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ORIGINAL PAPER

# Dependence of Colonization of the Large Intestine by *Candida* on the Treatment of Crohn's Disease

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Abstract

The aim of this study was to determine if there are quantitative differences in *Candida* fungi between pediatric patients with Crohn's disease (before and after exclusive enteral nutrition (EEN), and the biologic therapy with anti-tumor necrosis factor alpha – (IFX)), and healthy controls. DNA was isolated from fecal samples and PCR was used to determine the number of fungal cells. Both therapeutic interventions resulted in a statistically significant decrease in Pediatric Crohn's Disease Activity Index. The numbers of *Candida* decreased during both therapeutic intervention but the difference was statistically significant for the IFX intervention only ($p = 0.045$). Moreover, fungi population in both study groups declined during intervention when compared to the control group but the difference was significant before treatment only in the IFX group ($p = 0.013$). The total distribution of *Candida* with both IFX and EEN as well as in the control group differed significantly ($p = 0.01$) before treatment only. No correlation between the numbers of *Candida* and disease activity as well as the following biochemical parameters: serum iron concentration, protein or glucose level were found. It cannot be ruled out that, in combination with genetic and immunological disorders, fungi can contribute to the initiation of the disease process and perpetuation of active inflammation.

Key words: Crohn's disease; children; gut microbiota; biological treatment

## Introduction

Crohn's disease (CD) together with ulcerative colitis belongs to the group of disorders known as inflammatory bowel diseases (IBD). The etiology of Crohn's disease still remains not fully explained, although the changes in the composition and distribution of intestinal microbiome seem to play a crucial role in the development and persistence of inflammation in the gastrointestinal tract (Kostic et al. 2014; Scarpellini et al. 2015). It is still under debate if the changes in the microbiome in IBD are a cause or a consequence of inflammation.

Pediatric-onset CD is on the increase worldwide (Benchimol et al. 2017; Ng et al. 2017). Many young patients present with an extensive and aggressive course of the disease, which is a real therapeutic challenge. Treatment of CD is a complex, multistage process and depends on the type and clinical activity of the illness. ECCO/ESPGHAN guidelines recommend the usage of exclusive enteral nutrition (EEN) as a first-line therapy to induce remission in pediatric patients with mild to moderate CD (Ruemmele et al. 2014). EEN used as induction therapy should last from six to eight weeks and is usually based on standard, liquid, polymeric formulas. During this period, any other types of food are withdrawn and patients receive liquid diet orally or through a nasogastric tube, which covers full caloric and nutritional demand adjusted to the patient's requirements. Maintenance of remission can be achieved with thiopurines or methotrexate. This therapeutic approach is called conventional therapy.

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The biologic therapy with anti-tumor necrosis factor alpha (anti-TNFα) is recommended for treatment of patients with moderate to severe CD who didn't respond to conventional treatment (with thiopurines or methotrexate). Infliximab (IFX) is the biologic agent used most often as a first-line treatment in the pediatric population. Induction doses of 5 mg/kg are given intravenously in 0, 2 and 6 weeks mode, followed by maintenance infusions every eight weeks (Hyams et al. 2007).

The majority of IBD therapies are focused on controlling inflammation, but the question of how or whether the clinical status of patients is related to the microbiome remains unanswered. The human gastrointestinal tract contains a wide range of archaea, prokaryota, eukaryota and viruses.

As mentioned above, studies are available in the literature that concern changes in the bacterial flora of the human gastrointestinal tract in the course of IBD; however, there are few articles describing the study of anti-*Candida* antibodies and methods based on the fungi cultures (McKenzie et al. 1990; Standaert-Vitse et al. 2006; Standaert-Vitse et al. 2009). Hence, the primary objective of the present study was to determine if there are quantitative differences in *Candida* fungi (by quantitative real-time PCR (qPCR)) between pediatric patients with CD and healthy controls. Another aim of this study was to compare the quantitative differences in *Candida* fungi in the newly diagnosed CD during EEN and those who were qualified for biologic therapy and to find out whether these differences correlate with the selected biochemical and clinical parameters.

