Ph.D. Thesis

ADVERSE EVENTS ASSOCIATED WITH MANAGEMENT OF INFLAMMATORY BOWEL DISEASES: INFECTIONS AND SIDE EFFECTS

Anita Bálint M.D.

Supervisor: Tamás Molnár, M.D.,Ph.D, med.habil

1st Department of Medicine

University of Szeged

Szeged

2014

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1. ABBREVIATIONS

ApoA1: apolipoprotein A1

BMD: bone mineral density

BMI: body mass index

cAMP: Cyclic adenosine monophosphate

CD: Crohn's disease

CD40: cluster of differentiation

CDAI: Crohn's Disease Activity Index

CDP: *Clostridium difficile* positivity

CMV: cytomegalo virus

CRP: C-reactive protein

CyA: cyclosporine A

DHEA : dehydroepiandrosterone

DXA: Dual-energy X-ray absorptiometry

EIM : extraintestinal manifestations

ELISA: enzyme-linked immunosorbent assay

ESR: erythrocyte sedimentation rate

FC: faecal calprotectin

HDL: high-density lipoprotein

IBD: inflammatory bowel diseases

IBS: irritable bowel syndrome

LDL: low-density lipoprotein

LFA: lateral flow assay

MMP-9: matrix metalloproteinase-9

pMayo score: partial Mayo Score

PTH: parathyroid hormone

TNF-α : tumor necrosis factor

TSH: thyroid stimulating hormone

UC: ulcerative colitis

VZV: varicella zoster virus

2. LIST OF FULL PAPERS RELATED TO THE SUBJECT OF THE THESIS

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III. Farkas K*, Bálint A*, Valkusz Z, Szepes Z, Nagy F, Szűcs M, Bor R, Wittmann T, Molnár T. Bolus administration of steroid therapy is more favorable than the conventional use in preventing decrease of bone density and the increase of body fat percentage in patients with inflammatory bowel disease. J Crohn Colitis. 2014;8(9).992-7. IF: 3.562

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Number of full publications:	21
Impact factor related to the thesis:	10.54
Cumulative impact factor:	30.53

4. SUMMARY

Introduction. Inflammatory bowel diseases are chronic, multifactorial conditions characterised by immune-mediated inflammation of the gastrointestinal tract. Management of IBD represents a lifelong medical care as the disorder itself is chronic and permanent with periods of relapses and remissions. However, adverse events can modify conventional treatment of IBD. The **aim** of the thesis was to assess special situations in IBD as infections and side effects: (i) to assess *Clostridium difficile* infection among our outpatients with IBD in relapse, (ii) to assess *Blastocystis sp.* infection and skin manifestations connected it, (iii) to assess efficacy and safety of influenza vaccination, (iv) to compare bolus or conventional tapering of methylprednisolone for 12 weeks , and (v) to assess serum cholesterol, triglyceride and creatinine levels before, during, and after CyA therapy. **Patients and Methods.** We carried out 5 clinical studies connected to the topic. In the Study I, 90 outpatients with IBD who relapse were enrolled prospectively. Clinical data of patients were assessed, and stool cultures; in addition, blood and stool samples were obtained for the determination of serum biochemical factors, FC and faecal MMP-9. IBD patients in clinical remission were selected as a control group. In second study data of 80 patients with confirmed positive *Blastocystis* sp. infections were assessed retrospectively. Gastrointestinal and dermatological symptoms and the results of routine biochemical and haematological blood tests of enrolled patients were collected and analysed. In Study III, 156 immunocompromised IBD patients were vaccinated. 53 patients (control group) refused vaccination. Split virion vaccine and whole virion vaccine were used. Serum samples were obtained for pre- and postimmunisation antibody titres to influenza vaccine (A/California/7/2009 [H1N1], A/Victoria/361/2011 [H3N2], B/Wisconsin/1/2010–like B/Hubei-Wujiagang/158/2009). In Study IV, 19 IBD patients received intravenous methylprednisolone of 1 mg/kg for 5 days tapered by 4 mg per week. Patients were prospectively randomized in two groups. In "conventional" group (I) steroids were given daily. In "pulse" group (II) weekly dose of steroids were given on special days of the week. The BMI was measured before and after the corticosteroid therapy. Blood samples were collected to assess glucose level, electrolytes, cholesterol and triglycerides levels, inflammatory parameters, cortisol, osteocalcin and crosslaps

values. Total body composition analysis was performed at the beginning and at the end of the steroid therapy. In the last study, clinical data and serum cholesterol, triglyceride, creatinine levels of 72 patients were analysed and compared to a control group treated with infliximab. **Results.** We found that 56.6% of patients had positive microbiological results. *Clostridium difficile* A and B Toxins were verified as positive in 92.1% of these cases. Statistical analysis showed a significant difference between FC and MMP-9 values in patients in relapse and remission, but not in *Clostridium difficile* positive and negative cases. Our results revealed an association between previous antibiotic use and *Clostridium difficile* positivity. We discovered that 11.25% of our enrolled patients exhibited skin manifestations associated to *Blastocystis* sp., mainly on the females. The occurrence of *Blastocystis* sp. was 6% in symptomatic patients who required medical attendance in the time period between 2005 and 2013. In third study, postimmunisation titres of both influenza subtypes increased significantly after the administration of split virion vaccines compared to the controls and to those who received whole virion vaccine. The antibody titres of Influenza B also increased significantly in patients immunised with split vaccine and treated with anti TNF-α therapy. No serious side effects developed occurred after influenza vaccination, and the influenza-like symptoms did not differ significantly between vaccinated vs. control patients. The relapse of the disease was observed in only 10% of the patients, and was more common in vaccinated than in control subjects. In study IV, in Group I, BMI increased, total body bone density decreased significantly at the end of the steroid therapy. Body fat percent showed a tendency to be higher at the end of steroid therapy in Group I. Cholesterol level increased significantly in Group I patients. The decrease in serum cortisol level was more remarkable in Group I vs. Group II after steroid therapy. Less side-effect occurred in Group II vs. Group I. Lastly, in Study V elevated cholesterol levels were detected in 47.2% of the patients. Serum cholesterol levels were significantly increased during and after discontinuation of cyclosporine therapy compared to the time before use of the drug. However, cholesterol levels measured during cyclosporine therapy were significantly higher compared to the time after its discontinuation ($p<0.001$). Patients with drug-related side effects showed higher cholesterol levels after discontinuation of the therapy compared to those who did not experience any adverse events. **Conclusions.** The occurrences of *C.difficile* and *Candida* positivity were excessively high in patients in an acute relapse, which suggests the importance of intestinal microbiota in IBD and an important role in the relapse. FC and MMP-9 has no diagnostic value to differentiate between infection-induced and natural relapse. In addition, 11.25% of our enrolled patients exhibited skin manifestations associated to *Blastocystis* sp, that can make a differential diagnostic difficulties,

therefore stool analysis is recommended in case of cutaneous lesions of unknown origin. Results of Study III showed that split virion vaccines seem to be more effective than whole virion vaccines. Measuring the antibody responses is worthwhile in patients treated with immunosuppressants to determine the efficacy of influenza vaccination. Our results suggest that bolus tapering of corticosteroids may have more favourable short term outcome than conventional tapering that may revolutionize steroid therapy in IBD. Considering that significantly higher post-therapy cholesterol levels were more common in patients who developed drug-related complications, routine measurement of serum cholesterol may increase the safety of the drug.

5. INTRODUCTION AND AIMS

Inflammatory bowel diseases (IBD: Crohn's disease – CD – and ulcerative colitis – UC) are chronic, multifactorial conditions characterised by immune-mediated inflammation of the gastrointestinal tract with highest incidence of CD in North America, whereas top incidence of UC is in Europe (1,2). However, the incidence of IBD is increasing worldwide, including developing countries also (3). Etiology of IBD is not exactly understood; however, basically, it develops due to the combination of genetic and environmental risk factors that contributes to an individual's disease susceptibility. Previously in epidemiological and etiological studies already had many interest on genetics and environmental factors as racial/ethnic differences, smoking, western life-style or urbanization, other socioeconomic factors, dietary influence, geographical variations, perinatal factors, vaccinations and appendectomy (4,5,6). IBD may occur from early childhood to late adulthood, although more than three quarters of cases are currently diagnosed in the twenty-thirty years age population (2). Abdominal pain or cramp, diarrhea and rectal bleeding are the main complaints that permanently alter patients' quality of life. IBD may show many variations of symptoms, disease distribution and degree of activity at the onset. Continuous mucosal inflammation of the colon without granulomas on biopsy, affecting the rectum and a variable extent of the colon in continuity are presented in UC (7); worthy to note that incidence of pancolitis is increasing in adults as well as in children and this extension commonly related to severe disease course (8,9). Definition as well as diagnosis of CD depends on the finding of discontinuous and granulomatosus intestinal inflammation affecting any segment of gastrointestinal tract from mouth to anus. CD has been categorized by disease behavior as "non-stricturing, non-penetrating", „stricturing" and „penetrating" according to on presence or abcence of fistulas, abscess and strictures (10). Latella et al. defined (11) the crucial steps in the natural history of IBD as the occurrence of lesions, the manifestation and severity of symptoms, the need for surgery, the development of complications, disability and mortality; moreover, the main outcomes considered include disease activity and relapse, mucosal healing, need for corticosteroid therapy, hospitalizations, complications, surgery, post-operative recurrence, and mortality. In spite of the most of patients are able to live a normal, productive life, the health care and hospitalizations means a

life-long burden, moreover risk of disability pension is probably higher in young IBD patients as a recent prospective study showed (12). Degree of disability and/or productivity loss depends on general disability from disease activity or localized organic complications, treatment-related side effects, and problems related to surgery and hospitalizations (12, 13).

5.1. Keypoints in the management of IBD – infections and side effects

Management of IBD represents a lifelong medical care as the disorder itself is chronic and permanent with periods of relapses and remissions (14). However, some special situations modify conventional treatment of IBD like adverse events, infections, pregnancy, grown problems in childhood, nutritional difficulties, and malignancies (15,16).

Just as with the initial manifestation of IBD, the factors which cause subsequent relapses are also ambiguous in most cases; however, an infective agent may play a role in both the manifestation and the relapse (17). Risk of infections is increased evidently due to underlying disease and other comorbidities, malnutrition, surgical procedures and immunosuppressive therapy (18,19,20,21,22). *Clostridium difficile* is a pathogen which came to the front of interest due to high prevalence among IBD patients, and associated with increased mortality. Studies (23) had reported about altered intestinal microflora in IBD, in this manner consider to pay attention of avoidance of dysbiosis and microorganisms like *C.difficile* and *Candida* species that leads even a serious symptom and inflammation in special circumstances, thus had influence on optimization of therapy (Study I).

