

High-Normal C-Reactive Protein Levels Do Not Affect the Vitamin A Transport Complex in Serum of Children and Adolescents with Type 1 Diabetes

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ABSTRACT: Type 1 diabetes is associated with the presence of inflammation, which in turn affects parameters used to assess the vitamin A status. In the present study, we evaluated the influence of inflammatory status on retinol, retinol-binding protein 4 (RBP4), and transthyretin (TTR) in children and adolescents with type 1 diabetes. A total of 40 children with type 1 diabetes (median age, 14.2 y; median BMI-SDS, 0.53; median diabetes duration, 5.8 y; median HbA_{1c}, 7.3%) and 46 healthy subjects (median age, 12.8 y; median BMI-SDS, 0.34; median HbA_{1c}, 5.4%) were recruited. Serum levels of CRP were significantly elevated ($p = 0.005$) and retinol concentrations were significantly lower ($p = 0.02$) in children and adolescents with type 1 diabetes compared with healthy subjects. Serum RBP4 and TTR showed no differences between the groups. Healthy children with CRP levels above 0.6 mg/L had significant lower levels of retinol ($p = 0.03$). This was not observed in children with type 1 diabetes. The results suggest that, in contrast to healthy children, minor CRP elevation does not affect vitamin A transport complex in serum of children with type 1 diabetes. (*Pediatr Res* 62: 741–745, 2007)

Retinol and its metabolites are essential for normal growth, vision, differentiation of various cells, and resistance to infection (1). Plasma vitamin A levels are well maintained against variations in dietary intake due to tight regulation by synthesis of retinol-binding protein 4 (RBP4). RBP4 is a lipocalin specifically involved in the plasma transport of retinol with a molecular mass of 21 kD that is synthesized primarily by hepatocytes but also in the kidney, the adipose tissue, and several other organs (2,3). In plasma, RBP4 is mainly associated with transthyretin (TTR), a homo-tetramer with a molecular mass of 56 kD, preventing its filtration in the kidney (3–5).

Plasma levels of retinol are of limited value to evaluate the vitamin A status of an individual because of the tight homeostatic control of RBP4 in plasma as well as the strong influence of inflammation on its plasma levels. Only substantial degrees of deficiency or intoxication result in diagnostically relevant changes (1,6,7). A specific aspect in the determination of the extent of vitamin A deficiency based on reduced RBP4 plasma levels is the observation that, beside vitamin A deficiency, the inflammation-associated acute phase response

(APR) results in decreased plasma levels despite a sufficient vitamin A status (8). Recent studies have demonstrated significantly lower serum retinol level in patients with APR or trauma (7,9–12). The APR leads to a transient increase or decrease of plasma concentrations of different proteins due to changed hepatic protein synthesis, known as positive or negative acute phase proteins. C-reactive protein (CRP) belongs to the positive acute phase proteins with an inflammation-induced increase up to 1000-fold of its normal serum level and is used as an indicator for the extension of the APR (13). Both RBP4 and TTR, as negative acute phase proteins, are supposed to decrease during inflammation (6,13). Based on these difficulties, it has been suggested that the determination of the molar RBP4 to TTR index might help to discriminate between a deficiency and an inflammatory induced decrease of RBP4 (14). In inflammation, retinol, RBP4, and TTR levels decrease, whereas, in nutritional deficiency, only retinol and RBP4 are depressed (6).

Type 1 diabetes is one of the most common metabolic disorders in children and adolescents, with increasing incidence and decreasing age of onset. It is characterized by insulin deficiency due to destruction of pancreatic β -cells and by hyperglycemia (15–18). In addition, patients with type 1 diabetes have been considered at risk for deficiency in nutritional status of several micronutrients, e.g. vitamin A and carotenoids (19,20). Several studies have demonstrated an effect of type 1 diabetes on the vitamin A status in animals and humans. Decreased plasma concentrations of retinol, RBP4, and TTR were found in both streptozotocin-induced diabetic rats (21,22), and children and adults with type 1 diabetes (23–27). Studies reported as well increased plasma CRP concentrations in patients with type 1 diabetes (28). However, the effect of elevated CRP on components of the vitamin A transport complex is controversial. Therefore, the present study was conducted to examine the APR and the RBP4/TTR ratio to identify factors that influence the vitamin A metabolism in children with type 1 diabetes.