## Experimental

### Materials and Methods

**Patients.** We performed a single-center prospective study to examine the *Candida* population in CD patients hospitalized in University Children's Hospital in Krakow, Poland.

Patients aged 2 to 18 years diagnosed with CD according to the revised Porto criteria (Levine et al. 2014) were enrolled into two study groups.

The study protocol was approved by Jagiellonian University Ethics Committee – the decision no. 122.6120.68.2015. The informed consent was signed by patients' parents or legal guardians and by patients themselves if above 16 years of age.

Group 1 consisted of newly diagnosed children, who received EEN for the induction of remission. In this group, we collected two stool samples: the first one (N1) before any therapeutic intervention and the second (N2) 2 to 4 weeks after completing EEN. In a group 2, there were CD patients who failed to respond or stopped responding to conventional maintenance treatment (with thiopurines or methotrexate) and therefore were qualified for biologic therapy. Stool samples were collected prior to the first dose of IFX (Remsima®, Celltrion Healthcare, Incheon, Korea) (B1) and then 4 weeks after the 3rd induction dose (B2).

The exclusion criteria comprised the following: 1) age of patient below two years old or above 18 years of age; 2) treatment with antibiotics (including antimycotic antibiotics) and probiotics during the period of 3 months before collecting the stool sample; 3) confirmed infections of the gastrointestinal tract; 4) any active neoplastic diseases (particularly of the gastrointestinal tract); 5) confirmed immunodeficiency.

The control group consisted of healthy children who didn't meet the exclusion criteria. In this group, we collected one stool sample.

**Tests.** In all CD patients, we routinely checked hematological and biochemical parameters, collected stool samples and calculated the Pediatric Crohn's Disease Activity Index (PCDAI). All these tests were carried out at the University Children's Hospital in Krakow, Poland.

The stool samples were delivered to the Chair of Microbiology of the Jagiellonian University Medical College in deep-freeze conditions (–70°C).

**DNA extraction from the stool samples.** The frozen samples were thawed, precisely weighed (about 0.1 g of stool sample was used) and homogenized in 0.1 ml of saline. DNA extraction from all samples was performed using the Genomic Mini AX Stool Spin Kit (A&A Biotechnology, Gdańsk, Poland), according to the manufacturer's recommendations, with our own modification (Gosiewski et al. 2014; Salamon et al. 2018). After lysis of bacterial and fungal cells with lysozyme (Sigma-Aldrich, Poznań, Poland) (1 mg/ml) and lysostaphin (Sigma-Aldrich, Poznań, Poland) (0.1 mg/ml), the samples were incubated at 37°C for 20 min. Next, 200 µl 75 mM NaOH (Avantor Performance Materials, Gliwice, Poland) was added and the samples were incubated at 95°C for 10 min. After incubation, the samples were microcentrifuged (12 000 rpm, 10 min), supernatants were removed, and the pellets were resuspended in 500 µl of the buffer supplemented with β-mercaptoethanol (Sigma-Aldrich, Poznań, Poland). For each sample, lyticase (Sigma-Aldrich, Poznań, Poland) was added (0.1 mg/ml). The samples were incubated at 37°C for at least 30 min and microcentrifuged (12 000 rpm, 10 min). The next steps of DNA extraction were carried out according to A&A Biotechnology's procedure.

