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ORIGINAL PAPER

Intestinal microbiota in nephrotic children treated with immunosuppressive agents

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ABSTRACT

Aim of the study: The analysis of the effect of immunosuppressive therapy on gut microbiota in pediatric patients with stable idiopathic nephrotic syndrome (INS) treated with different protocols of immunosuppressive therapy and examined the changes in their gut microbiota components along with accompanying clinical symptoms.

Material and methods: The study consisted of 44 children with INS divided into 3 groups according to treatment protocols (group A: 18 children on cyclosporine A [CsA], 9 of which receiving additional glucocorticosteroids [GCS]; group B: 17 children on GCS; and group C: 9 children on cyclophosphamide [CYC] and GCS), along with 20 healthy children serving as controls. Intestinal microflora was analysed with microbiological diagnostics based on KyberStatus and KyberMyk tests. Additional laboratory blood and urine tests were performed along with history data and clinical symptoms analysis. Study was approved by local Ethical Committee. All caregivers gave an informed consent for participation.

Results: Total number of bacterial colonies was significantly lower in group A ($p < 0.001$) and group B children ($p = 0.04$) when compared to the healthy controls. Group C children had a significantly lower number of Bifidobacterium colonies than the controls ($p = 0.01$), while number of Candida colonies were significantly higher in group A subjects than in controls ($p = 0.01$). No significant correlation between clinical symptoms reported by patients and the therapy used was found. Degree of disbiosis did was not related to the patients' complaints either.

Conclusions: Immunosuppressed INS paediatric patients who are in remission had significantly unfavourable changes in their intestinal microbiota. Patient on chronic CsA therapy showed higher degree of dysbiosis.

KEY WORDS:

dysbiosis, idiopathic nephrotic syndrome, gut microbiota, immunosuppressive therapy, microbiological diagnostic.

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INTRODUCTION

Animal models show that immunosuppressive treatment can lead to microfloral disturbances or intestinal dysbiosis [1]. The potentially harmful effect of long-term dysbiosis on the human organism is still under debate. Some authors postulate that the detrimental action is associated with the loss of the protective function of probiotics, an increase in metabolic toxins, digestion and absorption impairment, and immune system disturbances [2, 3]. On the other hand, it has been proven that a properly functioning immune system maintains the intestinal microflora in physiological equilibrium [4]. We know from human and animal studies that the physiological microflora protects the intestinal ecosystem from pathological colonization and carcinogenesis [5]. It works closely with gut associated lymphoid tissue (GALT) as an important component of the human immune system. It affects metabolic functions related to nutrition, immunological function and intestinal barrier protection. The protective bacteria reduce oxidative stress and affect the maturation of endothelium by producing short chain fatty acids, vitamins B and K, and butyric acid [6]. The acidophilic bacteria maintain the pH of the small intestine and regulates gastrointestinal motility by synthesizing anti-diarrheal postbiotics (acetic acid, propionic acid, and butyric acid) [6]. Intestinal bacteria are involved in digestion, absorption of electrolytes and trace elements, metabolism of bile acids, lipid metabolism, and metabolism of nitrogen compounds. Furthermore, microbiota regulates angiogenesis and the development of intestinal microcirculation.

The clinical significance of microbiota imbalance was described in patients with bowel disease (enterocolitis, irritable bowel syndrome, inflammatory bowel disease, and pseudomembranous colitis) and systemic disorders (atopic dermatitis, rheumatoid arthritis, and fungal infections), as well as primary and acquired immunodeficiency (after radiotherapy and chemotherapy or organ transplantation). These imbalances were related not only to the disease itself, but also to the immunosuppressive treatment. Intestinal dysbiosis could be a source of selected clinical symptoms observed in immunocompromised patients and may influence the course of the disease. Paediatric relapsing idiopathic nephrotic syndrome (INS) patients show gut microbiota dysbiosis, characterized by a decreased proportion of butyric acid-producing bacteria and lower faecal butyric acid quantities, concomitant with reduced circulatory regulatory T cells (Tregs) [7].

In order to broaden the clinical knowledge concerning this issue, and to study the impact of immunosuppressive therapy on gut microbiota, we conducted a cross-sectional study on children with INS treated with different protocols of immunosuppressants.

