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ORIGINAL ARTICLE

Elevated serum neopterin levels in children with functional constipation: association with systemic proinflammatory cytokines

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Abstract

Background Functional constipation is a clinical problem with an incompletely understood etiology. Functional bowel diseases have been shown to be related to inflammation in many studies in adults. In this study, we aimed to evaluate leukocytes, C-reactive protein, proinflammatory and anti-inflammatory cytokines, and neopterin levels in children with functional constipation.

Methods Seventy-six children with constipation and 71 healthy controls (mean age 7.12 ± 3.46 years and 7.32 ± 4.33 years, respectively, $P = 0.991$) were included in the study. Leukocytes, C-reactive protein, interleukin (IL)-1β, IL-6, IL-10, IL-12, tumor necrosis factor-alpha (TNF-α) and neopterin levels were assessed in patients and healthy controls. Parameters were measured in the serum using enzyme-linked immunosorbent assay methods.

Results Mean IL-6 (20.31 ± 12.05 vs. 16.2 ± 10.25 pg/mL, respectively, $P = 0.003$), IL-12 (181.42 ± 133.45 vs. 135.6 ± 83.67 pg/mL, respectively, $P = 0.018$) and neopterin levels (2.08 ± 1.12 vs. 1.52 ± 1.02 pg/mL, respectively, $P = 0.001$) were significantly higher in constipated children than healthy controls. Leukocyte and thrombocyte counts, C-reactive protein, and IL-1β, IL-10 and TNF-α levels did not show any difference between the two groups.

Conclusions In this study, IL-6, IL-12 and neopterin levels of constipated patients were found to be higher than those of controls. These results indicate the presence of subclinical inflammation in children with functional constipation.

Keywords Children · Constipation · Cytokine · Inflammation · Neopterin

Introduction

Functional constipation is one of the most frequently seen functional gastrointestinal disorders in the pediatric age group. Although several clinical, pathophysiological, and epidemiological studies have been carried out, the cause of childhood functional constipation remains unclear. In a number of studies, it was shown that adult irritable bowel syndrome (IBS) patients have low-grade inflammation, namely, increased systemic cytokine levels and intestinal immune cell infiltration. There are a limited number of studies in which inflammation markers are investigated in children with IBS [1–3].

Severe colonic inflammation due to inflammatory bowel diseases has been shown to be a predisposing factor for cancer development [4, 5]. IBS and chronic constipation are functional colonic disorders, which cause subclinical systemic and local inflammation. They have also been found to be related to colon cancers in adult studies [6–8]. It is important to recognize and treat these early pathologic conditions to prevent future complications.

Neopterin, a pteridine derivative secreted by monocyte-derived macrophages in response to interferon-gamma (IFN-gamma) stimulation, is a reliable parameter to assess T helper-1 (Th-1) and IFN-gamma mediated immune activation. Neopterin measurement in body fluids provides information about cellular immune response level and also helps to estimate disease progression [9]. As an inflammatory parameter, high serum and/or urine neopterin concentrations

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have been detected in various infections [10, 11], obesity [12], autoimmune disorders, inflammatory diseases [13, 14] and malignancies [15–17]. Serum and/or fecal neopterin levels have also been investigated in different intestinal pathologies and shown to be elevated in patients with environmental enteropathy [18], giardiasis [19], acute appendicitis [20] and inflammatory bowel diseases [21, 22]. Plasma neopterin levels have been evaluated and increased neopterin levels have been detected in IBS patients in only one study in adults [23].

To the best of our knowledge, to date, no study has been conducted to investigate serum neopterin levels in children with functional constipation. The aim of this study was to assess serum neopterin concentrations in children with constipation, compare these levels with those in healthy controls and investigate the possible correlations between neopterin levels and proinflammatory cytokines.

Methods

Study population

This case–control study was carried at Dokuz Eylul University, Division of Pediatric Gastroenterology, Metabolism and Nutrition. Seventy-six children diagnosed with functional constipation according to Rome III diagnostic criteria (defecation frequency of < 3 times per week and ≥ 1 of the following criteria: fecal incontinence > 1 episode per week, large amount of stools that clog the toilet, painful defecation, withholding behavior, or abdominal or rectal fecal impaction on physical examination) for at least 2 months were enrolled in the study [24, 25]. Children with chronic diseases in addition to the complaint of constipation [celiac disease, hypothyroidism, cerebral palsy, malignancy, eating disorders (anorexia, etc.), metabolic diseases], children with a history of anticholinergic or antacid drug use or any other bowel disease (anatomic causes, Hirschsprung's disease, ileus) and those with previous abdominal surgery were excluded from the study.