Microbes have significant concern to differential diagnosis also, because some of them may provoke gut and extraintestinal symptoms similar to IBD. Immune-mediated inflammation in IBD leads to gastrointestinal symptoms primarily, although extraintestinal manifestations (EIM) may develop also. Varying symptoms of EIMs appear in 15-20% of UC patients and 20-40% of CD patients; despite of frequently occurrence, the therapy is often empirical (24, 25). The most common extraintestinal manifestations or extraintestinal complications beyond the IBD include arthropathies, mucocutaneous and ophthalmological manifestations, as well as conditions affecting the hepatobiliary system. Less frequent manifestations are pulmonary and neurological symptoms, but these disorders should be considered also as EIMs of IBD (26). Skin is the most common affected extraintestinal organ with varying cutaneous symptoms. Typical skin manifestations are the specific perianal or metastatic CD lesions, the reactive pyoderma gangrenosum, the IBD-associated erythema nodosum. Occurrence of the

drug-induced cutaneous manifestations as psoriasis-like, eczema-like lesions is progressive increasing. (27). EIMs may appear parallel with first signs of IBD, but any time during disease course. However, other intestinal disorders like *Blastocystis sp.* infection may associate with EIMs also that make a meaningful difficulty in differential diagnosis of IBD (28,29,30) (Study II).

Regards to infections, prevention is significantly important step of patient management including the recognition of risk factors for infection, the use of primary or secondary chemoprophylaxis, careful monitoring before and during use of immunomodulators and the vaccination and education of the patient (31). Patients with IBD on immunosuppressive therapy are at increased risk for infective diseases, some of which can be prevented by immunization. According to present standpoint, immunisation status should be control at the diagnosis, and vaccination should be performed before initiation of immunosuppressant therapy. In addition, VZV varicella (if there is no history of chickenpox, shingles, or VZV vaccination and VZV serology is negative), human papilloma virus, influenza (once a year), pneumococcal polysaccharide, hepatitis B (HBV seronegative cases) vaccines should be considered at the diagnosis (20). Respiratory infections are the most frequently reported complications in this population (32). Influenza infection is frequent and may have severe complications, however, it is a preventable modality, and thus vaccination is recommended (Study III). On the other hand, vaccination rates are relatively poor reflecting the low willingness of patients and medical doctors also. Numerous patients do not accept vaccination because of fear of side effects, and many doctors do not propose vaccine against influenza virus due to uncertain response to immunisation.

As it mentioned, immunosuppression can be the consequence of immunomodulatory therapy. Not just a risk of subsequent infection could occur in patients on immunosuppressants, but their use can result other side effects also. Pharmacological treatment of IBD includes the aminosalycilates (sulphasalazine, mesalazine), the corticosteroids, the immunosuppressants such as the thiopurine analogs (6-mercaptopurine and its pro-drug azathioprine), methotrexate, and the calcineurin inhibitors (cyclosporin and more recently tacrolimus), in addition the biological therapy (principally infliximab, adalimumab) depending on disease involvement and severity (33,34). Algorithms of medical management of CD and UC differ according to course and behaviour of diseases; however the goal of both therapies is reach and maintains the clinical and endoscopic remission (35,36). Corticosteroids still have an

important role in the management of acute episodes of IBD. Parenteral corticosteroids are usually the first treatment of choice for hospitalized patients with severe UC and CD (37). However, the therapeutic benefits are compromised by an extensive spectrum of adverse events; although in a recent years therapy strategies aimed to minimalize the side effects by careful drug administration and patient monitoring and using topically acting oral steroids (eg. budesonid) characterized by low systemic bioavailability (38). However, in case of moderate to severe disease activity use of classical systemic corticosteroids seems to be irreplaceable by new formulas, in this reason worth to study the response to different steroid administration types and their side effect profile. (Study IV)

Unfortunately, one third of UC patients is corticosteroid refractory, thus for them cyclosporine A, biological therapy or proctocolectomy is recommended in severe relapse (35). Adverse events of cyclosporine A that range from mild to severe, may lead even to discontinuation of the drug (Study V). Beyond treatment of acute relapses, patients should be monitored due to control short- and long-term effects of therapy.

5.2. Study I.: Role of the microorganism in the etiology and relapse of IBD

The triggering role of Clostridium difficile infection in the relapse of IBD and the clinical utility of faecal markers in the diagnosis of relapses with a different aetiology.

Outstanding concepts of etiology of IBD are the immune reaction to a persistent intestinal infection, existence of a defective mucosal barrier to luminal antigens and a dysregulated host immune response to ubiquitous antigens (39). Some animal experiments suggest a role played by bacteria in the pathogenesis of IBD: interleukin-10 knockout, germ-free mice do not develop colitis; however, interleukin-2-deficient mice develop spontaneous colitis after *Escherichia coli* exposure, but not with *Bacteroides vulgatus* exposure (40). Thus, compound of gut microflora has a significant importance. Intestinal microbiome consists of overall 100 trillion diverse microbes, including over 1100 prevalent species, but in each subject with at least 160 species (41). Studies on the intestinal microbiota has been progressed rapidly: investigations from one species (for example *Faecalibacterium prausnitzii, Escherichia coli*) (42, 43, 44) towards to diversity and interactions (45, 46, 47). Dysbiosis is a definitive change of the normal gut microflora with a disintegration of host-microbial mutualism. Qualitative

and quantitative changes in intestinal microbiome have been reported in patients with IBD. Reduced number of the phylia *Firmicutes*, particularly *Faecalibacterium prauznitzii*, and in opposite, inceased amount of the phylium *Bacteroides* have been noted in subjects with IBD (48). *Proteobacteria phylium* have been described to have a key role in IBD as an aggressor factor in initiation of chronic inflammation (49,50). Importance of dysbiosis in IBD is obvious, but more important question is whether how responds the host to dysbiosis and whether which was first the discrepant immune response or the dysbiosis? Interactions of human gut microbiome and host/intestinal mucosa have basic importance, nevertheless many associations exist with life-style and environmental factors that interfere with (51, 52).

Although the aetiology of IBD has not been unequivocally clarified, an altered intestinal immune response due to the interaction of several environmental and genetic factors is observable, which may lead to inadequate gut flora compound and function, thus making the intestine vulnerable to superinfections like the *Clostridium difficile* (*C.difficile*) infection (53,54,55). *C. difficile* came to the front since epidemiological data showed high prevalence of this bacterium (56,57) among IBD patients. Current data suggest that in patients with CD or UC, infection of this anaerobic bacterium occurs more frequently than in the general population (58,59,60). The significance of CDP (*C.difficile* positivity) manifests not only in its increasing incidence, growing antibiotic resistance and severe disease course, but also in the magnitude of the infections, which also leads to high health care costs. Expansion of CDP is more pronounced in IBD patients; moreover, its prevalence has been reported in 5 to 19% in patients with active disease (61,62). However, data from available prospective studies are limited.

The usual diagnostic check-up in case of a flare-up consists of faecal microbiological examination and an evaluation of inflammatory biochemical and stool biomarkers. None of these tests can immediately differentiate between infection-caused and natural relapse, and considering that most of the outpatients came from a long distance, microbiological validation of the stool sample is often delayed at the expense of treatment. On the other hand, in Hungary, *C. difficile* bacterium and toxin testing is not ordinary in every hospital, usually only clinical centres perform routine *C.difficile* testing procedures. A simply and quickly performing marker would be useful as strong point in the differentiation of an infective origin or exacerbation. Faecal calprotectin (FC) and matrixmetalloproteinase-9 (MMP-9) are markers that correlate with disease activity, but they cannot give a reliable differentiation in

the case of specific IBD. FC is a neutrophil leukocyte-derivate protein eligible for distinguishing IBD from functional disorders (63). In vitro resistance to degradation allows faecal samples to be assayed for reliable calprotectin determination. FC has a high sensitivity in the determination of IBD activity; however, elevated levels were also shown in intestinal infections, tumours and colon polyps, therefore its specificity is less favourable. MMP-9 is a Zn-binding neutral proteinase produced by polymorphonuclear cells. MMP-9 is one of the major contributors to the breakdown and reconstruction of the extracellular matrix (64). Faecal MMP-9 in UC patients is an excellent marker of disease activity and shows significant correlation with clinical and endoscopic scores (65).

5.3. Study II: Role of microorganism in the development of cutaneous lesions: a specific extraintestinal manifestation associated with an infective intestinal disorders

Don't Forget the Stool Examination! – Cutaneous and Gastrointestinal Manifestations of Blastocystis sp. Infection.

Out of IBD, gluten-sensitive enteropathy, polyposis syndromes and gastrointestinal malignancies are bowel diseases which are often accompanied by special extraintestinal cutaneous manifestations (66). Typical clinical scenario is a combination of non-specific gastrointestinal symptoms and recurrent non-specific skin abnormalities that cause the most commonly food allergy or small intestinal bacterial overgrowth. Testing for infective agents as *Helicobacter pylori* infection is also a common practice, although a microbiological stool examination should be one of the first diagnostic procedures in these cases. One of intestinal infections that provoke EIMs, first of all skin manifestations beside gastrointestinal symptoms is *Blastocystis* spp. infection (67). *Blastocystis* sp. is a protozoan genus with mitochondria-like organelles and at least one nucleus. It is an anaerobic microorganism; therefore, the mitochondria-like organelles presumably do not function in oxidative phosphorylation (68). This unicellular, obligate anaerobic protozoon, which resides mostly in the colon and the caecum, is one of the most common parasites in the human intestinal tract, and colonization has also been observed in many vertebrates. Incidence of *Blastocystis* sp. is varying worldwide. In healthy adults the prevalence has been reported to be between 30 and 50% in developing countries and between 1.5 and 10% in developed countries (69). *Blastocystis* sp.

infection commonly causes diarrhoea and abdominal pain, but extraintestinal symptoms, such as skin lesions, may also accompany this condition (70). On the other hand, it is not absolutely clear whether *Blastocystis* sp. is a commensal microorganism, harmless and only a cause of infection under special circumstances, or a true pathogen (71,72). Several studies reported skin manifestations, particularly urticaria, connected to this protozoon (73,74). *Blastocystis* sp. and *Giardia intestinalis* (75) are supposed to be the two most culpable protozoa on the basis of studies of the parasitological aetiology of urticaria. Urticaria may occur both IBD-associated cutaneous disorder and as skin side-effect of drugs used in the treatment of IBD (aminosalicylates, metronidazole, ciprofloxacin, azathioprine) (76).Thus, both IBD and *Blastocystis sp.* associated urticaria may occur presenting a differential diagnostic problem.

