Digestive Diseases and Sciences
https://doi.org/10.1007/s10620-020-06743-z

ORIGINAL ARTICLE

Differential Cytokine Expression in the Duodenum and Rectum of Children with Non-Immunoglobulin E-Mediated Cow's Milk Protein Allergy

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Received: 11 June 2020 / Accepted: 20 November 2020

Abstract

Background Cow's milk protein allergy (CMPA) is the most prevalent food allergy in children, and its pathogenesis remains poorly understood. It has been shown that the combination of genetic predisposition, perinatal factors, and intestinal imbalance of the immune response mediated by cytokines may play an essential role in CMPA pathogenesis.

Aim To characterize the gene expression of Th1, Th2, and Th17 cytokines in the duodenum and rectum in patients with CMPA.

Methods This is an observational, descriptive, cross-sectional, prospective study. We used specific IgE (ImmunoCAP®) in serum and biopsies from the rectum and duodenum for the detection of cytokine messenger RNA levels by real-time PCR in patients with a positive oral food challenge for CMPA. We analyzed the relative quantification of the gene expression of cytokines by real-time PCR, and we used the housekeeping gene GAPDH for normalization purposes.

Results Thirty children (13 male and 17 female) were evaluated. All patients had an open challenge for CMPA. IgE specific to casein, alfa-lactalbumin, and beta-lactoglobulin was negative in all patients. In terms of cytokine levels, the levels of TNFα, IL-6, IL-12 (Th1), IL-4, IL-10, IL-13 (Th2), and IL-17 were found to be higher in the rectum than in the duodenum ($p < 0.05$). IL-15 was found to be higher in the duodenum than in the rectum ($p < 0.05$).

Conclusions In the present study we observed that the immune response in CMPA seems to be mediated by a Th1, Th2, and Th17 cytokine profile, with the rectum being the main affected site.

Keywords Cow's milk protein allergy · Non-IgE-mediated cow′s milk protein allergy · Th1 · Th2 and Th17 cytokines

Introduction

Cow's milk protein allergy (CMPA) is defined as an immunological reaction to specific proteins present in cow's milk, manifesting itself through different gastrointestinal and non-gastrointestinal signs and symptoms [1, 2]. The prevalence of CMPA is around 2.5% in children younger than 1 year [3].

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Two immunological reactions play a leading role in CMPA, immunoglobulin E (IgE)-mediated and non-IgE-mediated, although some authors have also suggested a third group in which both mechanisms participate [4]. IgE-mediated reactions are characterized by acute onset (i.e., within 1 to 2 h after contact with the allergen) and are considered type 1 hypersensitivity reactions according to the Gell and Coombs classification [5].

These reactions are caused by differentiation from $CD4^+$ Th0 T lymphocytes to $CD4^+$ Th2, which is thought to be determined by an interaction between the antigens and the microenvironmental conditions (i.e., the communication between cytokines and antigen-presenting cells [APCs]) [5–7].

The immunological mechanisms involved in the non-IgE reactions are not well understood.

Published online: 12 January 2021

Some studies have suggested that in addition to the Th2-mediated immune responses mediated by production of IL-4, IL-5, and IL-13, other mechanisms may exist, such as alterations in intestinal motility, which are thought to be the result of an interaction between lymphocytes, mastocytes, and the enteric nervous system [5–8].

Fewer studies have shown the production of Th1 polarization cytokines (IFNγ, TNFα, IL-1β, and IL-6) in CMPA allergy [8]. The differentiation from $CD4^+$ lymphocytes to a Th1 response induced by IL-12 may also play a role in the pathogenesis [9].

Previous studies have shown increased production of IFNγ in biopsies of the duodenum in children with food allergies and CMPA, respectively [10, 11].

Another study recently reported higher levels of IL-6 and CCR4 (chemokine receptor) and decreased levels of IL-18 and IL-2 in duodenal biopsies of children with non-IgE-mediated CMPA [12].

Th17 polarization has not been documented in food allergy. IL-17 is one of the central cytokines produced by Th17 subpopulations. This family of cytokines comprises six different subtypes, from IL-17A to IL-17F. IL-17A and IL-17F share most of their amino acids; however, their functions differ from one another. Whereas IL-17A participates in inflammatory processes such as autoimmunity, cancer immunity, and immunity against bacterial and fungal infections, IL-17F is involved in mucosal immunity and in amplifying the Th2 response, so we deemed it as important to explore its response in CMPA patients [13].