METHODS

Subjects. The study included 40 Caucasian patients with type 1 diabetes who regularly attend the diabetes outpatient clinic of the University Hospital

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Abbreviations: APR, acute phase response; CRP, C-reactive protein; CV, coefficient of variability; RBP4, retinol-binding protein 4; TTR, transthyretin

for Children and Adolescents (University of Leipzig, Germany). The study included patients under 20 y of age with type 1 diabetes as defined by the American Diabetes Association (1998) and with no persistent microalbuminuria (defined as an albumin excretion rate $>20 \mu\text{g}/\text{min}$ in a minimum of three consecutive urine specimens), no retinopathy (examined by ophthalmoscopy according to the guidelines of International Society for Pediatric and Adolescent Diabetes, ISPAD), no celiac disease (monitored yearly by measurements of serum endomysium and gliadin antibodies and evaluation for clinical symptoms), and no other chronic diseases with the exception of autoimmune thyroiditis. Parameters such as age, gender, weight, height, duration of diabetes, blood pressure, stage of puberty according to Tanner (29), and smoking were recorded. Children and adolescents with type 1 diabetes were treated with basal-bolus regimens with combinations of short-/rapid-acting insulin and intermediate/long-acting insulin. Body mass index SD score (BMI-SDS) was calculated using the national reference data in Germany (30). The control group comprised 46 age- and gender-matched Caucasian healthy children and adolescents between the ages of 7 and 17 y. The healthy subjects were recruited along with regular public health service examinations in school classes from a study on anthropometric measurements ["Leipzig School Children's Project" (31,32)]. Parameters such as age, gender, weight, height, BMI-SDS (30), and stage of puberty according to Tanner (29) were recorded. Written informed consent was given according to their age by parents or adolescents. The Ethical Committee of the Medical Faculty of the University of Leipzig approved the project.

Measurement of laboratory parameters. Nonfasting venous blood was collected and serum was stored at -25°C until further analysis. Levels of serum cholesterol, HDL, LDL, and triglycerides were measured using commercially available enzymatic *in vitro* tests (Cholesterol CHOD-PAP, HDL-C Plus, LDL-C Plus, Triglycerides GPO-PAP, Hitachi, Roche Diagnostics, Penzberg, Germany). HbA_{1c} levels were determined by a commercial kit using a turbidimetric inhibition immunoassay (Tina Quant aHbA_{1c} , Roche Diagnostics). Normal range was 4.8–6.0% with an interassay coefficient of variability (CV) of 8.8% for values within the normal range and of 4.1% for values above the normal range. Serum creatinine was measured using a commercially available enzymatic test (Creatinine plus, Roche Diagnostics). CRP levels were determined using a high sensitive immunologic test (Tina-quant CRPLX, Roche Diagnostics) allowing measurement of serum CRP level until 0.2 mg/L.

Analytical determination of retinol, RBP4, and TTR in serum. Retinol was determined using a modified gradient reversed-phase HPLC-system (Waters, Eschborn, Germany) after organic extraction as previously described (33). Briefly, retinol was extracted twice with n-hexane from serum and separated on a C30 column ($5 \mu\text{m}$, $250 \times 4.6 \text{ mm}$; YMC, Wilmington, NC). The solvent system consisted of solvent A with methanol/water (90:10, vol/vol, 0.4 g/L ammonium acetate in H_2O) and solvent B with methanol/methyl-*tert*-butyl-ether/water (8:90:2, vol/vol/v, 0.1 g/L ammonium acetate in H_2O) at a column flow rate of 0.2 mL/min. Retinol was quantified by measuring the absorption at 325 nm by a photodiode array detector (Model 996, Waters) using an external retinol standard purchased from Sigma Chemical Co. (Deisenhofen, Germany). Results were compared with standard

reference material (SMR 968a fat soluble vitamins in human serum; National Institute of Standards and Technology, Gaithersburg, MD). The detection limit for retinol was 2.0 ng, the CV was 4%, and the recovery rate was above 95%. Serum concentrations of α -tocopherol were detected at 290 nm and quantified using an external standard (Sigma Chemical Co.) also by reversed-phase HPLC. Concentration of RBP4 in serum was quantitatively determined by ELISA as previously described (34). The intraassay CV was 2.9% and the CV for the interassay was 4.2%. TTR was measured using an adapted ELISA technique from the RBP4 procedure with an intraassay CV of 8.1% and an interassay CV of 8.8% (35).