5.4. Study III: Efficacy and safety of immunisation in immunocompromised IBD patients

Antibody and Cell-mediated Immune Response to Whole Virion and Split Virion Influenza Vaccine in Patients with Inflammatory Bowel Disease on Maintenance Immunosuppressive and Biological Therapy.

Influenza is one of the most common vaccine-preventable illness in adults and according to the latest statement of the European Crohn's Colitis Organization (20) on the prevention of opportunistic infections in IBD, and influenza vaccination is recommended for all patients with IBD on immunomodulators. Although several guidelines exist for the vaccination of patients with IBD, it still seems to be underused and the results of the studies on the immune response after vaccination are also conflicting (77).

One of the studies conducted in paediatric IBD patients (78) revealed that children receiving both infliximab and immunomodulators had a lower response to 2 influenza vaccine antigens (A/New Caledonia/20/99 and B/Hong Kong/330/2001) when compared with healthy controls. The prospective study of Lu et al. revealed a high prevalence of seroprotection in children and young adults with IBD, particularly against A strains (79). They did not detect difference among non-immunosuppressed and immunosuppressed patients. However, in adults, the types of immunosuppressive and biological therapies seem to influence the immune response to

vaccinations (80). Additionally, the majority of the studies aims to examine only the rate of seroprotection and does not consider the importance of the frequency of infections in vaccinated vs. not vaccinated patients.

Although vaccination against influenza is recommended, more and more information is needed in special clinical situations to make a proper decision according to adequate patient- and vaccine type selection.

5.5. Study IV: How to decease steroid side effects?

Bolus Administration of Steroid Therapy is More Favorable than the Conventional Use in Preventing Decrease of Bone Density and the Increase of Body Fat Percentage in Patients with Inflammatory Bowel Disease.

Use of steroids is associated with some well-known potential harmful side-effects; therefore oral steroids are recommended to be gradually tapered off and discontinued after 12 weeks in case of appropriate response to the parenteral therapy. Corticosteroid therapy is known to contribute to changes in body composition with the alteration of protein synthesis and degradation in skeletal muscle, resulting in decreased muscle mass and reduced fat-free mass. Steroids also lead to a reduction in the total body bone mineral density (BMD) (81). Therefore total body composition analysis is a useful method for quantification of multiple whole body and regional components, including bone mineral, fat, and lean soft tissue in patients treated with steroids. It gives a direct measurement of the percent body fat, muscle and bone (in grams) for the entire body and sub regions like the arm, leg, and trunk.

The optimal dose response for parenteral steroids in the treatment of severe attacks has not been clarified yet; dosages of methylprednisolone 40-60 mg or 1 mg/kg per day orally are the most frequently used regimen (37, 82) for flare up. Furthermore, no randomized trials have studied and even no guidelines have been developed by the European Crohn's and Colitis Organisation on taper schedules. After the induction of remission, methylprednisolone is usually tapered 8-16 mg weekly until a daily dose of 32 mg is reached followed by a tapering of 4 mg/wk. Tapering steroid regimen is most frequently carried out by administrating the drug daily, although alternate-day steroid management (given every other day) has also been a widely employed and effective mode of therapy for ages associating with fewer unpleasant side effects (83). The efficacy of "bolus-administered" corticosteroids when weekly dose of steroid

regimen is given on special days has not been previously examined in patients with IBD. The effect of a “short-term” 12-week course of corticosteroids on the metabolic processes and bone formation has not been well studied too; although these are some of the most important side effects should be considered.

5.6. Study V: An underdiagnosed common side effects of calcineurin inhibitor therapy

Long-term Increase in Serum Cholesterol Levels in Ulcerative Colitis Patients Treated with Cyclosporine: an Underdiagnosed Side Effect Frequently Associated with Other Drug-related Complications.

Flare-ups of UC can be very severe, requiring hospitalisation, and they can have life-threatening complications, such as toxic megacolon, perforation and severe bleeding in 15% of the cases (84). One third of the patients seem to be refractory to steroid therapy, but in these severe clinical situations possible alternatives to total proctocolectomy are cyclosporine A (CyA) and infliximab (13). Meta-analyses of the relatively limited amount of published data available have revealed a similar effectiveness profile for CyA and infliximab therapies (85), although the lower cost of CyA is undeniable (86). Cyclosporine A is a type of calcineurine-inhibitor which binds to immunophilins protein localized intracellularly. That complex attaches to calcineurin, thus inhibit its activation, and hence inhibiting T cell activation. Blocking transcription and expression of some cytokines (interleukin-2: IL-2, interleukin-4: IL-4, cluster of differentiation 40: CD40) and co-stimulatory molecules, T cell growth and differentiation interfere also (87). Since the short-term bowel-saving capacity of CyA is excellent, the remaining major issues are presented by long-term outcomes and the safety of CyA (88). Recently published studies have proven that the longer duration of CyA therapy and the concomitant use of azathioprine are associated with a significantly lower colectomy rate than expected, although the development of side effects still remains a problem (88,89).

The most well-known major adverse effects of CyA are nephrotoxicity and hepatotoxicity. The incidence of minor adverse reactions such as tremor, paraesthesia, malaise, headache, gingival hypertrophy and hypertrichosis varies between 31% and 51% (89). The manifestation of these side effects may lead to the discontinuation of the CyA therapy in a significant

proportion of patients, although the majority of the patients under regular monitoring of CyA blood levels are able to continue the therapy for more than one year for cases of psoriasis (90). An increase in cholesterol levels is a known side effect of CyA in transplant patients; however, we have limited data about the long-term influence on lipid metabolism in UC. Ballantyne et al. (91) measured lipoprotein levels in cyclosporine-treated patients undergoing heart or kidney transplantation. Significant increases in total (21%) and low-density lipoprotein cholesterol (31%) occurred only in the cyclosporine group of patients. Cyclosporine therapy by itself was found to affect plasma lipoprotein levels adversely by increasing total cholesterol levels.

5.7. Aims

1. To prospectively assess the frequency of *C.difficile* among our outpatients with IBD in relapse; in addition, to estimate the conduciveness of clinical and differential diagnoses of FC and MMP-9 in cases of both infection and non-infection.

2. To retrospectively assess the attributes of patients with a confirmed positive *Blastocystis sp*. infection who came to our clinics at the University of Szeged between 2005 and 2013, primarily to examine the occurrence of clinical gastrointestinal symptoms and skin manifestations.

3. To assess the antibody response to the seasonal influenza vaccine in patients with IBD treated with anti-TNF-α alone or combined with immunosuppressive therapy and to compare them with patients receiving non-immunosuppressive therapy. The secondary goal in this study was to compare the antibody response and the safety of whole virion and split influenza vaccines. The last aim was to evaluate the effects of the vaccines on IBD clinical activity. Last, but not least this study was also aimed to evaluate the acceptance of the vaccination in our patients and to assess the frequency of influenza infection in vaccinated vs. non vaccinated patients.

4. To compare the efficacy, the frequency of side effects and the changes in bone and lipid metabolism in IBD patients using bolus or conventional tapering of methylprednisolone for 12 weeks.

5. To prospectively assess serum cholesterol, triglyceride and creatinine levels before, during, and after CyA therapy in patients with severe, refractory UC and to examine the correlation between plasma lipoprotein levels and other side effects.

6. PATIENTS AND METHODS

6.1. Description of methods

6.1.1. Enzyme-linked immunosorbent assay

The basic immunology concept of enzyme-linked immunosorbent assay (ELISA) technique is a specific antigen- antibody binding. ELISA operates with enzyme-labelled antigens and antibodies to detect the biological molecules. Presence of antigen is indicated when chromogenic substrate for the enzyme yields make a visible colour change (92).

Fecal MMP-9 concentrations were measured using ELISA method (Quantikine MMP9 assay, R&D System, UK) in our clinical study. One gram of the sample was diluted and homogenised in 4 ml of an ice-cold buffer (0.15M NaCl + 20mM Tris-HCl, pH:8.3). The suspension was then centrifuged at 1500g for 10 min and the supernatant was recentrifuged at 10,000g for 10 min. The final supernatant was filtered and stored at -20˚C until analysed. Determination was in accordance of manufacturer's instructions.

From the collected sera samples, anti-influenza A virus IgG ELISA(Euroimmun, Germany) containing the following antigens "Texas" (H3N2), "Singapore" (H1N1) and "California" (H1N1) strains of influenza A virus was performed according to the manufacturer's recommendation. The results were evaluated quantitatively using calibration curve. From the above-mentioned sera specimens, anti-influenza B virus IgG ELISA (Euroimmun, Germany) was also set up. In this case, the antigen source was "Hongkong 5/72" strain of Influenza B virus. The results were also interpreted quantitatively.

6.1.2. Lateral flow assay

Fundamental mechanism of lateral flow assay (LFA) is the movement of fluid sample or its extract, along a strip of polymeric material though various zones where molecules are attached with more or less specific interacting with the analyte. Usage of labels made of

coloured or fluorescent nanoparticles are allowing the visualisation, and quantitation of response (93).

FC levels were measured by a quantitative LFA (Quantum Blue, Bühlmann Labortories, Switzerland). Samples for FC determination were stored at -20°C until analysed. Faecal specimens were thawed and prepared for a calprotectin assay as described by the manufacturer.

6.1.3. Microbiological examination of stool samples

A microbiological analysis (involving bacteria, fungi and, in reasonable cases, viruses and parasite examination) for the presence of an enteric pathogen was carried out at the Institute of Clinical Microbiology, Szeged. Laboratorial diagnosis of *C. difficile* was based on microscopic identification of bacterium; the detection of Toxin A and B was completed by using the ELISA method.

The laboratorial diagnosis of *Blastocystis* sp. colonization was based on directly microscopic detection of trophozoites and/or cyst forms. A culture of the organism from the stool was also performed in cases of microscopically negative direct smears. It was carried out using Boeck-Drbohlav-Locke-egg serum medium (94).

6.1.4 .Statistical analysis

The collected data were analysed statistically. $P<0.05$ was considered statistically significant. For the analysis, SPSS15.0 (SPSS Inc, Chicago, IL, USA) was used.

6.1.5. Ethical Considerations

The studies were approved by the Regional and Institutional Human Medical Biological Research Ethics Committee of the University of Szeged.

6.2. Patients and methods of clinical studies

Diagnosis was based on the Lennard-Jones Criteria (95), in addition disease phenotype was determined according to the Montreal Classification (96). The clinical activity of CD and UC were assessed using the Crohn's Disease Activity Index (CDAI) (97) and with a partial Mayo

Score (pMayo) (98), respectively. Relapse was defined as ≥150 points with an increase of ≥80 points in the CDAI in the case of CD and ≥3 points in the pMayo score in the case of UC.