MATERIAL AND METHODS

PATIENT RECRUITMENT AND ETHICAL CONSIDERATIONS

The study was designed as a cross-sectional clinical analysis of the intestinal microbiota in 44 individuals with INS on immunosuppressive therapy with a stable clinical course of the disease along with 20 healthy individuals all aged 2–18 years. The diagnosis of INS was based on criteria of International Study of Kidney Disease in Children [8]. This study was approved by the Ethics Committee at the Polish Mother's Memorial Hospital Research Institute. Written informed consents were obtained from parents of all participants. The study comprised faecal sample analysis from all 64 participants after dividing them into 4 groups (Table 1). Representing group A were 18 children with INS (7 females, 11 males; aged 2–14 years) treated with oral cyclosporine A (CsA) at a mean dose of 3.7 mg/kg/24 h given in two doses; 9 of the 18 children received prednisone (GCS) at a fixed dose ranging 10–15 mg/m^2/48 h. Group B consisted of 17 children with INS (8 females, 9 males; aged 2–17 years) treated with oral prednisone at a dose not exceeding 40 mg/m^2/48 h. In group C, 9 children (3 females, 6 males; aged 3–12 years) were treated with cyclophosphamide (CYC) at a dose of 2.5 mg/kg/24 h. All 9 children in group C received prednisone at a dose of 30–40 mg/m^2/48 h. Finally, 20 healthy children (7 females, 13 males; aged 2–15 years) served as controls in group D.

The primary inclusion criterion was INS with immunosuppressive therapy with a stable clinical course of the disease (groups A, B, and C). Exclusion criteria included exacerbation of INS; antibiotic or probiotic usage within two months prior to the study; chronic or acute intestinal disease affecting the intestinal flora. Clinical data and history were collected by a specially tailored questionnaire filled out for each patient at the time of the study, and also 6 months after the study for group A individuals. The questionnaire described the INS course with a special regard to the relapses and treatment, other co-morbidities, antibiotic and probiotic intake, as well as gastrointestinal and neurological symptoms and skin involvement. The severity of symptoms was assessed by children and/or their caregivers. For group A, further blood and urine samples were also collected, with specific consideration

TABLE 1. Description of the study groups and healthy controls

Characteristic	Group A	Group B	Group C	Group D (control)
Number of participants	18	17	9	20 healthy participants
Treatment	CsA + GCS	GCS	CYC + GCS	–

CsA – cyklosporine A, GCS – glicocorticosteroids, CYC – cyclophosphamide

for extended cholesterol, triglycerides, and albumin concentration testing.

COLLECTION OF STOOL SAMPLES AND DATA ANALYSIS

The intestinal microflora from the subjects was analysed with microbiological diagnostics based on KyberStatus and KyberMyk tests, which are developed by the Institute for Microecology (Herborn, Germany) [9]. These methods provide qualitative and quantitative description of intestinal bacterial and fungal species. Both tests assessed colony forming units (CFU) of microbiota per 1 gram of biological material. The material consisted of faecal samples, which were transported in special containers provided by the Institute of Microecology, 60-190 Poznań, 10 Sielska St. Samples were sent to the laboratory immediately after collection. Delivery time did not exceed 3 days.

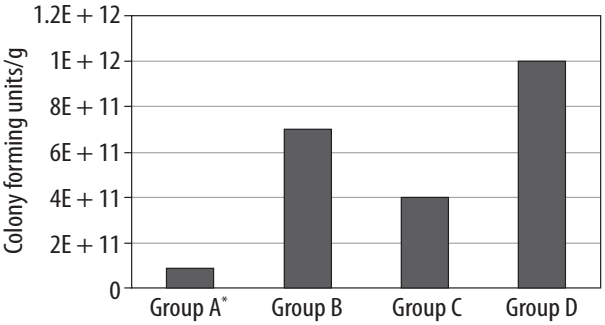
Collection of material was done from 8 different places after homogenizing the faecal sample collecting 0.25 g of feces for testing. The material collected was then placed in a 2,250 ml of sterile saline (10 : 1 dilution). The solution was vortexed and serially diluted with successive test tubes, obtaining a 10 : 8 series dilution.