Data analysis. Data are presented as medians with ranges. Statistical parameters were calculated using SPSS program 12.0 (SPSS Inc., Chicago, IL). Due to not normally distributed data, the nonparametric Mann-Whitney *U* test was used to test for significant differences between the groups. To test for associations between different parameters, Spearman's rho correlation was used. *p* Values <0.05 were considered for significance.

RESULTS

Anthropometric and clinical parameters. Anthropometric and clinical parameters of children and adolescents with and without type 1 diabetes are summarized in Table 1. Autoimmune thyroiditis was present in six children, thyroid function was normal under treatment in all patients. Serum concentrations of creatinine, cholesterol, triglycerides, and LDL cholesterol were within normal ranges in both the type 1 diabetes and control group. HDL cholesterol ($p = 0.0005$) and CRP ($p = 0.005$) were elevated in children with type 1 diabetes. In the type 1 diabetes group, significant positive correlations were found between HbA_{1c} and cholesterol ($r = 0.376$, $p = 0.02$) as well as between HbA_{1c} and LDL cholesterol ($r = 0.423$, $p = 0.006$). In contrast, the control group showed a positive correlation between CRP and BMI-SDS ($r = 0.351$, $p = 0.02$). Serum concentrations of α -tocopherol in children with type 1 diabetes were not lower when compared with controls (Table 1).

Retinol, RBP4, and TTR in serum. Serum concentrations of retinol were significantly lower in subjects with type 1 diabetes ($p = 0.02$). Lower retinol concentrations were also reflected by a decreased molar ratio of serum retinol to serum RBP4 ($p = 0.005$). In contrast to retinol, no significant differences in serum levels of RBP4 and TTR were observed.

Table 1. Anthropometric and clinical parameters of children and adolescents with type 1 diabetes and controls

	Type 1 diabetes mellitus	Controls	Significance (<i>p</i> value)
<i>n</i>	40	46	
Gender ratio (male/female)	24/16	27/19	
Age (y)	14.2 (5.40–18.4)	12.8 (7.18–17.8)	NS
BMI-SDS (kg/m^2)	0.53 (–1.52–2.22)	0.34 (–1.3–1.72)	NS
Diabetes duration (y)	5.75 (0.80–14.8)	—	—
Systolic blood pressure (mm Hg)	120 (93.0–147)	118 (105–140)	NS
Diastolic blood pressure (mm Hg)	65.5 (50.0–88.0)	70.0 (60.0–90.0)	$p = 0.0005$
HbA_{1c} (%)	7.30 (6.0–14.0)	5.36 (5.0–5.80)	$p = 0.0001$
Triglycerides (mmol/L)	0.82 (0.48–2.96)	0.96 (0.45–2.31)	NS
Cholesterol (mmol/L)	4.31 (2.72–6.46)	4.07 (2.97–6.53)	NS
LDL cholesterol (mmol/L)	2.38 (1.00–3.98)	2.19 (0.58–4.63)	NS
HDL cholesterol (mmol/L)	1.65 (1.01–2.41)	1.38 (0.94–2.19)	$p = 0.0005$
Creatinine ($\mu\text{mol}/\text{L}$)	57.0 (32.0–87.0)	n.d.	—
C-reactive protein (mg/L)	0.90 (0.40–16.9)	0.60 (0.20–4.40)	$p = 0.005$
α -Tocopherol ($\mu\text{mol}/\text{L}$)	18.3 (9.36–34.1)	16.2 (11.5–26.8)	$p = 0.02$

Data are presented as medians (range).

n.d., not determined; BMI-SDS, body mass index-standard deviation scores; HbA_{1c} , glycosylated hemoglobin; LDL, low density lipoproteins; HDL, high density lipoproteins.

Table 2. Parameters of the vitamin A transport complex in serum of children and adolescents with type 1 diabetes and of controls

	Type 1 diabetes mellitus (n = 40)	Controls (n = 46)	Significance (p value)
Serum retinol (μmol/L)	1.09 (0.72–2.65)	1.26 (0.81–1.75)	p = 0.02
Serum RBP4 (μmol/L)	1.11 (0.75–3.05)	1.16 (0.52–1.70)	NS
Retinol/RBP4 index*	0.89 (0.50–1.69)	1.11 (0.59–2.37)	p = 0.005
Serum TTR (μmol/L)	3.00 (1.42–9.91)	3.25 (2.12–6.44)	NS
RBP4/TTR index†	0.41 (0.17–1.51)	0.35 (0.09–0.71)	p = 0.01

Data are presented as medians (range).