Considering the poor understanding of CMPA pathogenesis, particularly the non-IgE-mediated subtype, this study aimed to describe the gene expression of Th1, Th2, and Th17 cytokines in the duodenum and rectum in children with non-IgE-mediated CMPA.

Materials and Methods

Subjects

We included children younger than 2 years of age with clinical suspicion of CMPA, based on signs and symptoms (irritability or excessive crying, abdominal distention, dyschezia, regurgitation, diarrhea, rectorrhagia, constipation, hematemesis, hematochezia respiratory symptoms, and eczema), who received care at the Department of Pediatric Gastroenterology and Nutrition at the National Institute of Pediatrics from October 2012 to July 2013. Children with systemic diseases, immunodeficiency, or malnutrition were excluded.

Once informed consent was obtained, the open cow's milk protein (CMP) challenge was performed to confirm the diagnosis. The open CMP challenge was performed as follows: once the patient was accepted to participate in the study, an extended hydrolyzed formula was initiated in patients who were formula-fed, and in breastfed patients, the mother was instructed to avoid dairy (exclusion diet). Patients were seen after a week, and those whose symptomatology disappeared were admitted to our department for further investigation. Initially, 1 mL of infant formula (non-hydrolyzed) was administered, followed by dose increases every 20 min in the following manner: 5, 10, 20, 40, 50, and 100 mL. During the observation period of 2 h 20 min, if the patient developed signs or symptoms, the test was stopped and was considered positive. Those patients who did not develop symptoms were followed by daily telephone calls, and if the symptoms occurred, we asked them to go to the hospital for a complete evaluation; otherwise, they were asked to come a week later for their clinical evaluation.

The challenge test was defined as negative if the patient did not develop the aforementioned signs or symptoms.

Once the diagnosis was confirmed, specific IgE (ImmunoCAP®) to α-lactalbumin, β-lactoglobulin, and casein was performed to determine whether the patient exhibited an IgE- or non-IgE-mediated response.

Tissue Sampling

Endoscopy and rectosigmoidoscopy were performed after the open oral challenge, once the diagnosis was confirmed, under general anesthesia at the Gastrointestinal Endoscopy Unit at the National Institute of Pediatrics, with a pediatric and neonatal endoscope and rectosigmoidoscope (Olympus América de México, S.A. de C.V.)

Endoscopy was performed by a single pediatric endoscopist. Biopsies were obtained at random sites, and in the case of macroscopic lesions, biopsies were taken at that site.

Relative Quantification of Gene Expression by Real-Time Polymerase Chain Reaction (PCR)

Biopsy tissues were placed in cryogenic vials along with 1 mL ribonucleic acid (RNA) stabilization solution (RNA later) and were kept at room temperature for 6 to 8 h. Thereafter, they were kept at −70 °C until the RNA extraction was performed. The total RNA was obtained from the duodenal and rectal biopsies by employing an RNA extraction kit (High Pure RNA Tissue Kit, Roche).

For q-PCR assays, quality control and determination of linearity and reproducibility were evaluated (VC < 10%). The mRNA relative quantification of target genes was conducted usihe LightCycler software 4.1, according to the 2-delta–delta Ct method. Table 1 shows the details of the primer designs and the number of the UPL probe (Universal ProbeLibrary, Roche Diagnostics, Mannheim, Germany) used for the RT-PCR assay.

