitamin D. The results from the vitamin D analysis has been reported previously [18]. Samples were prepared for the analysis according to Turpeinen et al. [19], with some modifications as earlier described in detail [18]. Briefly, 200 μl serum was mixed with 150 μl methanol:iso-propanol (80:20), containing 100 ng of retinoic acid as internal standard (Sigma Aldrich, Saint Louis, USA). After extraction twice with 2 ml hexane, the organic phases were evaporated and dissolved in 100 μl of methanol and transferred to vials. Retinol and retinoic acid were analyzed using an LC-MS system (Agilent 1260 Infinity Binary LC and Agilent 6120 Quadrupole LC/MS, Agilent Technologies, Santa Clara, California, USA). The LC-MS setup has previously been described in detail [18]. The instrument was operating in selected-ion monitoring mode: 269 for both retinol and retinoic acid. Retinol and retinoic acid were identified based on retention time.

A control serum sample was extracted and analyzed in every run. Study samples and control samples were prepared and analyzed in duplicates. Relative standard deviation (%RSD) for intra-assay precision was 4.7% for a serum sample with a mean retinol concentration of 116 $\mu\text{Mol/L}$. Relative standard deviation was 7.5% for between day variability. Retinol concentrations decreased from 116 $\mu\text{Mol/L}$ to 111 $\mu\text{Mol/L}$ after 24 hours in room temperature. After three freeze-thaw cycles, i.e. thawed in room temperature and frozen for 24 hours, retinol concentration was down to 99 $\mu\text{Mol/L}$.

Statistical analysis

For analysis of differences in background variables (Table 1), chi-square test was used. Mann-Whitney U test was used to compare group means of serum vitamin A concentrations due to non-parametric data (Figure 1). To analyse the trend for dose-response in figure 2 chi-square for linearity was used. Multiple logistic regression analysis was used to analyze the association between allergy and serum vitamin A levels, taking the following potential confounders into consideration: gender, siblings, breastfeeding, maternal allergy, paternal allergy and residence of school (Table 2). Statistical analyses were performed using IBM SPSS Statistics version 19 (IBM Corporation, New York) and a two-tailed p -value ≤ 0.05 was considered significant.

Ethical considerations

The study was approved by the local ethical committee in Umeå, Sweden (Dnr 09-110M) and was conducted according to the Declaration of Helsinki. At 13 years of age, the adolescents verbally approved their participation in skin prick tests and

collection and analysis of blood samples for vitamin A, and their parents provided written consent on behalf of the children. Participation was voluntary and the adolescents were informed that they were free to decline participation at any time, without stating the reason and that their decision would not lead to any disadvantage.

Results

Characteristics

The characteristics of the individuals selected from each group as well as the non-selected individuals, are shown in Table 1. Statistical analyses were performed to compare both selected with non-selected subjects as well as to compare cases and controls. For differences between selected and non-selected

subjects in the two groups, a larger percentage of selected non-allergic adolescents attended schools in the only major city of the region, Östersund, compared to the non-selected non-allergic adolescents (Table 1). No significant differences were found between selected and non-selected cases. For the comparisons between cases and controls: subjects with atopic rhinoconjunctivitis were more often males and had more often mothers with a history of allergy than selected non-allergic controls; also paternal allergic heredity tended to be higher in cases than in controls (Table 1).

Differences in serum vitamin A concentrations

Vitamin A was extracted and quantified in serum samples of 105 individuals representing the cases (n = 53) and controls (n = 52). The mean (SD) serum concentration of vitamin A was 176

Table 1: Characteristics of the study population.

	No allergy			Atopic rhinoconjunctivitis ¹			P ² cases vs controls
	Not selected	Selected	P ² selected vs not-selected	Not selected	Selected	P ² selected vs not-selected	
	(n = 280)	(n = 52)		(n = 76)	(n = 54)		
Female gender, %	54	60	ns	40	26	0.086	0.001
Have older siblings, %	58	71	0.08	66	59	ns	ns
Exclusive breast feeding ³ , %	70	69	ns	75	67	ns	ns
Maternal allergic heredity ⁴ , %	33	29	ns	60	47	0.12	0.05
Paternal allergic heredity ⁴ , %	32	37	ns	47	56	ns	0.06
Cat during first year in life ⁵ , %	14	14	ns	9	10	ns	ns
Dog during first year in life ⁵ , %	26	21	ns	16	10	ns	0.14
School in the city of Östersund ⁶ , %	42	64	0.004	50	49	ns	0.14

¹Defined as a positive skin prick test to an inhalant allergen, in combination with a positive answer to the question: "Have you had any signs of pollen allergy or allergy to furry pets during the last 12 months?"

²Pearsons Chi-Square test

³Exclusively breast feeding at 4-month-of-age.

⁴Defined as an affirmative answer to the question "Has the child's mother/father ever had asthma, hay fever or eczema?"; answered at 13-years-of-age.

⁵Answered at four years of age.