A total of 71 age- and sex-matched healthy children constituted the control group. None of the children had any history of drug usage, chronic systemic disease, malnutrition or obesity, previous constipation diagnosis or any gastrointestinal complaints.

Complete physical examination was performed on the patients and the controls. Socio-demographic data, duration of symptoms and accompanying symptoms were recorded in the patient group. Hardness of stool was evaluated with Bristol Stool Form Scale [26]. In this scoring system, stool hardness is numbered (according to adhesion and cracking property of stool) from hardest to softest between 1 and 7. The first two classes show constipation, the third and the fourth classes show normal defecation, whereas the last three classes indicate diarrhea [26].

Biochemical measurements

Venous blood samples were obtained from patients and healthy controls after an overnight fasting. Standard tubes with constant amount of K3-EDTA were used for complete blood count analyses. Complete blood count analyses were performed using a Coulter analyzer (LH-780, Beckman Coulter, Brea, CA, USA) with the impedance method (intra-assay variation coefficient 1.6%, inter-assay variation coefficient 1.6%). C-reactive protein levels were measured by turbidimetric method using an AU5800 analyzer (Beckman Coulter Inc., Brea, CA, USA). Blood samples for biochemical analysis were drawn in plain tubes. Blood samples were centrifuged at 1200 × *g* for 10 minutes and serum samples were removed from clots into clean Eppendorf tubes using plastic Pasteur pipettes. The samples were stored at – 80 °C until analysis.

Biochemical analysis

Serum neopterin (catalog no: CSB-EQ 027403HU, CUSABIO, Wuhan, China), tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6, IL-10 and IL-12 (catalog no: EK0525, EK0392, EK0410, EK0416 and EK0421, Boster Biological Technology Co. Ltd., Wuhan, China) levels were measured with the enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer's instructions. The sensitivity and detection ranges of the ELISA assays for inflammatory molecules are shown in parentheses as follows: neopterin (< 0.156 ng/mL, 0.625–40 ng/mL); TNF-α (< 1 pg/mL, 7.8–500 pg/mL); IL-1β (< 0.15 pg/mL, 3.9–250 pg/mL); IL-6 (< 0.3 pg/mL, 4.69–300 pg/mL); IL-10 (< 0.5 pg/mL, 3.4–250 pg/mL) and IL-12 (< 2 pg/mL, 7.8–500 pg/mL). The intra-assay assay variation (CV) was < 8% and the inter-assay CV was < 10%.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 20.0. Continuous and categorical variables were presented as mean ± standard deviation [median (25–75 percentiles)] and number (%), respectively. The Kolmogorov–Smirnov test was used to evaluate the normality of sample distribution. Mann–Whitney *U* test was used to compare the median values of the two groups (patients vs. healthy controls). Chi square test was used for the comparison of group ratios. Correlations between parameters were computed through the Spearman's

correlation analysis. All *P* values were two tailed, and group differences with $P < 0.05$ were considered to be statistically significant.

Results

Seventy-six patients (mean age 7.12 ± 3.46 years, median 7.0 and 25–75 percentile of 4.0–7.0 years) and 71 healthy children (mean age 7.32 ± 4.33 years, median 7.0 and 25–75 percentile of 3.0–7.0 years) were enrolled ($P = 0.991$). There was no significant difference between the patient and control groups regarding gender distribution (male/female ratio was 32/44 and 38/33 in patients and controls, respectively, $P = 0.188$). The mean value of Bristol Stool Scale in patients and in controls were 1.89 ± 0.52 and 3.65 ± 0.58, respectively ($P = 0.001$).

Mean IL-6, IL-12 and neopterin levels were significantly higher in children with constipation than healthy controls (Table 1). Although thrombocyte counts and mean C-reactive protein levels were higher in patients compared to the controls, the difference was not significant (Table 1). Leukocyte count and IL-1β, IL-10 and TNF-α levels did not show any difference between the two groups. Serum neopterin level was positively correlated with serum IL-6 and IL-12 levels in the patient group (Table 2). There was no significant correlation between serum neopterin levels and C-reactive protein, leukocyte, thrombocyte, IL-1β, IL-10 and TNF-α levels (Table 2). In the control group, serum neopterin level was only correlated with serum IL-6 levels (Table 2).