Table 1 Oligonucleotide sequences for real-time RT-PCR

Gene	NM GenBank	LEFT	RIGHT	UPL
TNFα	NM_000594.2	cgctccccaagaagacag	agaggctgaggaacaagcac	57
IL-6	NM_000600.3	gatgagtacaaaagtcctgatcca	ctgcagccactggttctgt	40
INF-γ	NM_000619.2	ggcattttgaagaattggaaag	tttggatgctctggtcatctt	21
IL-17F	NM_052872.3	ggcatcatcaatgaaaacca	tggggtcccaagtgacag	10
IL-4*	NM_000589.2, NM_172348.1	gaaacggctcgacaggaac	ctctggttggcttccttcac	57
IL-5	NM_000879.2	cactgaagaaatctttcagggaat	ccgtctttcttctccacacttt	47
IL-9	NM_000590.1	cttcctcatcaacaagatgcag	agagacaactggtcacattagcac	59
IL-10	NM_000572.2	cataaattagaggtctccaaaatcg	aaggggctgggtcagctat	45
IL-12A	NM_000882.2	cactcccaaaacctgctgag	tctcttcagaagtgcaagggta	50
IL-13	NM_002188.2	agccctcagggagctcat	ctccataccatgctgccatt	17
IL-15	NM_000585.3	cagatagccagcccatacaag	ggctatggcaaggggttt	46
GAPDH	NM_002046.3	agccacatcgctcagacac	gcccaatacgaccaaatcc	60

Assays were designed to detect both transcript isoforms, UPL (Universal ProbeLibrary Set, Human contains probes #1 to #90 of the 165 Probes@Roche)

Statistical Analysis

Statistical analysis was performed using SPSS software version 19.0 (IBM Corp., Armonk, NY, USA), and the graphs were constructed with Prism GraphPad software version 5 (GraphPad Software Inc., San Diego, CA, USA). Differences between cytokines were assessed by the Student *t* test, and descriptive statistics were used for the description of the demographic variables.

Results

Clinical and Demographic Characteristics

Thirty-six patients were evaluated for suspected cow's milk protein allergy, and only 30 had a positive open challenge test. These 30 were ultimately included in the analysis.

Of the 30 patients diagnosed with CMPA, the mean age was 4.03 months, 17 (56%) were female, five (16.6%) were delivered vaginally and 25 (83.3%) via caesarean section, and the first food given after delivery was formula in 20 (66.6%), and exclusively breast milk in 10 (33.3%).

With regard to the initial symptomatology reported, 27 (90%) had irritability or excessive crying, 25 (83%) had abdominal distention, 24 (80%) had dyschezia, 20 (66.7%) had regurgitation, eight (26.6%) had diarrhea, seven (23.3%) had rectorrhagia, six (20%) had constipation, two (6.67%) hematemesis, and one (3.3%) had hematochezia (Table 2).

All the patients tested negative for the specific IgE to cow's milk proteins (α-lactalbumin, β-lactoglobulin, and casein) by the ImmunoCAP® test.

With regard to the endoscopic findings, 62.5% presented normal duodenum, 33.3% nodular duodenitis, and 1% ulcerated duodenitis. Regarding the rectosigmoidoscopy findings, 75% were normal, 8.3% showed nodular proctitis, 12.5% undefined proctitis, and 4.2% anal fissure.

In the histopathological analysis, 20.9% presented normal duodenum, 70.8% presented < 20 eosinophils per high-power field (HPF), and 8.3% > 20% eosinophils per HPF. In the rectum, 25% were reported normal, 64.8% presented < 20 eosinophils per HPF, and 10.2% > 20 eosinophils per HPF.

Table 2 Demographic and clinical characteristics of patients with cow's milk protein allergy

Variable	Number (%)
Age	4.03 months
Gender	
Female	17 (56.7%)
Male	13 (43.3%)
Cesarean delivery	25 (83.3%)
Vaginal delivery	5 (16.6%)
Formula feed	20 (66.6%)
Breastfed	10 (33.3%)
Symptomatology	
Irritability	27 (90%)
Abdominal distension	24 (80%)
Dyschezia	20 (66.7%)
Regurgitation	20 (66.7%)
Diarrhea	8 (26.6%)
Rectorrhagia	7 (23.3%)
Constipation	6 (20%)
Hematemesis	2 (6.6%)
Hematochezia	1 (3.3%)

Relative Gene Expression of Th1 Cytokines in the Duodenum and Rectum in Patients with CMPA

The Th1 cytokines (TNFα, IL-6, IFNγ and IL-12) were quantified. TNFα gene expression was increased in rectal compared with duodenal biopsies ($p = 0.015$; Fig. 1a). IL-6 was found to be higher in rectal biopsies than in duodenal biopsies ($p = 0.041$; Fig. 1b). IFNγ gene expression was found to be higher in duodenal biopsies than in rectal biopsies, but the differences were not significant (Fig. 1c). IL-12 gene expression was increased in rectal compared with duodenal samples ($p = 0.008$; Fig. 1d).