6.2.1. Study I: Relapse of IBD and C. difficile

90 patients with symptoms of a flare-up were enrolled in our study with 49 control subjects in clinical remission from September, 2012 to July, 2013. All of them were outpatients at the First Department of Medicine, Szeged. The medical data of patients were collected prospectively. Clinical data, activity indexes, faecal specimens and serum samples were analysed of IBD patients who manifested worsening symptoms –indicating a potential flare-up – and of patients in remission. Blood samples were taken from all patients for the determination of CRP levels (C-reactive protein), platelet and leukocyte count, serum iron level and haemoglobin level. Faecal samples from every subject were sent to the microbiological laboratory of the Institute of Clinical Microbiology. The clinical characteristics of the patients are presented in Table 1. The activity of IBD and the presence of an enteric pathogen were carefully assessed at each visit, and laboratory findings were entered into the clinical records.

The collected data was analysed statistically, using Pearson's chi-square test, Pearson's chi-square test with Yates' continuity correction and Wilcoxon rank sum test with continuity correction.

Table 1. Clinical characteristics of patients.

	Relapsing group (n=90)	**Control group (n=49)**
Gender (male/female)	44/46	28/21
Age (years)	36.7 (16 to 79)	38.7 (19 to 75)
UC/CD	51/38	25/24
Disease duration (years)	6.9 (0 to 26)	10.2 (0 to 46)
Appendectomy (%)	10.8	3.8
Smoking (%)	37.7	16.1
Perianal involvement (%)	16.8	10.9

Extraintestinal manifestation (%)	42.7	22.9
Immunosuppression (%)	48.8	53
Gastricacid-suppressant agent (%)	27.7	16.6
Previous antibiotic use (%)	20	20.4
CDAI	227.9 (140 to 450)	71.04 (-5 to 127)
pMayo Score	5.2 (3 to 9)	0.56 (0 to 2)
FC (µg/g)	1292.5 (29 to 6800)	216.1 (29 to 1370)
Fecal MMP-9 (ng/mL)	11.8 (0 to 168.8)	3.5 (0 to 22.2)
CRP (mg/L)	23.5 (1 to 202.6)	6.5 (1 to 40.8)
Leucocytes (G/L)	10.05 (3.9 to 90.6)	7.06 (3.6 to 14.7)
Thrombocytes (G/L)	333.7 (169 to 657)	283.4 (88 to 602)
Serum iron (µmol/L)	11.2(1.9 to 25.3)	15.04 (4.4 to 33.1)
Hematocrit (%)	39 (21 to 49)	40 (33 to 47)

6.2.2. Study II: Clinical manifestations of Blastocystis sp. infection

The first part of our study determined the frequency of occurrence of a positive *Blastocystis* sp. infection among patients treated at the University of Szeged clinics between 2005 and 2013. A routine microscopic parasitological examination was performed on stool samples from 3255 patients, based on the patients' symptoms and complaints. Physicians requested stool examinations based on their assessment of the patients' clinical characteristics.

We subsequently assessed the frequency of *Blastocystis* sp. in 1471 patients at the First Department of Medicine with adult gastroenterology profiles who needed a stool protozoon examination between January 2005 and May 2013. In addition, data of 80 patients at the First Department of Medicine with confirmed positive *Blastocystis* sp. infections were assessed retrospectively (Table 2). Every patient of our cohort complained about different gastrointestinal symptoms, such as diarrhoea, abdominal pain, bloody stool, and meteorism. The results of routine biochemical and haematological blood tests of positive cases, as well as

the results of any relevant examinations performed during dermatological evaluations, were collected from the clinical database.

Table 2. Clinical data of *Blastocystis sp*. positive patients.

Number of patients	80
Gender (Male/female)	32/48
Age at *Blastocystis* sp. infection (years)	13-85 (Mean: 46.3)
The most frequent symptoms in enrolled patients:	Number of cases:
Abdominal pain	40
Blood in stool	17
Meteorism	15
Weight loss	8
Perianal symptoms	6
Mucus in stool	5
Vomitus	2
Fever	2
Skin manifestations	9

Symptoms, laboratory findings (elevated CRP, increased number of eosinophils, leucocytes and lymphocytes, etc.) and accompanying chronic diseases (lactose intolerance, IBD, gastroesophageal reflux disease, etc.) were statistically compared between patients with vs. without skin manifestations. Categorical data was analysed using Fischer's exact test, or one-sided Fischer's exact test. Continuous data was analysed using Student's t-test and Mann-Whitney U-test.

6.2.3. Study III: Efficacy of influenza vaccination

We conducted a multicentre, prospective cohort study from September 2012 through May 2013 in 4 Hungarian IBD centres (2nd Department of Medicine, Semmelweis University; 1st Department of Medicine, Semmelweis University; Military Hospital, Budapest; Integrated Szent Istvan and Szent László Hospital, Budapest). Patients with active IBD were excluded. At inclusion, influenza vaccination was offered to every patient attending the involved centres. Patients were randomized to two groups on the basis of the acceptance of the vaccination.

Patients refusing the vaccination served as control subjects. Patients and control subjects were followed up to 4 months to determine the clinical activity and the frequency of influenza infections. Clinical data and immunisation history in the previous 5 years was also obtained.

Patients who received vaccination were divided into two further groups: patients treated with aminosalycilates without immunosuppressive therapy and patients treated with immunomodulator and/or biological therapy for at least three month at the vaccination. Control subjects have received maintenance therapy with immunomodulator and/or biological therapy for at least three month at the vaccination.

The type of vaccine (whole virion or split virion vaccine) was randomly selected. The patients were scored and blood samples were also taken before and after the vaccination. Serum was collected at baseline (pre-vaccination) and 5-6 weeks after vaccination and it was stored at -20 ^{o}C until used. We compared the antibody titers of influenza A and B subtypes between vaccinated and control patients; between vaccinated patients receiving immunosuppressive (thiopurine, biological therapy) and non-immunosuppressive therapy (aminosalycilates); between vaccinated and control patients receiving immunosuppressive maintenance therapy and between patients vaccinated with whole virion and split vaccine receiving immunosuppressive therapy. Demographic and clinical characteristics, disease activities at the time of the vaccination and treatment types are summarized in Table 3.

Two non-live vaccines directed against the seasonal influenza virus A/California/7/2009 (H1N1), A/Victoria/361/2011 (H3N2), B/Wisconsin/1/2010–like B/Hubei-Wujiagang/158/2009 were used in the study. Inactivated, split virion vaccine (IDFlu9) and inactivated, whole virion vaccine (Fluval AB) were administrated depending on the random selection. The experimental use of whole virion vaccine, that is the first of the inactivated vaccine formulation, dates back to 1940s. Split virion is derived by disrupting whole virus particles with detergents thus being less immunogenic than whole virion vaccines (99). Whole virion vaccine was administered intramuscularly; split virion vaccine was administered by intradermal route.

Table 3. Clinical characteristics of patients.

	All patients (n=209)	**Fluval AB vaccinated (n=57)**	**IDFlu9 vaccinated (n=99)**	**Controls (n=53)**
Male/Female	96/113	30/27	42/57	24/29
Mean age at diagnosis (years)	28.6	27.2	28.3	30.7
Median disease duration (years)	9	9	9	7
CD/UC	127/82	46/11	52/47	29/24
Disease location				
- L1	25	10	10	5
- L2	46	14	20	12
- L3	54	21	22	11
- L4	2	1	0	1
Disease behaviour				
- B1	41	14	19	8
- B2	24	9	12	3
- B3	62	23	21	18
Extent of UC				
- E1	21	3	11	7
- E2	28	5	16	7
- E3	33	3	20	10
Therapy				
- Aminosalycilates	21	5	6	10
- Thiopurines	27	7	14	6
- Anti TNF-α	26	10	12	4
- Combined thiopurines and anti TNF-α	62	20	20	22
Mean CDAI	120.3	108.4	133.2	137.5
Mean pMayo score	1.8	1.7	1.6	2.1

We assessed cell-mediated immune response after vaccination and also compared in patients treated with and without immunosuppressants. The patients were contacted by phone every

week throughout 16 weeks. During the phone calls, data from each patient were collected using a standardised questionnaire. The patients were interviewed about the change in clinical activity and the development of local and systemic adverse reaction.

Categorical data were analysed using Pearson's chi-square test and Fisher's exact test. The effects of the vaccination on the antibody and cell-mediated immune response were examined with multivariate analysis of variance (MANOVA) models with time as repeated measures (within-subject) factor and the types of the vaccines, the immunosuppressive status, the vaccinated status, the different therapies and the development of side-effects and influenza-like symptoms as between-subject factors. Pairwise comparisons were performed on estimated marginal means by considering the presence or absence of interaction; p-values were corrected with the Holm-Sidak method.

6.2.4. Study IV: Bolus administration of steroid therapy

This single-center, prospective, randomized trial was carried out from November 2011 to February 2013 on consecutive patients with acute exacerbation of IBD and not being on steroid therapy admitted to our clinic. Twenty patients were enrolled in the study (Table 4). The median CDAI and partial Mayo score were 184 and 6 in CD and UC at the time of the enrolment. None of the patients received oral corticosteroid at the time or at least 6 months before the enrolment. On admission a complete blood chemistry including erythrocyte sedimentation rate (ESR), CRP, serum glucose, electrolytes, liver and renal function, cholesterol, triglycerides, blood count, serum cortisol, calcium, dehydroepiandrosterone (DHEA), thyroid stimulating hormone (TSH), parathyroid hormone (PTH), ß-crosslaps and osteocalcin levels were performed before starting steroid therapy. The mean value of CRP, ESR, leukocytes and thrombocytes before steroid therapy were 16 mg/l, 21.5 mm/h, 8.828 G/l and 322 G/l. Flexible colonoscopy or sigmoidoscopy with biopsies was carried out only in patient with relevance to therapy, however, it was not essential for inclusion into the trial. Thus, 3 CD and 13 UC patients underwent colonoscopy at inclusion. DXA total body composition analysis was also performed at the beginning of the study to determine the fat and fat-free component of the body.

Patients eligible for iv. steroid therapy received methylprednisolone dosage 1 mg/kg for 5 days. After iv. therapy, patients were prospectively randomized in two groups. In "conventional" group (Group I) methylprednisolone was given daily while in "bolus-

administered" group (Group II) weekly dose of steroids were given on special days of the week. 64 mg/day methylprednisolone dose at the first week in Group I was equal to 150 mg/day given in the first 3 days in Group II. Finally, both groups received the same methylprednisolone dose and it was tapered by 4 mg per week in both groups. The two different types of methylprednisolone dosages are detailed in Table 5. Follow up appointments were done every two weeks. These visits involved the assessment of the clinical activities by the determination of CDAI and pMayo scores. Patients were asked about side effects, the BMI was determined and the waist and hip circumferences were also measured. Laboratory assessment (including inflammatory parameters, electrolytes, glucose level, liver and renal function, and blood count) was carried out every four weeks. Detailed laboratory parameters (DHEA, THS, PTH, serum cortisol, serum ß-crosslaps and osteocalcin levels) and DXA for total body composition analysis were performed at week 0 and week 12.