For the KyberStatus test 50 µl of each dilution were plated onto enriched or selective agar media. Viable bacterial cell counts in feces were enumerated on the following media: 5% sheep blood agar (BioMerieux) for total bacterial count, Schaedler agar (Heipha) for anaerobic *Bacteroides*, DIC agar (Heipha) for *Bifidobacteria*, CPS® (BioMerieux) for *Enterococcus*, *Escherichia coli*, *Enterobacteriaceae* spp., and *Pseudomonas* spp., Endo's medium (Heipha) for *E. coli Biovare*, SPM® (Heipha) for anaerobic *Clostridium,* and Rogosa medium with peroxidase and TMB (Heipha) for detecting *Lactobacilli,* and determining the proportion producing H_2O_2.

The cultures were grown under appropriate conditions; the plates were incubated under aerobic (24 h) or anoxic (48 h) conditions at 37°C.

In order to determine the presence and quantify molds and *Candida* spp. in the faecal samples, KyberMyk test was carried out. 0.25 gram samples of feces were diluted in 2.5 ml of Trypsin-EDTA with 25 µl penicillin/streptomycin to inhibit bacterial growth. After homogenization, the solution was placed for 15 minutes in the incubator at 37°C. Then, 400 µl aliquot was transferred to 1.6 ml PBS for rinsing. Two separate volumes of 100 µl each were transferred from the PBS solution into 2 separate Sabouraud agar media with chloramphenicol (BioMerieux). Both plates were incubated for 48 h; one at 37°C for 48 h, while the other at room temperature. This allowed to distinguish mushrooms from pathologic fungi. Identification of *Candida* was done using the chromogenic CHROMagar Candida media (Becton Dickinson).

Additional laboratory tests for children treated with CsA included FBC, kidney function tests, and urinalysis. The CsA concentrations in blood were determined by EMIT (Synevo).



*A – CsA, B – GCS, C – CYC, D – healthy controls; * significantly different when compared to controls – group A: p < 0.001, group B: p = 0.04, group C: p = 0.3*

FIGURE 1. Maximum number of bacterial growth in the study groups

DATA ANALYSIS

The main parameters analysed were the differences in the quantities of particular strains of bacteria and fungi. Results obtained from each group were compared with other groups' results, including the control. Data obtained from the questionnaire was also analysed. Shapiro-Wilk test was used to assess the normality of distribution of variables. The median and 25–75 interquartiles defined the qualitative variables. Mann-Whitney test, Yates-corrected χ^2 test, Fisher's exact test, and Kruskal-Wallis test were used to evaluate differences between groups. Correlation was assessed by Spearman rank correlation coefficient. A p-value < 0.05 was considered significant. Calculations were done using STATISTICA 7.0 software.

RESULTS

MICROBIOTA

Total number of bacterial colonies

The total number of bacterial colonies was lowest in children treated with CsA and significantly different when compared with the healthy controls ($p < 0.001$). Children treated only with GCS also had a significantly lower number of colonies ($p = 0.04$), while the CYC-treated children did not show a significant difference when compared with the controls ($p = 0.3$). The significance of this data was confirmed by Mann-Whitney test ($p = 0.05$) as shown in Figure 1.

When individual study groups A, B, and C were compared with each other, the total number of bacterial colonies was significantly lower in group A – CsA in comparison to group B – GCS ($p = 0.007$), as well as when compared with group C – CYC ($p = 0.04$). There was no

TABLE 2. Number of bacterial and *Candida* colonies (CFU/g)

Colony	Group A			Group B			Group C			Group D (control)		
	Q1	Median	Q3	Q1	Median	Q3	Q1	Median	Q3	Q1	Median	Q3
Non-pathogenic *E.coli*	2×10^7	2.5×10^7	5×10^7	9×10^6	1×10^8	6×10^8	1×10^7	6×10^7	2×10^8	5×10^5	2×10^7	7×10^7
Enterococcus	4×10^5	2×10^6	6×10^6	3×10^5	4×10^6	4×10^7	2×10^4	1×10^6	4×10^6	2×10^4	1×10^5	2.5×10^7
Bifidobacterium	3×10^8	6.5×10^8	4×10^9	1×10^9	2×10^9	3×10^9	4×10^8	9×10^8	1×10^9	1.5×10^9	2×10^9	4×10^9
Bacteroides	2×10^9	3×10^9	4×10^9	2×10^9	4×10^9	6×10^9	8×10^8	2×10^9	4×10^9	2×10^9	3.5×10^9	5×10^9
Lactobacillus	2×10^4	4×10^5	2×10^6	2×10^4	1×10^5	2×10^6	1×10^5	1×10^6	8×10^6	8×10^4	2×10^5	1.5×10^6
Lactob. H_2O_2	0	0	9×10^4	0	0	2×10^5	0	0	8×10^4	0	1.5×10^4	7×10^5
Clostridium	0	3×10^4	3×10^5	0	0	1×10^5	0	2×10^4	8×10^5	0	0	5×10^4
Candida	0	0	5×10^4	0	0	3×10^3	0	0	0	0	0	0
Total number of bacteria, CFU/g	1×10^{10}	2×10^{10} (+)	4×10^{10}	2×10^{10}	1×10^{11} (+)	2×10^{11}	3×10^{10}	2×10^{11}	3×10^{11}	8.5×10^{10}	2×10^{11}	4×10^{11}