Relative Gene Expression of Th2 Cytokines in the Duodenum and Rectum in Patients with CMPA

Various Th2 cytokines were analyzed. Higher Th2 expression (IL-4, IL-10, IL-13) was seen in the rectum than in the duodenum of patients with CMPA, with statistically significant differences ($p = 0.004$, $p = 0.024$, and $p = 0.006$, respectively; Fig. 2a, d, and e). No significant differences were found between IL-5 and IL-9 in patients with CMPA, as shown in Fig. 1b, c. Conversely, IL-15 was higher in the duodenum than the rectum ($p = 0.03$; Fig. 2f).

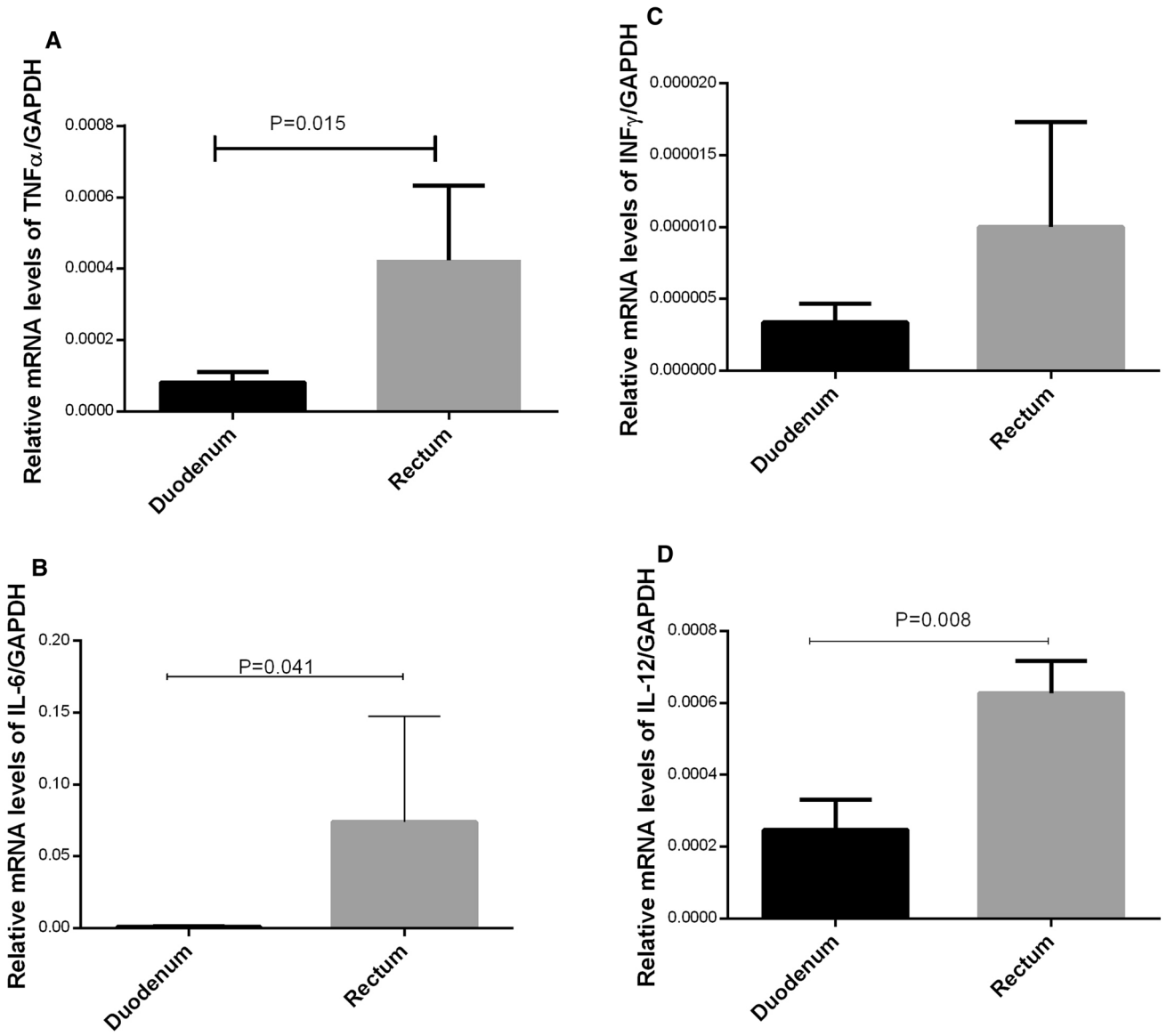

Fig. 1 Relative gene expression of Th1 cytokines in the duodenum and rectum in patients with CMPA. RT-qPCR was performed to assess mRNA levels in colonic mucosa biopsies from CMPA patients. Bars show means with standard error of the mean for **a** TNFα, **b** IL-6, **c** IFNγ, and **d** IL-12 transcript levels with GAPDH as housekeeping gene determined by 2 Δ ΔCt. Statistical significance was considered when p value was < 0.05

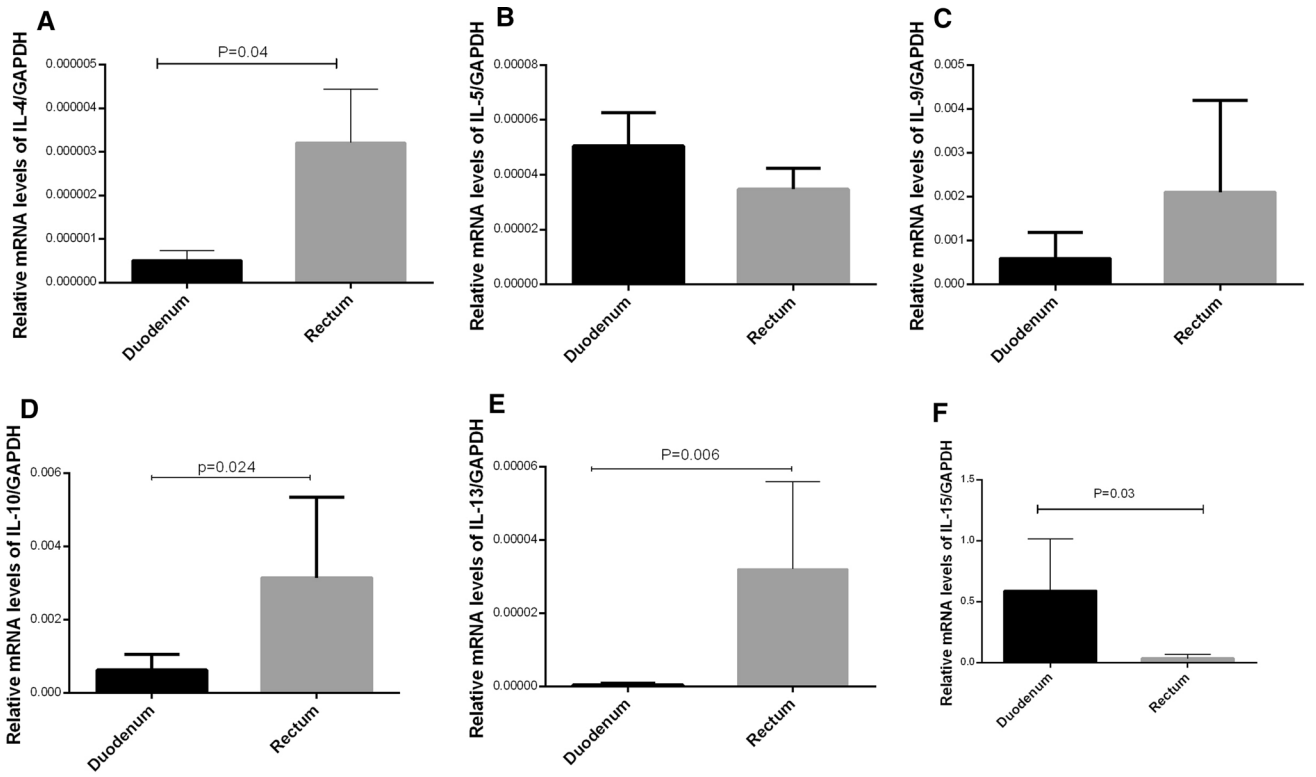

Fig. 2 Relative gene expression of Th2 cytokines in the duodenum and rectum in patients with CMPA. RT-qPCR was performed to assess mRNA levels in colonic mucosa biopsies from CMPA patients. Bars show means with standard error of the mean for **a** IL-4, **b** IL-5, **c** IL-9, **d** IL-10, **e** IL-13, and **f** IL-15 transcript levels with GAPDH as housekeeping gene determined by 2 Δ ΔCt. Statistical significance was considered when p value was < 0.05

Relative Gene Expression of Th17 Cytokines in the Duodenum and Rectum in Patients with CMPA

We found significantly higher expression of IL-17 in the rectum than in the duodenum of these patients ($p < 0.038$; Fig. 3).