Table 4. Clinical characteristics of the enrolled patients

	Group I (n=9)	**Group II (n=10)**
Mean age at the diagnosis (years)	34.3	30.3
Mean disease duration (years)	5.2	6.2
CD/UC	3/6	3/7
Female/male	4/5	2/8
Location/extension		
- **Ileal**	1	2
- **Colonic**	2	1
- **Ileocolonic**	-	-
- **Extensive colitis**	3	5
- **Left-sided colitis**	3	2
- **Proctitis**	-	-
Concomitant therapy		
- **5-ASA**	4	6
- **Budesonide**	0	2
- **Azathioprine**	3	4
- **Metronidazole**	2	1

The Student's *t*-test was employed to compare continuous variables. Multivariate analysis with stepwise logistic regression by SPSS software was performed to investigate the parameters with a possible influence on clinical outcome, such as age, gender, location of disease, duration of disease, concomitant immunosuppressive therapy. The differences between the two groups were performed by mixed effects ANOVA model for repeated measures. The results were corrected using a Bonferroni-Holm method for multiple testing.

Table 5. Methylprednisolone dosages in the conventional and the bolus administration groups

Conventional administration								
Week/day	1	2	3	4	5	6	7	Total dose
1	64	64	64	64	64	64	64	448
2	48	48	48	48	48	48	48	336
3	32	32	32	32	32	32	32	224
4	28	28	28	28	28	28	28	196
5	24	24	24	24	24	24	24	168
6	20	20	20	20	20	20	20	140
7	16	16	16	16	16	16	16	112
8	12	12	12	12	12	12	12	84
9	8	8	8	8	8	8	8	56
10	4	4	4	4	4	4	4	28
11	2	2	2	2	2	2	2	14
12	0	0	0	0	0	0	0	0

Bolus administration								
Week/day	1	2	3	4	5	6	7	Total dose
1	150	0	150	0	150	0	0	450
2	112	0	112	0	112	0	0	336
3	75	0	75	0	75	0	0	225
4	98	0	98	0	0	0	0	196
5	84	0	84	0	0	0	0	168
6	70	0	70	0	0	0	0	140
7	112	0	0	0	0	0	0	112
8	84	0	0	0	0	0	0	84
9	56	0	0	0	0	0	0	56
10	28	0	0	0	0	0	0	28
11	12	0	0	0	0	0	0	12
12	0	0	0	0	0	0	0	0

6.2.5. Study V: Side effects of cyclosporine A therapy

72 patients suffering from severe, steroid-refractory UC were enrolled in our study from our tertiary clinic between January 1998 and June 2009. In the acute phase, intravenous cyclosporine was administered at the initial dose of 5 mg/kg for seven days, then, depending on serum levels of the drug, the dosage was modified (to 2-4 mg/kg). Subsequently, oral treatment was administered at a mean dose of 4.7 mg/kg, adjusted according to serum levels of the drug, and co-administration of azathioprine, at a dose of 2 mg/kg, was also started in naïve patients. Patient data were collected prospectively. Patient demographic data are detailed in Table 6.

Cyclosporine serum levels were regularly monitored during application of the therapy. The normal range of fasting levels and of 2-hours-post-dose levels were determined, based on transplant data, to be between 100 and 200 μg/L and between 800 and 1,400 μg/L, respectively. Our UC patients had received concomitant steroid therapy, but this was tapered off within 3 months after starting cyclosporine therapy.

Total cholesterol, triglyceride and creatinine levels had been collected prospectively before, then at the time of and after administration of the cyclosporine therapy. The normal total cholesterol level was defined as less than 200 mg/dL, normal triglyceride level as less than 150 mg/dL and normal serum creatinine in the range of 0.6-1 mg/dL. Cholesterol and triglyceride levels were measured at months 3, 6 and 12 after discontinuation of the cyclosporine therapy.

All patients were regularly monitored: in monthly follow-up visits during the CyA therapy and every second month after discontinuation. The activity of UC and the presence of adverse events were carefully assessed at each visit, and laboratory findings were entered into the clinical record. The side effects that led to discontinuation of the therapy were categorised as major, while others were categorised as minor. To compare results and eliminate additive effects of corticosteroids on the lipid profile, we used control patients treated with infliximab and concomittant steroids. There were 24 patients in the control group, with similar clinical characteristics to the cyclosporine-treated group (Table 7.)

Categorical data were statistically analysed using Pearson's chi-square test, Fischer's exact test, or one-sided Fischer's exact test. The effects of cyclosporine therapy on serum cholesterol, triglyceride and creatinine levels were examined with mixed-design variance analysis (ANOVA) models with time as repeated measures (within-subject) factor and both group and side effects as between-subject factors. Pairwise comparisons were performed on estimated marginal means by considering the presence or absence of interaction; p-values were corrected by the Holm-Sidak method.

Table 6. The demographics of the patients treated with cyclosporine

Gender(female/male)	39/33
BMI (kg/m²)	23.6 (15.2-38.3)
Mean age at diagnosis (years)	31.8 (14-69)
Mean disease duration (years)	13.5 (3-42)
Left-sided colitis/extensive colitis	27/45
Previous corticosteroid therapy	39
Concomitant azathioprine	26
Mean age at start of cyclosporine therapy (years)	40.3 (15-72)
Mean disease duration at the beginning of cyclosporine therapy(years)	8.6 (0-40)
Mean duration of cyclosporine therapy(months)	9.6(0.1-60)
Mean dose of cyclosporine/bwkg (mg)	4.7
Mean trough level of cyclosporine (µg/ l)	193.18
Mean peak level of cyclosporine (µg/l)	866.04

Table 7. The demographics of the control group.

Gender(female/male)	14/11
Mean age at diagnosis (years)	30.8 (15-52)
Left-sided colitis/extensive colitis	10/15
Concomitant azathioprine	16
Mean age at start of cyclosporine therapy (years)	39.2 (19-67)
Mean disease duration at the beginning of cyclosporine therapy(years)	9.2 (1-32)
Mean duration of biological therapy(months)	12

7. RESULTS

7.1. Study I: Relapse of IBD and *C. difficile*

7.2.1. Infection rates and patient follow-up

Out of the 139 enrolled patients, 76 subjects were diagnosed with UC and 63 with CD. More patients with UC participated in the relapse group than CD patients (51 vs. 38 subjects). The number of CD and UC patients were approximately equal (24 vs. 25 subjects) in the control group. The mean value of the CDAI was 227.89 points (in the range of: 160 to 450) and the mean value of the pMayo in the case of UC was 5.21 (in the range of: 3 to 9) points. The most frequent disease locations in the relapse cohort were left sided or pancolitis in the case of UC, and pure colonic and the ileocolonic in the case of CD. Half of the patients were on immunomodulatory therapy in the relapse group (48.8%) and also in the control group (53.1%).

Bacteria or fungi were identified in 51 of 90 faecal samples taken from relapsing patients. On the other hand, 14 patients had positive microbiological stool findings in the control group in remission. Overall, *C. difficile* Toxins was found in 40.3% of the cases with IBD. 46 participants with positivity of *C. difficile* A and B Toxins had been isolated among the patients in relapse (51.1%), while CDP was observed in 10 patients (20.4%) in the remission group. Other causes of microbiological positivity in the relapse group were due to the presence of *Candida* species (seven subjects with *C. albicans*, one with *C. glabrata* and one with *Saccharomyces cerevisiae*) in all except one subject, who had a *Salmonella* infection. *Candida* subspecies occurred in five patients in remission (four with *C. albicans* and one with *C. glabrata* and *Geotrichum candidum* simultaneously). In five cases we found simultaneously presence of *C.difficile and Candida* in the relapse group and in two cases in the control group (Table 8.). A significant difference was revealed between the relapse and the control group regarding the microbiological findings and positive cases of *C. difficile* (where $p=0.0027$ and $p<0.001$). 21 subjects in the relapse group had previous CDP in their medical history, although only 12 of the currently infected patients were positive in the past. Eight patients had recurrent or sustained (over 4 weeks) CDP. 10 of the control subjects had CDP previously, but only four of currently *C. difficile*-positive subjects had a medical history of CDP. We did not find a difference between CD and UC regarding positive microbiological examination findings or CDP (30 subjects with UC and 27 with CD). There was no correlation between a medical history of previous CDP positivity and a current *C.difficile* positivity (where $p=0.21$).

Table 8. Infection rates among patients with relapse and remission. Mixed infections occurred in assessed faecal samples, not just in relapse cohort, but in patients with remission also.

	Relapsing group (n=90)	**Control group (n=49)**
Positive microbiological findings	51	14
***C.difficile* Toxin A, B**	46	10
Candida	8	5
Mixed *C.difficile* and *Candida*	5	2
Salmonella	1	0

There was no significant connection between disease extension, EIMs, perianal involvement, smoking, age, gender, disease duration, immunosuppression and proton-pump inhibitor use and CDP. Previous antibiotic use was similar in both groups (20% in the relapse group and 20.4% in the control group), although it was more frequent among *C.difficile*-positive subjects. Therefore, previous antibiotic use was shown to have a connection to CDP (p=0.033), but other assessment factors did not predict the presence of *Cl.difficile* Toxins.

Patients in whom *C. difficile* Toxins had been detected in the relapse group were treated with metronidazole. A combination of antibiotics was used, or a substitute was used for metronidazole (rifaximin, ciprofloxacine or vancomycin) in case of metronidazole inefficiency, or if repeated stool examinations continued to be positive. Three patients needed hospitalisation in a specialist Department of Infectology due to a therapy-resistant case of CDP. However, reasonable administration of a supplementary therapy or a modification of the maintained treatment was sufficient in 65.9% of the cases (oral or topical corticosteroids, 5-ASA and anti-TNF-therapy). 16 subjects required hospitalisation because of a flare-up. 46 participants who relapsed achieved a clinical remission within 8 weeks; the effectiveness of the therapy was not associated with initial levels of FC or MMP-9, neither in the relapse cases, nor in the CDP cases.