Table 1 Complete blood analysis, serum inflammatory markers, pro- and anti-inflammatory cytokines and neopterin levels of patients and controls

Variables	Patients ($n = 76$)	Controls ($n = 71$)	*P* value*
Leukocytes ($\times 10^3/\mu L$)	8.24 ± 2.38	7.95 ± 1.67	0.402
	8.1 (6.35–9.45)	8.0 (6.75–9.35)	
Thrombocytes ($\times 10^3/\mu L$)	318.56 ± 83.29	293.33 ± 70.61	0.052
	299.0 (264.0–358.0)	289.5 (233.0–356.0)	
CRP (mg/L)[a]	3.75 ± 4.38	1.77 ± 1.08	0.361
	2.0 (0.8–5.5)	1.0 (1.0–3.0)	
IL-1β (pg/mL)[a]	25.16 ± 21.53	25.42 ± 17.71	0.235
	19.86 (9.12–32.41)	20.37 (10.43–33.32)	
IL-6 (pg/mL)[a]	20.31 ± 12.05	16.2 ± 10.25	**0.003**
	16.34 (12.87–22.29)	11.93 (9.54–20.11)	
IL-10 (pg/mL)[a]	65.19 ± 46.85	66.09 ± 47.62	0.289
	52.58 (38.34–78.83)	54.49 (37.43–81.03)	
IL-12 (pg/mL)[a]	181.42 ± 133.45	135.6 ± 83.67	**0.018**
	137.63 (87.43–249.52)	111.59 (76.48–171.54)	
TNF-α (pg/mL)[a]	23.65 ± 11.28	21.67 ± 9.07	0.711
	20.0 (15.97–26.01)	19.59 (15.97–25.19)	
Neopterin (ng/mL)	2.08 ± 1.12	1.52 ± 1.02	**0.001**
	1.79 (1.33–2.35)	1.13 (0.89–1.59)	

Values are presented as mean ± standard deviation and median (25–75 percentiles). *CRP* C-reactive protein, *IL* interleukin, *TNF-α* tumor necrosis factor-alpha. *$P < 0.05$ is significant and represented in bold characters. [a]Mean corpuscular volume

Table 2 Correlation between serum neopterin levels and inflammatory markers in the patient group ($n = 76$)

Neopterin	Leukocytes	Thrombocytes	CRP	IL-1β	IL-6	IL-10	IL-12	TNF-α
Patient group	0.133	0.140	0.161	0.194	**0.281***	0.111	**0.335**†	0.114
Control group	0.100	0.023	0.072	0.095	**0.204***	0.066	− 0.111	0.087

CRP C-reactive protein, *IL* interleukin, *TNF-α* tumor necrosis factor-alpha. *Correlation is significant at the 0.05 level. †Correlation is significant at the 0.01 level

Discussion

In this study, proinflammatory and anti-inflammatory cytokines and neopterin levels in children with functional constipation were compared with the levels in healthy controls. The results of the present study showed that serum neopterin and IL-6 and IL-12 levels were higher in constipated children than in healthy controls. To the best of our knowledge, this is the first study in the literature to investigate serum neopterin concentrations in children with functional constipation.

There are a number of studies conducted with adults evaluating the systemic or mucosal cytokine profiles in IBS patients. Moreover, three systematic reviews (one is also a meta-analysis) on these clinical studies have been published [27–29]. The results of the systematic reviews, meta-analysis and recently published studies showed that proinflammatory cytokines (IL-1β, IL-5, IL-6, IL-8, IL-12, IL-13 and TNF-α) and C-reactive protein levels (a nonspecific marker of inflammation) were higher in adult IBS patients or diarrhea-dominant adult IBS patients compared to the control groups in most of the published studies [27–31]. On the other hand, IL-10 levels, as a mostly used anti-inflammatory cytokine, did not differ between the patient and control groups in most of these studies. In general, these studies were different from each other regarding study design and IBS subgroups.

Three studies have been carried out in childhood IBS patients in the literature [1–3]. Similar to the studies in adults, pediatric studies have also revealed conflicting results. In the first study, Hua et al. compared 35 IBS patients with 25 healthy children using *Escherichia coli* lipopolysaccharide (LPS) stimulation on peripheral blood mononuclear cell cultures [1]. They showed that children with IBS had lower IL-10 levels at baseline and after LPS stimulation compared to healthy controls. TNF-α and IL-6 levels were not different between the two groups [1]. The authors concluded that defects in immune modulation may contribute to IBS in children [1]. The second study was carried out in Chernobyl, in children who were exposed to low dose radiation with or without IBS symptoms (75 and 20 children, respectively). Higher IL-4 (an anti-inflammatory cytokine) levels and lower IFN-gamma (a proinflammatory cytokine) levels were found in children with IBS symptoms compared to those without IBS symptoms [2]. The authors concluded that children residing in a contaminated area may have shifted from Th-1 to Th-2 immune deviation and differential expression of IL-4 and IFN-gamma. In the third pediatric study, Vázquez-Frias et al. detected lower IL-10 levels and higher IL-12 and TGF-β levels in IBS patients compared to the control group (with 15 children in each group). Similar to the findings of our study, TNF-α levels were not different in the two groups [3].