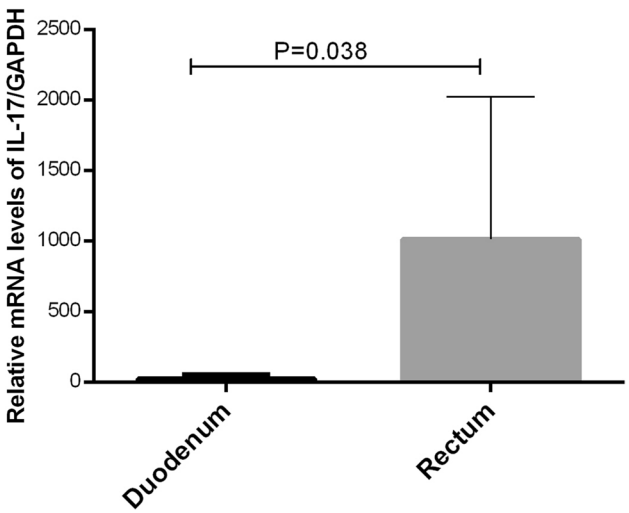

Fig. 3 Relative gene expression of IL-17 (TH17) cytokines in the duodenum and rectum in patients with CMPA. RT-qPCR was performed to assess mRNA levels in colonic mucosa biopsies from CMPA patients. Bars show means with standard error of the mean of IL-17 transcript levels with GAPDH as housekeeping gene determined by 2 Δ ΔCt. Statistical significance was considered when p value was < 0.05

Discussion

In the present study we showed differential cytokine expression in the duodenum and rectum of children with non-IgE-mediated cow's milk protein allergy (CMPA). We detected elevation of TNFα, IL-6, and IL-12 gene expression in the rectum compared with the duodenum ($p < 0.05$) in patients with CMPA. IFNγ gene expression was found to be higher in duodenal biopsies than in rectal biopsies, but the differences were not significant.

As for the Th2 cytokines, the gene expression of IL-4, IL-10, and IL-13 was found to be significantly elevated in the rectum. IL-5 and IL-9 gene expression was detected in the duodenum and rectum in CMPA patients, but no significant differences were observed. Conversely, IL-15 was higher in the duodenum than in the rectum.

Increased gene expression of pro-inflammatory cytokines Th1 (TNFα, IL-6, and IL-12) and TH2 (IL-4, IL-10, IL-13) may be related to a higher level of expression by inflammatory infiltrates such as eosinophils, neutrophils, and T-cell lymphocytes as the main cellular sources. Additional studies are required for the co-localization and identification of Th1 and Th2 populations and their total or partial involvement in CMPA pathogenesis.

We also detected a statistically significant difference in the expression of IL-17 in the rectum versus the duodenum for Th17 polarization.

Even though this is a descriptive study, the findings are of interest for additional studies investigating Th subsets and their cytokines involved in the pathophysiology and immune response of cow's milk protein allergy. The role of IL-17 in food allergies, particularly in CMPA, has not been well documented in humans.

A previous study reported increased production of IL-6, IL12, and IL-18 in the serum, as well as increased levels of TNFα in the presence of diarrhea and fever in children who underwent the CMP challenge [14]. Although the cellular source of production and synthesis of these cytokines is different, it is important to mention that, in accordance with our results, there is a correlation with the synthesis at the local and systemic levels.

In another study [15], in children with CMPA treated with immunotherapy, serum cytokines were studied before and after treatment, and an increased level of IL-6 was found after treatment. The authors concluded that this elevation might be due to the patients' desensitization and tolerance to CMP; however, one limitation of the study was that the patients' symptomatology after 6 months of orally administered immunotherapy treatment was not reported.