7.2.2. Laboratory Findings

The mean values of CRP in the relapse and the control group were 23.52 mg/L (in the range of: 1 to 202.6 mg/L) and 6.54 mg/L (in the range of: 1 to 40.8 mg/L), respectively. CRP was slightly higher in those with CD than in those with UC (the mean values of which are: CD: 26.02 mg/L vs. UC: 21.01 mg/L), but on the other hand, this difference was greater in the control group (the mean values of which are: CD: 9.37 mg/L vs. UC: 3.56 mg/L). The mean values of the leukocyte

count, the thrombocytes, the serum Fe^{+} and the hematocrit levels were 10.05 G/L, 333.6 G/L, 11.2 μmol/L and 39% in the relapse group.

7.2.3. Fecal calprotectin and matrix-metalloproteinase-9 levels

The mean value of FC in those in relapse was significantly higher compared to the control group (with a mean value of: 1292.5 μg/g vs.216.11 μg/g; where $p<0.001$). Interestingly, FC showed greater values in relapsing patients without CDP (with the mean value of: 1457 μg/g) vs. relapsing patients with positive microbiological stool examination (with the mean value of: 1152 μg/g), but in the Wilcox rank test with continuity correction, there was no significant difference between a *C. difficile* positive and negative population (Table 9.).

Similarly to FC, faecal MMP-9 levels were higher in the relapse group (11.77 ng/mL; in the range of: 0 to 168.79 ng/ml) compared to the control group (3.46 ng/mL; in the range of: 0 to 22.29 ng/mL; where $p=0.001$). We found a definite difference between the mean values in cases of CDP and microbiological negative cases (8.08 ng/ml vs.16.42 ng/ml) in the relapse cohort; however, statistical analysis did not confirm the difference (Table 9.).

Table 9. The mean values of FC and MMP-9.

	Mean value of FC (μg/g)	**Mean value of MMP-9 (ng/ml)**
Relapsing group	1292.5	11.8
...with infection	1152	8.07
...without infection	1457	16.4
Control group	216.1	3.46

7.2. Study II: Clinical manifestations of *Blastocystis sp.* infection

7.2.1.Frequency of Blastocystis spp.

4567 faecal samples from 3255 patients from the clinics at the University of Szeged were analysed at the Department of Clinical Microbiology, Szeged, between January 01, 2005 and May 01, 2013. *Blastocystis sp.* infection was discovered in 275 of 4567 faecal samples (196 of 3255 patients) – the occurrence of *Blastocystis sp.* infection was 6% in the symptomatic patients who required medical attendance in that period.

Eighty of 1471 patients tested positive for *Blastocystis* sp. at our clinic during this 8-year period, representing 5.4% of all stool samples sent during that time from our clinic to be examined for the presence of protozoa.

7.2.2. Results of stool microbiological examination

Of the faecal specimens, 41.1% contained few *Blastocystis* sp. cells, 5.5% of specimens contained a moderate amount and 53.4% contained a high number of the parasites. In 18.75% of the cases other microorganisms were also present besides *Blastocystis* sp., such as *Campylobacter jejuni*, *Campylobacter lari* and *Clostridium difficile*, among fungi *Candida albicans*, *Candida glabrata* and *Geotrichum candidum*, as well as other parasites such as *Entamoeba histolytica* and *Entamoeba coli* (Table 10). Seven of those 15 patients had great number of *Blastocystis* sp. cells in stool samples, and 6 individuals had low numbers of this form, therefore the number of *Blastocystis* sp. cells in co-infections did not make a difference. However, every subject had symptoms that improved after antimicrobial therapy. Only one patient with gastrointestinal co-infection (*C. glabrata*) had skin symptoms.

Table 10. Microorganisms detected in the same stool samples together with *Blastocystis* sp.

Microorganism in stool:	**Number of cases**
Campylobacter jejuni	3
Candida albicans	3
Clostridium difficile A and/or B toxin(s)	2
Campylobacter lari	1
Non-toxin-producing *Clostridium difficile*	1
Candida glabrata	1
Geotrichum candidum	1
Entamoeba coli	1
Entamoeba histolytica and *Clostridium difficile* A and/or B toxin(s)	1
Entamoeba histolytica with *Geotrichum candidum*	1

7.2.3. Blastocystis sp. and skin manifestations

Nine out of 80 people had accompanying skin manifestations. We did not find a statistically significant difference regarding age and gender between individuals with vs. without skin manifestations (p=0.73 and p=0.34, respectively).

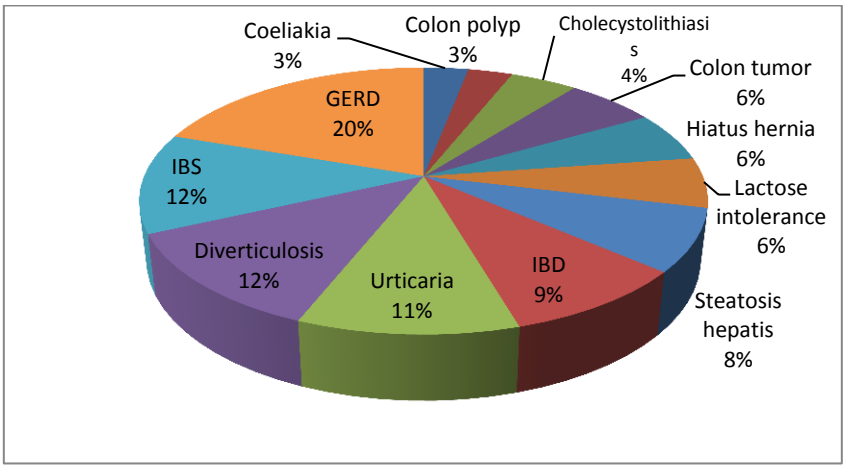
73.75% of the 80 patients indicated that they had gastrointestinal symptoms: 40 patients complained of abdominal pain, 17 with blood in their stool, while other symptoms, such as meteorism (15 subjects), weigh loss (8 subjects), perianal pain or itching (6 subjects), passing stool with mucus (5 subjects), vomiting (2 subjects) and fever (2 subjects) were less frequent. The prevalence of abdominal pain in the cohort without skin lesions was higher compared to those patients with skin problems (p=0.007).

Full blood count, including thrombocytes, leucocytes (eosinophils, basophils, neutrophils, etc.), was within a normal range, except for CRP, which showed elevated levels (at a mean value of 13.89 mg/l, in a range between 1 and 89.9 mg/l) at admission.

Significant differences were revealed between patients with and without skin manifestations regarding laboratory findings, namely the value of CRP (p=0.038), leucocytes (p=0.024), neutrophil granulocytes and its percentage ratio (p=0.007, p=0.012), thrombocytes (p=0.002) and RDW (Red blood cell Distribution Width /RDW/; p=0.025) were significantly higher in patients with skin manifestations. The value of lymphocyte (p=0.011) and monocytes (p=0.023, p=0.011) and their percentage ratios were higher in subjects without skin lesions. Interestingly, we didn't find a significant difference in eosinophil counts.

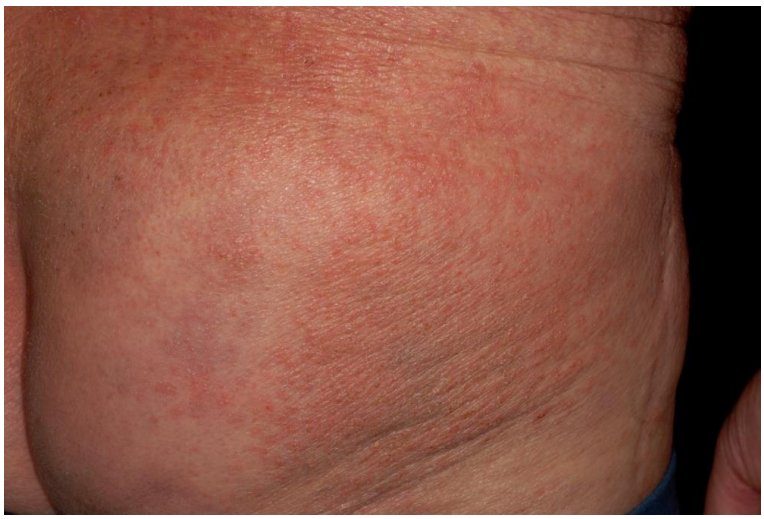
The diagnosed accompanying conditions (e.g. gastroesophageal reflux disease /GERD/, weight loss, and hepatomegaly) of a *Blastocystis* sp. infection are summarised in Figure 1. Prevalence of IBD was 9% among *Blastocystis* sp. positive patients. Weight loss, lactose intolerance and GERD were significantly more common in patients with skin lesions vs. without skin manifestations (p=0.04, p=0.04, p=0.02, respectively). Seven patients were treated with antibiotics previously, due to other infections (prostatitis, pharyngitis, etc.) No association was found between previous antibiotic use and the occurrence of skin lesions.

Figure 1. Common conditions associated with *Blastocystis* sp. positivity in our patients



Skin manifestations (Picture 1.) occurred in 9 patients, predominantly with females (7 out of 9 patients). Those were urticariform – itching, tiny rashes 1-2 mm in diameter – in 5 patients, and reddish-brown infiltrated papules, sometimes with hyperaemic and irregular borders, in 3 subjects. In one patient a nutritive allergy was found by a dermatologist. Only one patient with UC had skin rashes.

Picture 1. Numerous, 5-10 mm diameter sized, sporadically confluent, itching, urticariform-papular skin lesion on back and gluteal region.



Eight patients with cutaneous symptoms received metronidazole, although 3 needed an additional antimicrobial agent due to co-infection (e.g. *Borrelia burgdorferi*, *Mycoplasma pneumoniae* and *Escherichia coli* infections.) One patient had doxycycline therapy. With regard to gastrointestinal

symptoms and skin manifestations, all patients became asymptomatic after antimicrobial treatment.

40 of the patients had persistent gastrointestinal symptoms, both with and without skin lesions, justifying the initiation of antimicrobial therapy. We used metronidazole in 92.5% of the cases, but 10 patients needed combined therapy or a switched antimicrobial therapy, using mainly rifaximin or sulphametoxazole and trimethoprim due to synchronous infections and the ineffectiveness of metronidazole.

7.3. Study III: Efficacy of influenza vaccination

7.3.1. Patient characteristics

209 IBD patients (127 with CD, 82 with UC) were eligible and enrolled in the study. 156 patients received influenza vaccination, while 53 patients (control group) refused the vaccine – the willing to vaccination was 66.3%. Whole virion vaccine was given to 57; split vaccine was given to 99 patients. The mean age of the vaccinated patients was 27.9 years; 84 were women, 72 were men. In the control group the mean age was 30.7 years, 29 were women, 24 were men. Of the 156 vaccinated patients, 98 had CD, 58 had UC. Median disease duration was 9 years for CD (IQR 5-13), and 9 years for UC (IQR 4-15.8). Of the control subjects, 29 had CD and 24 had UC. Median disease duration was 7 years for both CD (IQR 5-14), and for UC (IQR 4.5-12).