**Quantitative real-time PCR (qPCR).** *Candida* spp. in the fecal samples were quantified by qPCR, as described by Gosiewski et al. (2014). To detect specific DNA sequences, ready-to-use JumpStart TaqReadyMix (Sigma-Aldrich, Poznań, Poland) kit, fluorescently

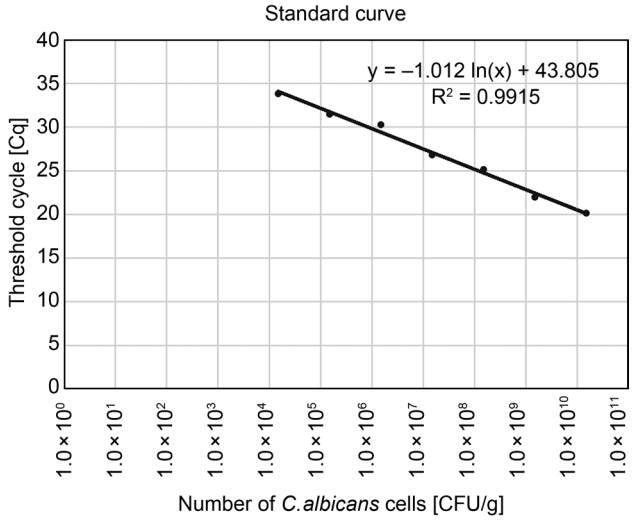


Fig. 1. A RT-PCR standard curve by plotting the threshold cycle (Cq) versus the number of *C. albicans* ATCC10231 (CFU/g). The DNA was amplified with the primers labeled with FAM.

FAM dye labelled probe (FAM-5'-TTAACCTAC-TAAATAGTGCTGCTAGC-3'-BHQ1) and pairs of specific primers (Genomed, Warszawa, Poland): (F) 5'-TTGGTGGAGTGATTTGTCTGCT-3; (R) 5'-TCTAAGGGCATCACAGACCTG-3' (Genomed) for *Candida* were used (Sugita et al. 2012). A standard curve was prepared. DNA from the given numbers of C. *albicans* ATCC10231 was added in serial dilutions from $10^1$ to $10^7$ cells (in a volume of 1 ml saline ~ 1 g) to a series of qPCRs. The reactions were carried out in a CFX96 thermocycler (BioRad, California, USA). A standard curve from these data is shown in Fig. 1. Detection and quantitation were linear over the range of the DNA concentrations examined. To determine the number of *Candida* cells, the fluorescent signals detected from DNA of stool samples (in duplicate) in the linear range of the assay were averaged and compared to the standard curve.

**Statistical analysis.** Descriptive statistics were calculated for quantitative variables. In the case of variables following a normal distribution, means and standard deviations were presented. For variables demonstrating distribution other than normal, medians and interquartile ranges were used. The Mann-Whitney test was used to compare the differences between the two study groups and control. The Kruskal-Wallis test was applied for comparisons for all study groups. In the latter case, multiple comparisons of mean ranks for all groups were performed in order to assess the differences between pairs of groups. The correlation between variables was assessed with Spearman's rank correlation coefficient. Statistical analysis was carried out with the Statistica 13.1 (StatSoft, Inc. Tulsa, Oklahoma, USA) software.

## Results

A total of 61 patients were enrolled in this study. Table I contains the baseline patient characteristics. The control group consisted of eight girls and nine boys, aged on average 140.76 months (± 34.58). Both therapeutic interventions resulted in a statistically significant decrease in disease activity assessed according to PCDAI. In group 2, the mean PCDAI was 47.5 points (ranged from 5 to 60 points) before induction therapy and decreased to a mean of 9.04 (ranged from 0 to 20) points ($p = 0.00$). In group 1, the mean initial PCDAI was 32.03 points (ranged from 0 to 65) and dropped to a mean of 5.93 (ranged from 0 to 57.5) points ($p = 0.00$).

The DNA sequences isolated from all 139 fecal samples were analyzed using qPCR. The presence of *Candida* DNA was assessed quantitatively by qPCR (Fig. 1 and Fig. 2).