(+) significantly different when compared to controls

statistically significant difference regarding this variable between groups B and C ($p = 0.5$).

Detailed analysis of bacterial colonies

CYC-treated children showed a significantly lower number of *Bifidobacterium* colonies when compared with controls ($p = 0.01$), and also when compared with the GCS-only group ($p = 0.04$).

The number of *Bacteroides* sp., *Lactobacillus*, and H_2O_2 producing *Lactobacillus* sp. CFU in groups A, B, and C was not significantly different when compared with the controls. Likewise, the amounts of CFU of *E. coli* and *Enterococcus* sp. showed no significant differences between any of the study groups. Similar results were obtained regarding the *Clostridium* sp. content.

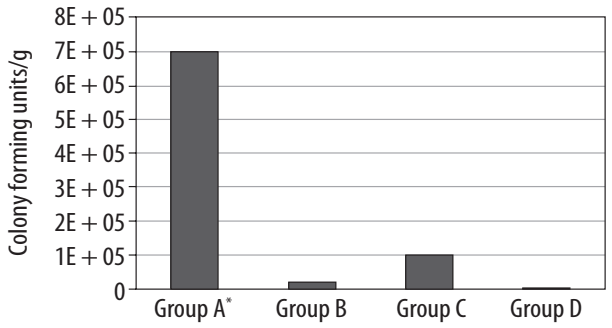
In the analysis of proteolytic bacteria and molds, we found no significant differences between children with INS and healthy children. However, there was a tendency for overgrowth of other proteolytic bacteria in the CsA group when compared with the controls ($p = 0.07$), with a similar tendency observed in the GCS group ($p = 0.06$). CFUs of *E. coli Biovare*, *Proteus* sp., and *Pseudomonas* sp. were comparable.

Fungi

Group (A) subjects (CsA) had a significantly higher amount of *Candida* sp. colonies when compared with the controls ($p = 0.01$) (Table 2, Fig. 2). Groups B and C did not show a significant difference when compared with the control group.

CLINICAL SYMPTOMS ANALYSIS AND LABORATORY TESTS

After analysing the clinical questionnaires, we found that all three groups of children with INS reported symptoms related to gastrointestinal tract, skin disorders, mood changes and anxiety, and tendency to nervous-



*A – CsA, B – GCS, C – CYC, D – healthy controls; * significantly different when compared to controls p < 0.05 – group A: p = 0.01; group B: p = 0.07; group C: p = 0.4*

FIGURE 2. Maximum number of *Candida* sp. growth in the study groups

TABLE 3. Percentage of children with proteolytic bacterial overgrowth

Proteolytic bacteria and mold		Group A (CsA)	Group B (GCS)	Group C (CYC)	Group D (control)
		n = 18 (%)	*n* = 17 (%)	*n* = 9 (%)	*n* = 20 (%)
E. coli Biovare	Normal growth	16 (89)	13 (76.5)	8 (89)	19 (95)
	Overgrowth	2 (11)	4 (23.5)	1 (11)	1 (5)
Proteus sp.	Normal growth	17 (94.4)	17 (100)	9 (100)	20 (100)
	Overgrowth	1 (5.6)	0	0	0
Pseudomonas sp.	Normal growth	18 (100)	17 (100)	9 (100)	18 (90)
	Overgrowth	0	0	0	2 (10)
Other proteolytic bacteria	Normal growth	13 (72.2)	12 (70.6)	8 (89)	19 (95)
	Overgrowth	5 (27.8)	5 (29.4)	1 (11)	1 (5)
Molds	Normal growth	16 (88.9)	13 (76.5)	8 (88)	20 (100)
	Overgrowth	1 (5.6)	4 (23.5)	1 (11)	0