Of the 156 vaccinated patients 115 patients received immunosuppressive therapy. The non-immunosuppressive group of vaccinated subjects was composed of 41 patients. Of the 53 control subjects immunosuppressive therapy was given to 32 patients. Twenty-one patients were free of immunosuppressive therapy. 8.3% of the patients are regularly vaccinated against seasonal influenza virus. 39 patients (21.5%) received the last vaccination at the a year, 25 patients (13.8%) within 3 years and 3 patients (1.7%) within 5 years. 63% of the patients received the last vaccination more than 5 years ago.

7.3.2. Antibody titers for Influenza A and B subtypes

The values of preimmunisation antibody levels of influenza A and B titers varied between 33.8-341.3 RU/ml and 45.8-248.7 RU/ml, respectively. The antibody values of the postimmunisation levels of influenza A and B titers varied between 43.9-301.5 RU/ml and 58.2-216.9 RU/ml. The postimmunisation antibody titers of Influenza A and B subtypes significantly increased in patients immunized with split virion vaccines (mean increase of antibody levels was 13.8 RU/ml for Influenza A and 17.4 RU/ml for Influenza B) compared to control subjects (mean increase of antibody levels was 9.4 RU/ml for Influenza A and 7.3 RU/ml for Influenza B) ($p=0.045$ for Influenza A and $p=0.03$ for Influenza B). Although the increasing tendency of titers measured at week 6 was also observed in control patients it was not significant.

The antibody titers of Influenza A and B significantly increased after the administration of split vaccine compared to whole virion vaccines ($p=0.03$ for Influenza A and $p<0.001$ for Influenza B). The postimmunisation antibody titers of Influenza B also increased significantly after administrating split vs. whole virion vaccine in patients treated with anti TNF-α ($p=0.002$). However, the study was not powered to directly compare the alterations in the postimmunisation antibody titers in vaccinated and control patients treated with thiopurines and anti-TNF-α alone or in combination.

7.3.4. Effect of vaccination on IBD activity

During the 4 months follow-up period, 1 of the control subjects and 21 of the vaccinated patients (8 CD, 13 UC) developed a flare up with an increased diarrhea or bloody stool. The mean CDAI at the flare up was 273 points, the mean pMayo score was 4 points. This was observed with both types of vaccine; however, of the 21 patients with flare up, 15 received IDFlu9 split virion vaccine. The flare up developed a mean of 6 weeks after the vaccination. These 21 patients were followed up for 4 months additionally. Absence of flare was observed in 3 weeks. Disease activity resolved spontaneously in 11 patients, 2 patients needed corticosteroids, 5 antibiotics. Three patients remitted after the following biological therapy.

7.3.5. Side effects and development of influenza-like symptoms

26 patients developed local reaction (pain, redness, warmth, swelling) after vaccination, 32 had systemic symptoms (shivering, subfebrility, fever, fatigue, malaise, headache, muscle pain) during the first week. The most common local reaction after any type of vaccine was redness at

the injection site, reported by 11%. The local reactions disappeared within 48 hours in every case. The most common systemic reactions were common cold and sore throat in 39.7 and 28.8% of the patients. Systemic reactions resolved within a week.

Upper respiratory tract infection like symptoms more frequently occurred within the first week in vaccinated vs. control patients (31.4% vs. 9.4% p=0.002). During the further follow-up, no difference was shown between the two groups. Influenza-like symptoms occurred in 7 of the vaccinated and 1 of the control patients in the first 4 weeks, and 6 of the vaccinated and 3 of the controls between the 5 and 16 weeks. These influenza-like symptoms developed in 12 CD and 5 UC patients and all but one was on immunosuppressive therapy in both vaccinated and control group. Local and systemic reactions were more common in patients vaccinated with IDFlu9 split vaccine vs. Fluval AB (p<0.001). The local and systemic side effects developed after the influenza vaccination are summarized in Table 11.

Table 11. Local and systemic adverse events after vacctination.

Effect	**Fluval AB (n=57)**	**IDFlu9 (n=99)**
Local reaction		
Any reaction, n (%)	2 (3.5)	24 (24.2)
Pain, n (%)	1 (1.8)	11 (11.1)
Redness, n (%)	1 (1.8)	16 (16.2)
Swelling, n (%)	1 (1.8)	14 (14.1)
Itching, n (%)		6 (6.1)
Systemic reaction		
Any reaction, n (%)	28 (59.1)	70 (70.7)
Shivering, n (%)	0	3 (3.0)
Fever ≥38^{o}C, n (%)	0	16 (16.2)
Unusual fatigue, n (%)	1 (1.8)	2 (2.0)
Malaise, n (%)	2 (3.5)	13 (13.1)
Headache, n (%)	4 (7.0)	18 (18.1)
Muscle pain, n (%)	5 (8.8)	11 (11.1)
Arthralgia, n (%)	1 (1.8)	7 (7.0)

Sore throat, n (%)	15 (26.3)	30 (30.3)
Common cold, n (%)	15 (26.3)	47 (47.5)
Sneeze, n (%)	12 (21.1)	22 (22.2)
Coughing, n (%)	6 (10.5)	21 (21.2)
Subfebrility, n (%)	10 (17.5)	8 (8.0)
Vertigo, n (%)	0	4 (4.0)

7.4. Study IV: Bolus administration of steroid therapy

7.4.1. Patient characteristics, clinical response

During the study period, 20 patients with IBD (5 with CD, 15 with UC) were enrolled. At day 5, all but one patient achieved clinical remission. One patient proved to be refractory to intravenous steroid and needed rescue therapy therefore she was excluded because of treatment failure. Methylprednisolone was tapered weekly and stopped at week 12 in these 19 patients who could complete the study. The clinical characteristics of the participated 19 patients are presented in Table 10. Ten patients had already been diagnosed with IBD, while the remaining 9 patients were at disease onset. Although the male/female ratio was higher in Group II, baseline clinical characteristics of patients did not differ significantly between the two treatment groups. CDAI and pMayo score showed decreasing pattern in both groups during the steroid therapy.

CDAI and pMayo score decreased to a median value of 21 and 0 (median CDAI 35, median partial Mayo score 0 in Group I and 12 and 0 in Group II) at the end of the steroid therapy. Median CDAI and pMayo scores during the „conventional" and the „bolus" methylprednisolone treatment periods are indicated in Table 12. The mean value of CRP, ESR, leukocytes and thrombocytes after steroid therapy were 7.4 mg/l, 9.7 mm/h, 7.935 G/l and 238 G/l. The effects of bolus therapy on the clinical and laboratory parameters of disease activity did not differ from the conventional administration. The patients in both groups had not relapsed at the discontinuation of steroid therapy.

Table 12. Median CDAI and pMayo scores during the „conventional" and the „bolus" methylprednisolone treatment periods

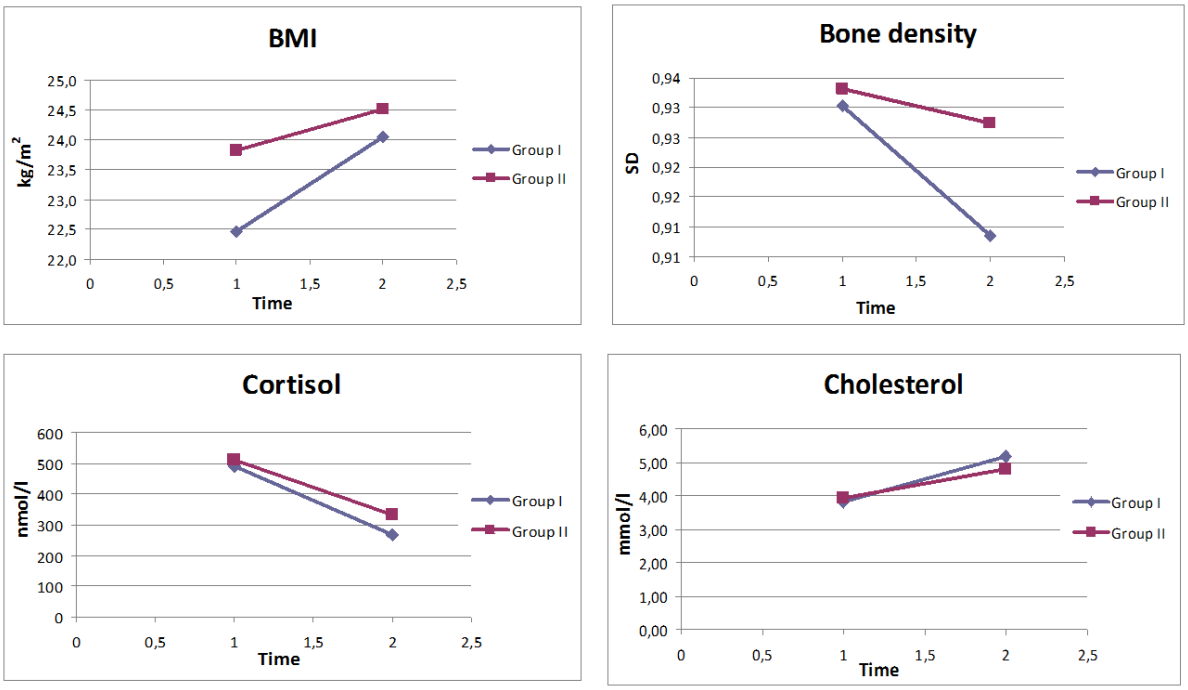
	Group I		**Group II**	
Weeks	Median CDAI	Median pMayo	Median CDAI	Median pMayo
0	204	6.2	164	5.1
2	184	5	152	4
4	146	3.4	126	2
6	86	1	90	0
8	72	0	64	0
10	68	0	48	0
12	35	0	12	0

7.4.2. Changes in adrenal glands hormone secretion, in the lipid and bone metabolism after methylprednisolone therapy

In Group I, BMI increased significantly at the end of the steroid therapy (p=0.008). In Group II, no difference was observed in BMI before and after the steroid therapy. Total body composition analysis showed significant decrease in BMD in Group I (p=0.032). Body fat percent showed a tendency to be higher at the end of steroid therapy in Group I, although the difference was not significant.

Considering the laboratory parameters, serum cholesterol level increased significantly in Group I patients after steroid therapy (p=0.028). The decrease in serum cortisol level was more remarkable in Group I vs. Group II after steroid therapy (p=0.02 and p=0.055). Figure 2 summarizes the significant changes in the examined parameters.

Figure 2: Significant changes in the examined parameters after 12-week methylprednisolone therapy in Group I and II



No changes were detected in the waist and hip circumference, T and Z scores, electrolytes, liver and renal function, serum glucose, serum calcium, triglyceride, DHEA, TSH, PTH and ß-crosslaps before and after the steroid therapy neither in Group I, nor in Group II.