* Retinol/RBP4 index is the molar ratio of serum retinol to serum RBP4.

† The RBP4/TTR index is the molar ratio of serum RBP4 to serum TTR.

However, the molar ratio of RBP4 to TTR was significantly higher in patients with type 1 diabetes (p = 0.01, Table 2).

Neither retinol nor RBP4 and TTR showed any significant correlation to CRP. Furthermore, we found no association between levels of CRP and HbA_{1c} or duration of diabetes. To investigate the effect of CRP on plasma vitamin A complex, a cut-off value of 0.6 mg/L established by Abraham *et al.* (9) was used to allocate children and adolescents into two groups. In the type 1 diabetic group retinol, RBP4 and TTR were not different, but there was a significant difference in the control group for retinol (p = 0.03) (Table 3). Considering in both groups only children and adolescents with CRP concentrations below 0.6 mg/L, subjects with type 1 diabetes showed significant lower levels of retinol (p = 0.02). However, this was not evident in children and adolescents with CRP values above 0.6 mg/L (Table 3).

As shown in Figure 1A, retinol correlated positively with age (type 1 diabetes: r = 0.431, p = 0.005; controls: r = 0.493; p = 0.001). Furthermore, in children with type 1 diabetes, retinol and RBP4 correlated positively with serum triglycerides (retinol, p = 0.008; RBP4, p = 0.008), cholesterol (retinol, p = 0.002; RBP4, p = 0.003) and LDL cholesterol (retinol, p = 0.003; RBP4, p = 0.02). TTR showed no significant correlations with lipid parameters. Among the children and adolescents with type 1 diabetes, no correlations were found between HbA_{1c} and retinol, RBP4 and TTR (Fig 1B–D), or between the duration of the diabetes disease and

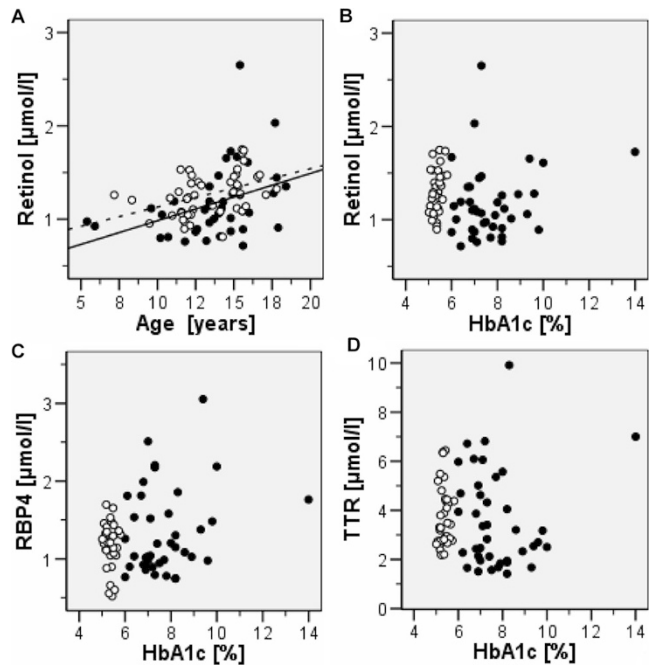


Figure 1. (A) Correlation (Spearman’s rho) of serum retinol (determined by HPLC) and age in children and adolescents with and without type 1 diabetes. Serum concentrations of retinol were significantly higher in children and adolescents with type 1 diabetes (●) than in controls (○, p = 0.02). Furthermore, serum retinol concentration increased with age in children and adolescents with (black line, r = 0.431; p = 0.005) and without diabetes (dotted line, r = 0.493; p = 0.001). (B–D) Correlations (Spearman’s rho) of serum retinol, RBP4, TTR, and HbA_{1c} in children and adolescents with and without type 1 diabetes. No correlations between serum retinol, RBP4, and HbA_{1c} in children and adolescents with type 1 diabetes (●) and in controls (○) were observed.

retinol and TTR. Statistical analysis revealed no influence on retinol, RBP4, and TTR in serum regarding diabetes duration and metabolic control as estimated with HbA_{1c} below and above 8%. There were no differences between boys and girls in any parameters.