The numbers of *Candida* decreased during the therapeutic intervention in both groups (Fig. 2). This

Table I
Baseline patient characteristics.

| Characteristics | Biologic therapy – IFX (n = 13) | EEN (n = 48) | Control group (n = 17) |
|---|---|---|---|
| Male:Female, n (%) | 7 (54%):6 (46%) | 29 (60%):19 (40%) | 9 (53%):8 (47%) |
| Age at diagnosis, months; mean (± SD) | 137 (± 48.15) | 160.27 (± 37.11) | N/A |
| Age at initial treatment, months; mean (± SD) | 157.15 (± 45.16) | 160.27 (± 37.11) | N/A |
| Weight, kg; mean (± SD) | 41.97 (± 16.3) | 40.93 (± 14.05) | 42.8 (± 17.2) |
| Height, cm; mean (± SD) | 149.95 (± 20.31) | 155.3 (± 19.1) | 148.7 (± 18.8) |
| BMI, kg/m$^2$; mean (± SD) | 17.89 (± 3.62) | 16.4 (± 2.92) | 18.3 (± 3.8) |
| PCDAI-1; mean (± SD) | 47.5 (± 16.43) | 32.03 (± 15.01) | N/A |
| PCDAI-2; mean (± SD) | 9.04 (± 6.5) | 5.93 (± 11.36) | N/A |

EEN – exclusive enteral nutrition; N/A – not applicable; PCDAI (Pediatric Crohn's Disease Activity Index): 1 – prior to therapeutic intervention, 2 – after therapeutic intervention

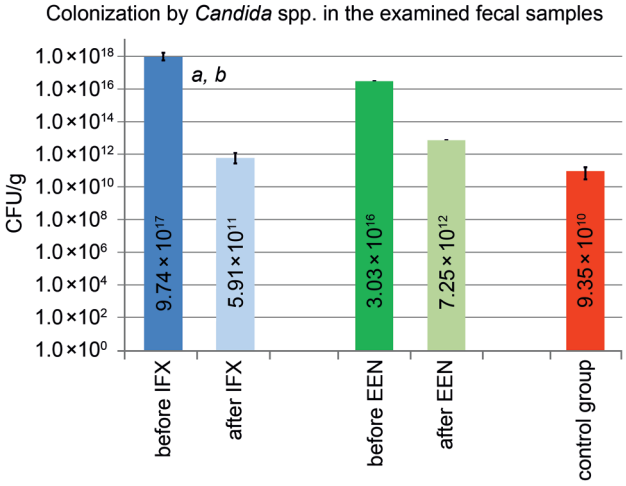


Fig. 2. Quantitative assessment of fungi of the genus *Candida* using qPCR in the stool of patients with CD before and after biologic (IFX) and exclusive enteral nutrition (EEN) treatments and control group.

a – significant differences between children with CD and the control group; b – significant differences between children with CD before and after biologic treatment.

difference was statistically significant in group 2 (IFX intervention) ($p = 0.045$) but not in a group 1 (EEN intervention) ($p = 0.626$). Additionally, fungi population in both studied groups declined during intervention when compared to the control group but the difference was significant before treatment only in IFX group ($p = 0.013$) and in the EEN group, it was close to statistical significance ($p = 0.056$) (Fig. 2).

The total distribution of *Candida* in the large intestine contents before treatment with both IFX and EEN as well as in the control group differed significantly ($p = 0.01$). The total fungal distribution after treatment in the three groups was statistically insignificant ($p = 0.39$).

We didn't find any correlation between the numbers of *Candida* and disease activity as well as with such biochemical parameters as serum iron concentration, protein or glucose level.

## Discussion

The increasing occurrence of Crohn's disease and the decreasing age of patients stimulate researchers to find out the causes behind this illness (Benchimol et al. 2017; Ng et al. 2017). Although, until now, it has not been possible to associate a particular microorganism with CD etiology, microbial participation is still considered crucial, besides genetic and immunological disorders, for induction or intensification of inflammation in the gastrointestinal tract (Gosiewski et al. 2012; Wright et al. 2015). However, there is still insufficient knowledge concerning the role of fungi in the course of CD, as well as the impact of the treatment on gastrointestinal colonization with fungi of the genus *Candida* in IBD patients.