CsA – cyklosporine A, GCS – glicocorticosteroids, CYC – cyclophosphamide; normal growth < 10^4, overgrowth > 10^4 CFU of microbiota per 1 gram of stool

TABLE 4. Clinical complaints in the idiopathic nephritic syndrome children as reported by their parents

Symptom		Group A (CsA)		Group B (GCS)		Group C (CYC)	
		n = 18	%	*n* = 17	%	*n* = 9	%
Gastrointestinal symptoms	Present	12	66.7	11	64.7	6	66.7
	Absent	6	33.3	6	35.3	3	33.3
Mood disorders and anxiety	Present	11	61.1	8	47.1	4	44.4
	Absent	7	38.9	9	52.9	5	55.6
Skin problems	Present	12	66.7	9	52.9	3	33.3
	Absent	6	33.3	8	47.1	6	66.7
Tendency to nervousness	Present	15	83.3	9	52.9	6	66.7
	Absent	3	16.7	8	47.1	3	33.3

CsA – cyklosporine A, GCS – glicocorticosteroids, CYC – cyclophosphamide; no statistically significant differences in these symptoms across the groups ($p > 0.05$)

ness. No significant differences were observed between different protocols of immunosuppression. Almost 67% of children treated with CsA complained of gastrointestinal symptoms (i.e. constipation, flatulence, dyspepsia, abdominal pain, compulsive hunger or irritability), with similar percentages of children in groups B (64.7%) and C (66.7%) suffering from such symptoms. Mood disorders and anxiety were reported by 60% of group A patients, and almost half of the patients in groups B and C. Skin problems appeared in about two-thirds, half, and third of the children in groups A, B, and C respectively. 83% of group A children showed tendencies to nervousness, compared to 53% in group B, and 67% in group C (Table 4). There were no statistically significant differences in these symptoms across all three INS groups ($p > 0.05$).

Selected biochemical parameters were analysed in the INS groups. Some children in group B had elevated levels of WBCs. Cholesterol levels were also elevated in some children from all three groups. The rest of the laboratory test values were within the laboratory norms (Table 5). All patients were in remission, and no acute kidney injury was present during the analysis.

DISCUSSION

The main finding of our study is that immunosuppressive therapy in patients with INS may induce unfavourable changes in gut microbiota. Different protocols had a slightly different influence on specific microbiota profile. We postulate that this observation might be important for patients from a clinical point of view.

Microorganisms inhabiting the human gastrointestinal tract are in a close symbiotic relation with the human organism. Microbiota perform a variety of functions necessary to maintain homeostasis [2, 5]. Gut microbiota affect the immune system by creating an acidic profile, hydrogen peroxide, antibiotic-like substances, bacteriocins, function peptides, proteases, and nutrients. They also play a role in the local immune system by stimulating GALT to produce sIgA. Some authors reported a favourable effect of the digestive tract physiological flora on carcinogenesis [2, 5, 10]. There is definitely an interdependency between intestinal microorganisms and the immune system. Gut bacteria stimulate development and proper function of immune system components, where-

TABLE 5. Selected clinical and biochemical parameters in the study groups

Parameter	Group A	Group B	Group C
Demographics	18 Children: 7 F; 11 M Age: 2–14 years	17 Children: 8 F; 9 M Age: 2–17 years	9 Children: 3 F; 6 M Age: 3–12 years
Course of the disease	Steroid-dependent (16) Steroid-resistant (2)	Steroid-sensitive	Steroid-dependent
Number of relapses	> 3	< 2	> 3
CsA dose (mg/kg/24 h)	3.7	–	–
GCS dose (mg/m^2/48 h)	10–15 (only 9 children)	30–40	30–40
CYC dose (mg/kg/24 h)	–	–	2.5
Haemoglobin (mg/dl)	12.9 (11–15.1)	13.9 (11.7–17.1)	13.35 (12–14.2)
Haematocrit (%)	38 (33–43.5)	40.3 (35.6–45.8)	39.9 (34–47)
Creatinine (mg/dl)	0.46 (0.15–0.67)	0.43 (0.2–0.78)	0.37 (0.32–0.46)
Urea (mg/dl)	24 (13–43)	28 (18–35)	29 (22–36)
Uric acid (mg/dl)	4.7 (3–7.4)	3.5 (2.9–8.3)	3.9 (3.8–4)
Cholesterol (mg/dl)	186 (159–310)	198 (134–261)	202 (158.4–247)
Thrombocytes (mm^{-3})	381,000 (262,000–515,000)	380,500 (226,000–458,000)	332,000 (208,000–396,000)
WBCs (mm^{-3})	7,290 (5,330–1,2350)	10,140 (6,280–20,000)	7,140 (4,840–7,470)