DISCUSSION

In our study, some differences regarding the characteristics between the study and control subjects are observed. Healthy children in the control group had higher diastolic blood pressure. Despite these elevated diastolic blood pressures, all

Table 3. Parameters of the vitamin A transport complex in serum allocated by a serum CRP cut-off value of 0.6 mg/L in children and adolescents with type 1 diabetes and controls

CRP (mg/L)	Type 1 diabetes mellitus (n = 40)		Controls (n = 46)	
	<0.6 (n = 16)	>0.6 (n = 24)	<0.6 (n = 30)	>0.6 (n = 16)
Serum retinol (μmol/L)	1.17 (0.81–1.73)‡	1.03 (0.72–2.65)	1.31 (0.81–1.75)‡	1.11 (0.90–1.63)‡,§
Serum RBP4 (μmol/L)	1.01 (0.75–1.99)	1.18 (0.75–3.05)	1.16 (0.52–1.70)	1.17 (0.60–1.53)
Retinol/RBP4 index*	1.01 (0.63–1.69)	0.86 (0.50–1.62)	1.15 (0.73–2.37)	1.07 (0.59–2.27)
Serum TTR (μmol/L)	3.35 (1.57–7.00)	2.86 (1.42–9.91)	3.26 (2.12–6.44)	3.07 (2.18–5.48)
RBP4/TTR index†	0.40 (0.17–0.96)	0.44 (0.21–1.51)	0.35 (0.09–0.71)	0.35 (0.14–0.56)

Data are presented as medians (range).

* Retinol/RBP4 index is the molar ratio of serum retinol to serum RBP4.

† The RBP4/TTR index is the molar ratio of serum RBP4 to serum TTR.

‡ p = 0.02.

§ p = 0.03.

measured blood pressures were within the normal ranges and no hypertension was present. Moreover, our study showed, like others have, that HDL cholesterol is elevated in type 1 diabetes (36,37). One possible explanation for increased HDL cholesterol in type 1 diabetes is peripheral hyperinsulinemia caused by insulin-mediated lipoprotein-lipase activity.

RBP4 has recently gained considerable attention due to its possible link to insulin resistance and type 2 diabetes (38–40). The exact mechanism between elevated RBP4 levels in serum and type 2 diabetes is still controversial. Contrary to type 2 diabetes, there is compelling evidence that patients with type 1 diabetes have reduced serum levels of retinol, RBP4, and TTR (19,21,23). However, it is necessary to validate those results by considering factors that might influence RBP4 levels as well. Major effects on plasma RBP4 levels are caused by inflammation, infection, and the resulting APR (7–9,42). Since patients with type 1 diabetes have increased levels of inflammatory markers, this increased inflammatory status might contribute to changes in the vitamin A transport complex in plasma (28,43).

In agreement with previous investigations, serum retinol concentrations were significantly lower in children and adolescents with type 1 diabetes when compared with those of control subjects. However, RBP4 and TTR levels were not different between the diabetes and control group. Based on published data and on our observation that α -tocopherol levels in serum of children and adolescents with type 1 diabetes were not lower than in controls, it is very unlikely that an insufficient vitamin A intake or an impaired absorption of lipophilic components might cause these changes (24,44). There are further investigations necessary to estimate the significant lower α -tocopherol serum levels of the control group.

The lipophilic retinol is transported in serum by binding to the RBP4-TTR complex (1). Therefore, changes in plasma RBP4 and TTR concentrations may influence the serum retinol level. Dietary vitamin A deficiency leads to a reduction of retinol and RBP4 levels, whereas TTR remains unchanged. This results in a lower molar ratio of RBP4 to TTR; at this, a cut-off of 0.36 is used to define vitamin A deficiency (1,5,6,45). During inflammation, both RBP4 and TTR levels are depressed due to APR resulting in an unchanged ratio at lower absolute levels (6). CRP as a sensitive indicator of an ongoing APR can be used to avoid misclassification in studies, which evaluate the nutritional status of retinol (9,13). In the present study, we demonstrate significantly higher CRP serum levels in children and adolescents with type 1 diabetes indicating APR. This APR may contribute to lower serum retinol levels in children with type 1 diabetes as has been also observed in young children (9). However, this APR would not only change the levels of retinol but also of RBP4 and TTR. In our study, we observed not only no differences in absolute levels of the two transport proteins but also no correlation between CRP and retinol, RBP4 and TTR, which is in contrast to other studies (11,46). This lack of correlation could be a consequence of the relative small size of the study group with CRP serum values within a narrow range. On the other hand, these results also suggest that neither dietary vitamin A deficiency nor an elevated inflammatory status might cause de-