Compared to these pediatric studies, our study included a greater number of children in each group (76 and 71 children). We evaluated proinflammatory and anti-inflammatory cytokines and found significantly higher IL-6 and IL-12 levels in constipated children. However, leukocyte and thrombocyte counts, C-reactive protein, and IL-1β, IL-10 and TNF-α levels were not different between the two groups in our study.

Serum neopterin levels were significantly higher in our patient group compared to healthy controls. Neopterin is secreted from monocyte-derived active macrophages in a state of inflammation. As previously mentioned, elevated serum and/or fecal neopterin levels have been shown in several intestinal pathologies [18–22]. Plasma neopterin levels have been evaluated in IBS patients in only one adult study performed by Clarke et al [23]. In this study, the authors stated that plasma neopterin measurement was used as a general immune activation marker [32] and also as a sensitive biomarker of bowel disease activity, as described before [33]. They showed that plasma neopterin levels were considerably increased in the patient group compared to the control group [23]. However, it was not specified whether these patients had diarrhea- or constipation-dominant irritable bowel disease.

We investigated serum neopterin levels in constipated children with different reasons. Firstly, circulating neopterin measurement is accepted as a general immune activation marker and commonly used in studies on inflammation-related disorders including intestinal system disorders [23, 32, 33]. Secondly, although biopsy findings are inconclusive to demonstrate whether IBS patients have higher monocyte–macrophage infiltration in their colonic mucosal biopsies compared to healthy controls [34, 35], some previous studies have shown significantly higher levels of monocyte–macrophage related cytokines in IBS patients compared to healthy controls [36, 37]. These findings support the possible roles of these cells in functional bowel disorders. Increased neopterin levels in our study confirm that monocyte–macrophage activation is seen in these functional bowel disorders, even in the pediatric age group. Finally, whereas all of the previous studies investigated neopterin levels in dominantly diarrhea-related intestinal disorders [18, 19, 21–23, 33], our study is the first to document systemic neopterin status in constipated patients.

The exact mechanism of low-grade inflammation in patients with functional bowel disorders remains unclear. Intestinal dysbiosis and food-related reactions such as food allergy have been proposed to cause low-grade inflammation and altered permeability in IBS [38, 39]. Some changes in intestinal microbial composition have been documented in

constipated patients in recent studies [39–41]. Endogenous triggers such as serotonin, histamine, proteases and eicosanoids may increase intestinal permeability, either directly or via the stimulation of neurons of the enteric nervous system. Therefore, these pathways may be considered as mechanisms of inflammation [38, 42, 43]. Since these mechanisms are mostly studied in adults and diarrhea-dominant IBS patients, further studies are needed to clarify the possible mechanisms in other patient groups.

One major limitation of our study is the lack of prospective follow-up of our patients. Thus, we did not evaluate the neopterin and other cytokine levels after treatment of constipation. The second limitation is the relatively low number of patients studied. This will impact the statistical significance of the data. The third limitation is the lack of colonic mucosal biopsies of our patients. For this reason, we could not evaluate the local inflammation status in these patients. Although there are controversial results regarding the role of cow's milk and other food allergies in childhood constipation [43–47], the lack of evaluation of our patients for food allergies is an another limitation of the present study. The last limitation is that the relationship between constipation severity and neopterin levels was not assessed.

In conclusion, IL-6, IL-12 and neopterin levels were found to be high in children with functional constipation in this study. These results support the presence of low-grade inflammation in patients with functional constipation. Prospective studies are warranted to evaluate the effect of treatment on the inflammatory markers and the long-term effect of low-grade systemic inflammation in these patients.

Author contributions CC contributed to concept and design, and acquisition of data. EU contributed to acquisition of data and drafting the article. TK contributed to acquisition of data, analysis and interpretation of data. NA contributed to concept and design, acquisition of data, analysis and interpretation of data, revising it critically for important intellectual content and final approval of the version to be published.

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Compliance with ethical standards

Ethical approval All procedures performed in study involving the patients were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was started after the approval of the Ethics Committee of Dokuz Eylul University Faculty of Medicine (number of ethical approval: 2012/30-01). Written informed consent was obtained from parents of all patients included in the study.

Conflict of interest All authors declared that they have no conflict of interest.

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