⁶Residents of school, city of Östersund or in the more urban surroundings, at 13 years of age.

ns: p > 0.20

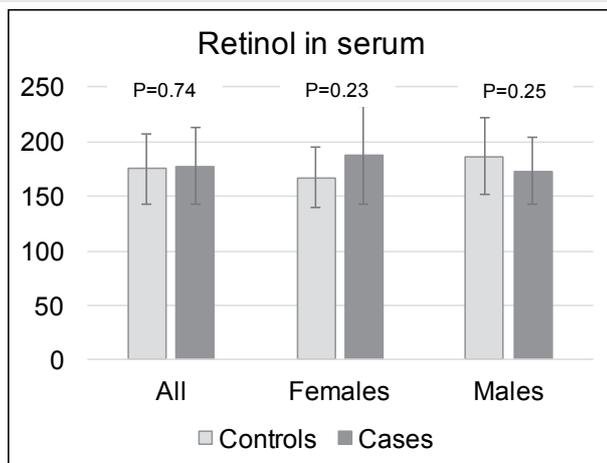


Figure 1: Mean concentration of vitamin A in serum in cases and controls, all and separately for males and females. Controls are shown in light grey and cases in dark grey. Number of subjects in each group: all controls n=52, all cases n=53, female controls n=31, female cases n=14, male controls n=21, male cases n=39. P-values obtained by Mann-Whitney U-test.

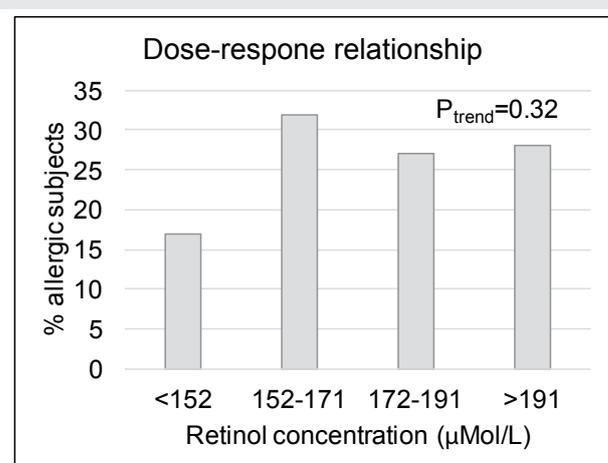


Figure 2: Dose-response relationship between vitamin A concentrations in serum and atopic rhinoconjunctivitis. % allergic subjects: proportion of allergic subjects in each quartile of vitamin A concentrations. P_{trend}=0.32: P-value for trend analysis obtained with Chi-square test and linear-by-linear association for proportion of subjects with allergy in the four quartiles.

(34) $\mu\text{Mol/L}$ for the group as a whole. No significant differences in vitamin A serum concentrations were noted between cases and controls, 177 (35) $\mu\text{Mol/L}$ vs 175 (32) $\mu\text{Mol/L}$ respectively, $p > 0.2$, (Figure 1). As the distribution of males and females were unevenly distributed among the cases and controls the analysis was also performed for males and females separately; the mean serum concentration of vitamin A in serum was $178 \pm 33 \mu\text{Mol/L}$ for males and 173 ± 35 for females ($p = 0.26$). No significant differences in vitamin A serum concentrations were noted between cases and controls for neither female nor males (Figure 1).

Multiple logistic regressions

Multiple logistic regression was used to analyse the association between serum vitamin A concentration and allergy diagnosis at age 13, either as crude odds ratio, or adjusted for gender, siblings, breastfeeding, maternal allergy, paternal allergy and residence of school. Serum levels of vitamin A was not a risk factor for being allergic at 13 years of age in neither the crude nor the adjusted model (Table 2). The logistic regression models were also performed separately for males and females. For females, there was a tendency for a higher risk for atopic rhinoconjunctivitis for higher vitamin A levels in serum. This association was not found for male subjects (Table 2).

Of the 53 selected subjects with atopic rhinoconjunctivitis, 19 subjects had both asthma and rhinoconjunctivitis. The logistic regression analyses were performed stratified for 'having asthma' and 'not having asthma'. No significant difference in serum vitamin A levels was found between cases with or without asthma and controls (data not shown).

Dose-response relationship

To analyze if there was a dose-dependent difference between vitamin A and allergy, all subjects were divided in four groups depending on vitamin A status. Proportions of allergic subjects in each vitamin A group are displayed in Figure 2. We found no significant difference in prevalence of allergy between the four groups stratified according to serum vitamin A concentration (Chi-square for linearity, Figure 2).