CsA – cyklosporine A, GCS – glicocorticosteroids, CYC – cyclophosphamide; data is given as the median, quartile values are given in brackets

as the immune system protects against translocation of bacteria to the bloodstream, where these organisms may cause disease [6, 11].

The relationship between intestinal microflora and GALT was proven in animal studies. Animals deprived of microbiota in a sterile environment had undeveloped GALT and only single B lymphocytes [12, 13]. Normal development of GALT was restored after colonization by physiological microflora. Specific intestinal microflora stimulated production of natural antibodies which are part of non-specific immunity. Probiotics showed the ability to recover intercellular tight junctions disrupted by pro-inflammatory cytokines (TNF-α, INF-g) [12, 13].

In addition to their immunological effects on the host organism, gut microbiota also play critical roles in certain metabolic processes. Intestinal microorganisms influence host metabolic function by producing vitamins B_1, B_2, B_{12}, and K, which are absorbed from the gastrointestinal tract. Microbiota are involved in host lipid metabolism by converting cholesterol to coprostanol, degrading bile acids and converting bilirubin to urobilinogen. Changes in microbiota may interfere with the metabolic pathways of these substances leading to long-term clinical consequences.

Changes in intestinal flora composition depend on environmental factors and acquired diseases. In acute diseases, microbiological balance is restored shortly after detrimental factors subside. In chronic diseases, the recovery of proper intestinal flora is more difficult. Adverse effects of antibiotics and selected immunosuppressive drugs on intestinal flora were observed in animal models [10, 14]. Negative consequences of CYC, CsA, and GCS administration on the microbiota have been proven in previous studies on mice [1, 15]. In our study, we confirmed an imbalance of microbiota induced by immunosuppressive drugs. A diminished total number of bacterial colonies was observed in CsA and GCS-only-treated subjects.

There are few studies examining disturbances of intestinal flora during immunosuppressive treatment, and those available are comprised of case reports and animal model studies [16, 17]. Kaur *et al.* showed that CsA disturbed intestinal microbiota in mouse model increased the risk of *Clostridium difficile* (proteolytic bacteria) colonization of the gastrointestinal tract [17]. An increase in *Clostridium difficile* occurred with diminished protection of commensal bacteria with a particular decrease in *Bifidobacterium* colonies [18]. In our study, we assessed *Clostridium* sp. growth and reported no significant increase in the *Clostridium* sp. colonies in any of the groups.

Gut colonization with *Candida* is a common complication following any antibiotic therapy. In our study the CsA-treated group showed significant increase in *Candida* colonies, without antibiotic intake. It might be related to the significant decrease in total number of protective bacteria colonies, as reported above.

In children treated with CYC, there was a decrease in the number of *Bifidobacterium* colonies. However, the low number of patients in this group might have influenced this observation. On the other hand, the CYC therapy protocol is shorter than the one for CsA, thus the changes might be less prominent over time. Samonis *et al.* reported increased gastrointestinal tract colonization by yeasts in mice on CYC with neutropenia [14]. Shenderov *et al.* found a negative influence of CYC on gut microbiota in rats [19]. Disturbances in intestinal flora were found only

when neutropenia occurred. Administering probiotics to re-establish normal gut microbiota resulted in resolution of neutropenia, thus the authors postulated that the severe disturbances of intestinal flora by CYC use might be related to neutropenia [19].