Research by Sokol et al. (2017) demonstrated a significant increase in the *Candida albicans* population size in patients with IBD in the period of disease exacerbation compared to the period of remission and healthy people. However, the authors did not analyze the influence of the treatment type on the observed change in mycobiota. Similar results were obtained by Standaert-Vitse et al. (2009) where CD patients were more frequently and more heavily colonized by *C. albicans* than the patients in the control group. As it was found in our study, the group undergoing biologic therapy showed significantly higher colonization with *Candida* spp. when compared to healthy children (Fig. 2). Additionally, the patients qualified for biologic therapy exhibited higher numbers of *Candida* fungal cells than the children qualified for nutritional treatment (Table I, Fig. 2); but, the difference between them was not statistically significant. It has to be noted that the clinical activity of the disease in patients qualified for biologic therapy was greater as compared to the group treated with EEN. A further difference was the disease duration, which was longer in the IFX group. It is also telling that these patients stayed longer in the hospital for anti-inflammatory and immunosuppressive treatment and also, in the past, could have undergone antibiotherapy more often. This is an implication that, with the increase in the activity of the disease and its duration, the numbers of *Candida* fungi also increased, which was also confirmed by Li et al. (2014). Moreover, the researchers found that in the inflamed colonic mucosa *Candida* fungi were more numerous than in non-inflamed areas and that the number of fungal cells correlated with overexpression of proinflammatory cytokines, i.e., TNF-α and IFN-γ. It might have been the cause behind the loss of tolerance to commensal fungi among the patients with CD, which resulted in immune disorders as a consequence of continuous induction of an inflammatory response (Li et al. 2014). A confirmation of this hypothesis can be the study by Iliev et al. (2012), which found that mice with Dectin-1 deficiency (it's a receptor responsible for recognition of fungal β-glucan) are substantially more susceptible to developing intestinal inflammation in comparison with mice without the gene knockout encoding this receptor. Furthermore, animals with disorders within Dectin-1 triggered the release of excessive amounts of TNF-α, IFN-γ and IL-17 against fungi within intestinal microbiota, including *Candida* spp. It is also worth noting that administering of antimycotic fluconazole to mice resulted in alleviating the symptoms of the disease (Iliev et al. 2012). These observations could confirm our results, in which the number of *Candida*, before the start of biologic therapy, were significantly higher than

two weeks after its completion ($p = 0.045$) (Fig. 2). The strong anti-inflammatory activity of IFX enables the healing of the intestinal mucosa, which has probably reduced the gastrointestinal colonization with fungi. This effect was less visible in the case of EEN treatment when the reduction in the number of fungi was on the borderline of significance ($p = 0.056$) (Fig. 2).

Zwolinska-Wcislo et al. (2009) demonstrated, in an animal model, similar observations as regards the effectiveness of treatment of patients with inflammatory bowel diseases using antifungal agents and a significant clinical improvement following treatment with fluconazole.

Our research demonstrated that the IFX therapy translated into a statistically significant reduction in the number of fungi of the genus *Candida*, which following induction therapy was comparable to the number of fungi colonizing the gastrointestinal tract in healthy children (Fig. 2).

A large number of *Candida* fungi in newly diagnosed patients (EEN group) and those with very high disease activity (IFX group) may be the result of a long-term disease process but may also indicate the participation of fungi in the pathogenesis of CD. It cannot be ruled out that in combination with genetic and immunological disorders, fungi can contribute not only to the initiation of the disease process but also play a role in maintaining an active inflammation. None of the applied therapeutic interventions has a documented direct effect on mycobiome. However, both methods of treatment have a proven effect on the so-called mucosal healing. It can be presumed that the reduction in the number of *Candida* is an indicator of recovery and improvement of the defensive role of the mucosal barrier.

This study provides additional information to the multifactorial nature of CD and may contribute to the modification of therapeutic approach.

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**Conflict of interest**

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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