Ozen et al. [16] showed increased levels of TNFα and decreased levels of transforming growth factor-beta (TGF-β) in samples of colonic biopsies from subjects with CMPA, similar to what we found in our study, where TNFα was higher in rectal than duodenal biopsies.

To determine the gene expression of Th17 mediators, we found increased gene expression of IL-17 in rectal compared to duodenal biopsies in patients with CMPA, which likely explains the presence of symptomatology such as dyschezia rectorrhagia, and irritability.

In allergic processes, IL-17 has been shown to contribute to eosinophil recruitment. In our study, 75% of the children presented eosinophils in the rectum, consistent with IL-17 found on this site.

Żbikowska-Gotz et al. [17] found significantly increased production of IL-17 in serum and activated neutrophils in adults with food hypersensitivity, while Dhuban et al. [18] demonstrated decreased synthesis of this cytokine in children with food allergies as compared with healthy controls.

However, it should be noted that in both studies, the cytokines were obtained from the serum, while in the present study the cytokines were obtained directly from the duodenal and rectal mucosae. Moreover, it is essential to note that there are different subtypes of IL-17 that can be involved in both pro-inflammatory and regulatory processes. While both our study and that by Zbikowska-Gotz [17] report the type of IL-17, IL-17F, and IL-17A, respectively, both with similar functions, Dhuban [18] does not mention the subtype, which may explain the differences in expression, in addition to the measurement site, which can generate additional bias. To the best of our knowledge, our study is the first to measure IL-17 in rectal tissue in children with CMPA.

The expression of some Th2 cytokines was higher (IL-4, IL-10, IL-13) in the rectum than in the duodenum of patients with CMPA, with statistically significant differences, while IL-15 was higher in the duodenum. We did not find a clear explanation for the difference in expression of this subgroup of cytokines; however, we consider that additional studies should be carried out to clarify this.

Hakonarson et al. [19] similarly demonstrated the presence and autologous upregulation of Th2 type cytokines and their respective receptors in atopic asthmatic sensitized airway smooth muscle. IL-10, IL-4, and IL-13 are known to be potent stimulators of mastocytes and basophils.

In allergic individuals, a Th2 response will be favored, which will lead to eosinophil proliferation and thus increased production of IgE, resulting in severe reactions such as asthma or anaphylaxis [20].

The pathogenesis of CMPA remains poorly understood. Our study demonstrates that in children younger than 2 years, the allergic response follows the typical Th2 pathway but also a TH1 and Th17 response. Instead, we propose a mechanism whereby IL-17F triggers the immunological response in the rectum in children with CMPA, which further amplifies the Th2 response—and even the Th1 response—along with an increase in its cytokines, leading to the classic intestinal symptomatology including rectorrhagia, constipation, and abdominal distention.

The present study suggests the possible role of Th1, Th2, and Th17 cytokines in the pathophysiology of CMPA, and it represents a new strategy in the identification of novel therapeutic targets. This transverse study is limited to a Mexican population, and since diet is related to the function and expression of these cytokines, further studies are needed in other populations with a larger number of individuals, and with pertinent characterization of dietary patterns. The limitations of our study are the number of patients studied and the absence of a control group; however, the latter cannot be studied for ethical reasons.

Moreover, translational studies and mechanistic studies are required, since there is some evidence that cytokines could play a role in the pathogenesis of CMPA.

In addition, because of the poor understanding of the non-IgE-mediated CMPA pathogenesis, the diagnostic methods are scant and have low sensitivity and specificity.

Conclusion

In the present study, we analyzed differential gene expression of Th1, Th2, and Th17 cytokines in the duodenum and rectum in patients with CMPA less than 2 years old, and we observed that the immune response seems to be mediated by this profile of cytokine, with the rectum being the main affected site.

Acknowledgments We thank the Molecular Allergy Laboratory in Mexico City, especially Elizabeth Figueroa for all her support in the collection and processing of Specific IgE samples (Immunocap®).

Funding This work was supported by funds from the Inflammatory Bowel Disease Clinic at the National Institute of Medical Sciences and Nutrition.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval The present study was approved by the research and bioethics committee of the National Institute of Pediatrics in Mexico City. We complied with the declaration of Helsinki version 2008. Informed written consent was obtained from all patients' parents.

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