Our study did not confirm a significant increase in proteolytic bacterial colonies, although groups A and B showed overgrowth tendencies regarding in some proteolytic bacteria as we have mentioned in the results section. These bacteria may increase fermentation processes in the gastrointestinal tract and lead to inflammation [18, 20]. The GCS influence on microbiota has been described in previous studies. GCS may trigger inflammation of gastric mucosa, which affects the digestion process. Treatment with GCS increases the risk of carbohydrate metabolism disturbances. It may be related to hunger attacks and craving for sweets reported in our questionnaires. Siew *et al.* investigated the influence of GCS on intestinal flora in the course of nonspecific enteritis. In Crohn's disease they found a decrease in *Bacteroides* and an increase in invasive *E. coli* colonies. In ulcerative colitis, a low number of *Bifidobacterium* colonies was observed [21]. On the other hand, the authors reported that GCS administered together with probiotics may improve microbiota profile by decreasing intestinal mucosa inflammatory status. Microbiota status, pH in the colon, GCS dosage and method of administration were factors influencing effectiveness of the therapy of colitis [14, 22, 23]. Siew *et al.* also found that GCS together with probiotics at a dose of 3.6 billion CFU, promoted synthesis of anti-inflammatory cytokines (IL-10), inhibited synthesis of pro-inflammatory cytokines (IL-6, IL-12p40) and TLR expression by intestinal dendritic cells [21]. There are no such data in children with INS.

GCS may also disturb gut microflora by increasing susceptibility to mycosis colonization. The tendency to increase in yeast-like fungi in steroid-treated patients may be due to a decrease in the total number of bacterial colonies. This was confirmed in a mouse model [15]. We did not confirm this hypothesis in vivo in children.

Tsuji *et al.* looked at the relationship between gut microbiota dysbiosis and INS from a different perspective. They examined relapsing INS patients before treatment, and found out that these patients showed gut dysbiosis along with a reduced count of circulatory Tregs [7]. We did not measure the circulatory Treg count, and we compared different treatment protocols in patients during remission, so the two studies cannot be compared to full extent. However, looking at the results from both studies, we suppose that the changes in the gut microbiota can be attributed to the immunosuppressive treatment, as well as the course of the disease itself.

We think that a future study that can analyse a wider variety of INS patient groups (e.g. INS patients on immunosuppressive therapy, INS patients not receiving immunosuppressants, and healthy controls) would be extremely valuable, as such a study would have the potential to show to what extent each factor (the disease itself vs. the immunosuppressive therapy) affects the gut microbiota in those children.

One of the limiting factors to our study was collecting eligible patients. The population of children with INS consists mostly of patients with steroid sensitive, frequently relapsing clinical course. The relapses are usually triggered by upper respiratory tract infections with frequent antibiotic use. Due to this fact, collection of antibiotic-naive patients (to exclude their influence) was a challenge. Thus, restricted inclusion criteria led to a rather low number of participants in our study groups. Moreover, the smaller pool of patients restricted the ability to collect enough subjects receiving only one drug. Thus, half of the children in the CsA group were also receiving GCS, while all the CYC group children received GCS as well. On the other hand, INS children may have disturbances in microbiota because of more frequent antibiotic usage. Long-term antibiotic usage has proven its influence, but there are conflicting data how long the influence is sustained. In our study we relied on other author observations and suggestions from lab test manufacturers to exclude possible changes induced by recent antibiotic administration.

The method of microbiota assessment was based on non-invasive stool testing in order to avoid unnecessary harm to the immunosuppressed patients. However, it gave results of the average composition of bacterial flora, without distinction between different parts of the gastrointestinal tract [6, 11].

Despite these limitations, our study has its own robust aspects. All patients were in remission during the analyses, with no acute kidney injury and without any chronic or acute intestinal disease either. No antibiotics or probiotics were used by any of the subjects within two months prior to the study.

CONCLUSIONS

In conclusion, our study showed that immunosuppression used in childhood INS had unfavourable influence on the intestinal microbiota. The most severe changes were detected in the CsA-treated subjects as they had the lowest total number of bacterial colonies, and the largest growth of *Candida* sp. colonies. Total number of bacterial colonies was significantly diminished in the GCS-only group as well, while CYC-treated subjects had the lowest number of *Bifidobacterium* colonies. Clinical gastrointestinal symptoms were not significantly attributed to a specific immunosuppressive agent or to the degree of dysbiosis.

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DISCLOSURE

The authors declare no conflict of interest.

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