Discussion

Serum levels of vitamin A were measured in 105 adolescents at the age of 13 years, living in the County of Jämtland in Northern Sweden. The subjects were followed from birth onwards with regular skin prick tests and questionnaires

probing for allergic symptoms. From a well-defined group of children with allergy diagnosis based on questionnaire and skin prick test, we randomly selected cases with atopic rhinoconjunctivitis, i.e. subjects with a positive prick test to one or more airborne allergen (cat, dog, horse, birch or timothy) together with symptoms from the eye or nose in contact with furred animals or pollen. Moreover, we randomly selected non-sensitized, non-allergic controls, i.e. subjects without sensitization and symptoms at 13 years of age or in any of the previous follow-ups. No differences in serum vitamin A levels were found between adolescents with atopic rhinoconjunctivitis and healthy controls of the same age, in neither the crude nor the adjusted logistic regression model. Since the distribution of the two genders were different in the cases and the controls, with more males in the cases than in the controls, the logistic regression models were also performed separately for females and males. For females only, vitamin A tended to be associated with an increased risk for having atopic rhinoconjunctivitis. This was not shown for males separately. Hence, it seems likely that there might be a gender difference in the association between vitamin A and allergy. When the mean values of vitamin A in cases and controls was visualized with a bar chart, a slightly higher mean vitamin A level were seen in female cases than in female controls, however this difference was not statistically significant.

Previous studies on vitamin A in serum and allergy have shown mainly inverse associations [13,14,20–24], but also no associations [25–29], between vitamin A in serum and asthma. The findings of low levels of vitamin A in subjects with asthma may either be due to a casual effect of vitamin A on asthma or simply be a consequence of the disease. Factors involved in asthma exacerbations, such as airway inflammation and pulmonary infections, have been shown to increase cellular demand or urinary excretion of vitamin A and hence result in decreased serum concentrations of vitamin A.

Most of the previous studies have been focusing on asthma which has mainly been diagnosed as recurrent cough and/or wheezing, sometimes together with previous demonstration of improvement of symptoms in response to asthma medication. In young children, asthma is often diagnosed as wheezing, which may also be a sign of respiratory infection. Furthermore, asthma symptoms in adults are often triggered by viral infections. As this birth cohort used questionnaires for symptoms and did not have any objective method i.e. lung function test, we considered the diagnostic tools not sharp enough for a clear allergic asthma diagnose. Instead we chose to study allergic rhinoconjunctivitis, defined as coherently reported symptoms

Table 2: Estimated odds ratios and 95% CIs of association between serum vitamin A and atopic rhinoconjunctivitis in 13-year-old adolescents

	Model 1			Model 2 ¹		
	Cases/controls	Unadjusted OR (95% CI)	P	Cases/controls	Adjusted OR (95% CI)	P
All subjects	53/52	1.002 (0.990 – 1.013)	0.76	53/51	1.001 (0.989 – 1.014)	0.81 ¹
Females	14/31	1.017 (0.998-1.036)	0.084	14/28	1.023 (0.998-1.048)	0.069 ¹
Males	39/21	0.988 (0.971-1.004)	0.146	36/19	0.986 (0.968-1.005)	0.154 ¹

Odds ratios were calculated with binary logistic regressions between allergic subjects and non-allergic, non-sensitized controls.

¹Adjusted for gender, siblings, breastfeeding, maternal allergy, paternal allergy and residence of school.

and sensitization to airborne allergens. Allergy is defined as symptoms in contact with the allergen and no symptoms when the allergen is removed. Sensitization to airborne allergens is an important piece of the puzzle and will further strengthen an allergy diagnose. Rhinoconjunctivitis could be well diagnosed from skin prick tests and questionnaires in this study, since parents to children at this age know for sure if the children have allergic reactions in contact with cat, dog or horse or if the children get allergic symptoms during some part of the summer (allergic reactions to i.e. pollen).

We have previously shown that neither serum levels of 25-hydroxy vitamin D nor proportions of long chain polyunsaturated fatty acids (LCPUFAs) in serum were associated with allergy in the same study population cohort in a cross-sectional analysis of the same cohort at 13 years of age [17,18]. On the other hand, we found that higher proportions of LCPUFAs in the cord blood of both the n-3 and n-6 families, were associated with being allergic at 13 years of age in this cohort [16]. This suggests that perinatal exposure to fatty acids and perhaps other factors, such as fat-soluble vitamins, may influence immune maturation and thereby subsequent allergy development. Unfortunately, we had no possibility to analyze vitamin A in the cord serum samples due to low amount of remaining cord serum.

A few studies have assessed the relation between early life exposure to vitamin A and allergy later in life. A randomized clinical trial in a vitamin A-deficient population in Nepal found no relationship between supplementation with vitamin A early in life and child asthma at nine to 23 years of life in more than 5000 subjects [30]. An observational study of 200 subjects analyzed vitamin A levels in serum at birth at two, four and twelve months of age, and at five and eleven years of age. Allergy was assessed at five, eleven and 20 years of age [22]. Overall, the study found no association between serum vitamin A levels and atopic disease. The only significant relation was an inverse association between vitamin A status at 2 months of age and atopic allergy at 20 years of age [22]. In a recent study, serum carotenoids analysed at various time points during early childhood were related to parental reported asthma at five years of age [31]. Carotenoids have antioxidant capacity and are also precursors for vitamin A. The study found no associations between serum carotenoids early in life and the risk of asthma at five years of age [31].

Conclusion

We found no association between levels of vitamin A in serum and atopic rhinoconjunctivitis at 13 years of age in adolescents, selected from a population-based cohort followed from birth onwards and with well-defined allergic manifestations.

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