pressed serum retinol concentrations in children and adolescents with type 1 diabetes. The mechanism explaining this observation may lie in type 1 diabetic specific alteration in vitamin A metabolism. One explanation might be the observed impaired release of the ternary retinol-RBP4-TTR complex by hepatocytes into plasma of streptozotocin-induced type 1 diabetic rats (22). Interestingly, using a limit of 0.6 mg/L (9), subjects of the control group with CRP serum levels above this showed significantly decreased retinol serum concentration compared with controls who had CRP concentrations below 0.6 mg/L. This could be related with the not significant higher mean age of healthy children and adolescents with CRP level below 0.6 mg/L (13.9 ± 2.2 versus 12.5 ± 2.4) according to the strong correlation of retinol with age demonstrated in Figure 1. Otherwise, this may indicate multiple processes, which affect the vitamin A status in patients with type 1 diabetes. Therefore, the single effect of elevated CRP levels is not supporting the described hypothesis above. In contrast, in healthy subjects the influence of minor inflammation was detectable and supported other results (7,9).

Recent studies have demonstrated higher ratios of RBP4 to TTR under selected pathologic conditions. Both the molar ratios of RBP4/TTR and serum CRP levels are increased in HIV-1 seropositive women as well as in extremely low birth weight infants with bronchopulmonary dysplasia (11,46). In accordance to this, our individuals with type 1 diabetes showed an increased molar ratio of RBP4 to TTR. It is likely that these findings are related to disease concomitant inflammations indicated by elevated serum CRP level. Inflammation causes decreased TTR and RBP4 plasma levels which leads to an unchanged RBP4/TTR molar ratio (6). In addition to the elevated RBP4/TTR ratio, it has been suggested that TTR is more sensitive to inflammation than RBP4 and shows a stronger decrease during the APR with the possible consequence of a higher RBP4/TTR molar ratio (13,46). Nevertheless, in the present study we could not find an inverse correlation of serum TTR with elevated serum CRP level or lower TTR level in subjects of both groups with CRP level above 0.6 mg/L. Baeten *et al.* (11) demonstrated that a HIV-1 infection was associated with a higher RBP4/TTR ratio independently from CRP. This implies additional effects of HIV-1 infection on the vitamin A status next to those resulting from inflammatory reactions. Comparison between the subjects in the study of Baeten *et al.* and our study shows similar serum CRP levels in HIV-seronegative women (interquartile range, 0.40–2.78 mg/L) and children with or without type 1 diabetes in our study (interquartile ranges: in controls, 0.40–0.83 mg/L, and in children with type 1 diabetes, 0.50–1.83 mg/L); in HIV-seropositive women, CRP levels are higher (interquartile range, 1.20–10.60 mg/L). In previous studies, RBP4/TTR ratios were unchanged or often decreased (47), which was explained by modest TTR changes due to inflammation compared with RBP4. It was supposed that the differential suppression of RBP4 in respect to TTR as result of differential inflammatory reaction is in adults not as intense as in children (11). Our results demonstrate that, also in children with type 1 diabetes, the RBP4/TTR molar ratio increases. Therefore, suppression of RBP4 and TTR during inflammation may not be different between adults and children.

In conclusion, these results verify previous investigations of patients with type 1 diabetes, who had significantly lower retinol concentrations compared with healthy subjects. Furthermore, in accordance to recent publications in this study the molar ratio of RBP4 to TTR was increased in children and adolescents with type 1 diabetes. Contrary to other studies, plasma levels of both transport proteins, RBP4 and TTR, did not differ between the diabetic and control group. Thus, vitamin A deficiency and elevated CRP are not primarily responsible for the decreased serum retinol concentrations in children with type 1 diabetes. Other factors seem to exist, which influence vitamin A metabolism in young patients with type 1 diabetes.

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