7.4.3. Steroid-related side effects

The most common side effects occurring during the therapy were the Cushingoid appearance, development of acne, fatigue, gastrointestinal complaints. Side effects were presented in 5/9 (55.6%) vs. 4/10 (40%) of the patient in Group I and II. Cushingoid appearance did not occur in Group II. The side effects are summarized in Table 13.

Table 13. Side effects developed in patients in Group I and II

Group I		Group II	
	Side effect		**Side effect**
Patient 1	Cushingoid appearance	**Patient 1**	Acnes
Patient 2	Cushingoid appearance	**Patient 2**	Acnes
Patient 3	Acnes	**Patient 3**	Arthralgia
Patient 4	Acnes Arthralgia	**Patient 4**	Fatigue Hypertension
Patient 5	Cushingoid appearance Arthralgia Fatigue Nausea Stomatitis		

7.5. Study V: Side effects of cyclosporine A therapy

The average duration of cyclosporine therapy was 9.6 months. The mean oral dose of cyclosporine was 4.7 mg/kg, the mean fasting level of cyclosporine was 193.18 μg/L and the mean 2 hours-post-dose level was 866.04 μg/L.

Table 14. Mean levels of serum total cholesterol, triglyceride and creatinine.

	Before cyclosporine therapy	**During cyclosporine therapy**	**After cyclosporine therapy**
Mean level of serum total cholesterol (mmol/l)	172.9	235.5	196.2
Mean level of serum total triglyceride (mmol/l)	127.4	155.7	112.4
Mean level of serum creatinine (μmol/l)	0,81	0.83	0.78

Side effects occurred in 52 patients (72.2%) during the therapy (Figure 3.). The most frequent side effects were hypertension (15.23%), tremor (13.8%), hypertrichosis (9.72%), myalgia and muscle cramping (11.1% and 4.16%, respectively) and numbness of legs (5.5%). Nephrotoxicity or hepatotoxicity occurred in 6 patients (8.33%). Increased serum cholesterol and triglyceride levels were detected in 47.2% and 19.4% of the patients, respectively. Major side effects resulting in discontinuation of the cyclosporine therapy occurred in 21 patients. The most frequent major side effects were adverse muscle reactions (47.6%) and hypertension (38.1%), followed by gastrointestinal side effects including liver enzyme abnormalities (23.8%) and skin side effects (14.3%). Major side effects are detailed in Figure 4.

Figure 3. Side effects occured in our enrolled patients.

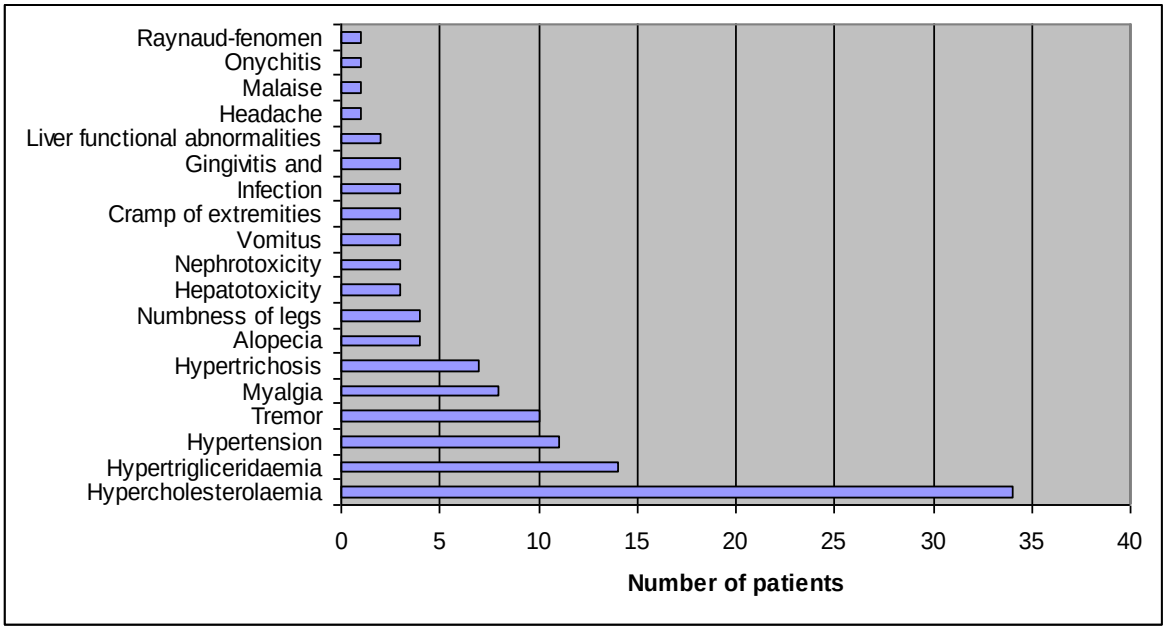
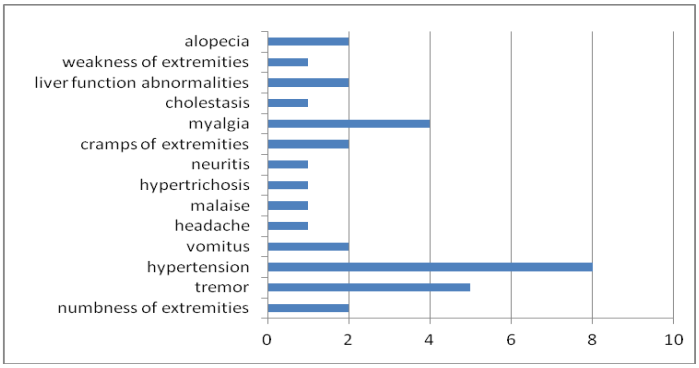


Figure 4. The major side effects during cyclosporine therapy.



The mean value of serum cholesterol levels (Table 14.) measured before starting the cyclosporine therapy was 172.9 mg/dL (range: 84.9 -331.6 mg/dL) and in the control group, it was 200 mg/dL (115.8-370.6 mg/dL). During the therapy, serum cholesterol levels increased to the mean value of 235.5 mg/dL (range: 134.4-370.6 mg/dL), while this value remained within normal range in the control group (200 mg/dL, range: 137.8-301.5 mg/dL). After discontinuation of the cyclosporine therapy, serum cholesterol levels decreased to the mean value of 196 mg/dL (range: 88-323.2 mg/dL) (Figure 5.). Decreased levels of serum cholesterol were detected in patients treated with combined infliximab-steroid group after cyclosporine therapy (190.3 mg/dL; range: 110.8-277.6 mg/dL). Statistically, serum cholesterol levels increased significantly during the therapy and remained higher for one year after the discontinuation of CyA ($p<0.001$, $p<0.001$). Furthermore, serum cholesterol levels measured during the cyclosporine therapy were significantly higher than those after the discontinuation of the drug ($p<0.001$). In the cyclosporine group, cholesterol levels were considerably higher during therapy than in the control group ($p<0.001$).

Cholesterol levels measured after cyclosporine therapy were found to be significantly higher in patients with an adverse reaction ($p= 0.045$) vs. patients without adverse reactions. In both groups, serum cholesterol levels showed a significant elevation at the time of the cyclosporine therapy ($p<0.001$) and afterwards ($p<0.001$; Figure 6.). No difference was found between patients who had only minor side effects during therapy and those who had none, but those patients who had major adverse effects had higher cholesterol levels after therapy ($p=0.013$).

Figure 5. Serum cholesterol levels during study period.

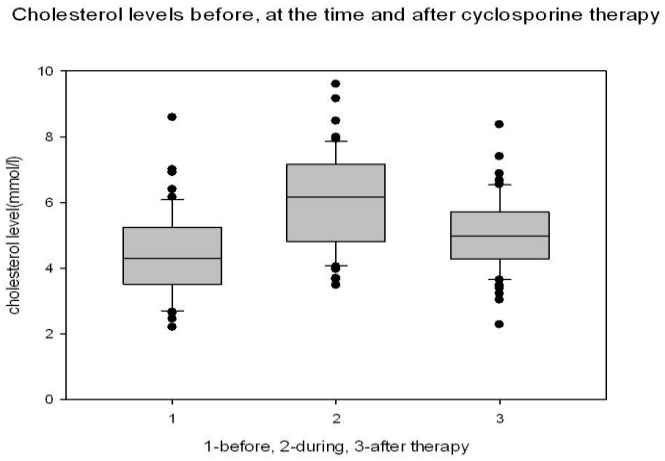
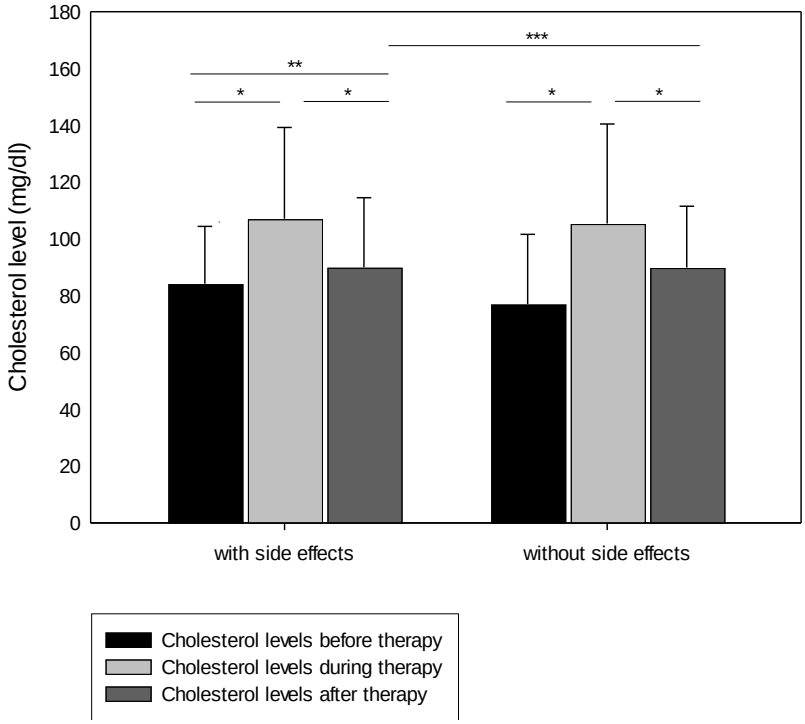


Figure 6. Cholesterol levels after cyclosporine therapy were significantly higher in patients with an adverse reaction vs. patients without adverse reactions.



*: p<0.001; **: p=0.015; *** : p= 0.045

Elevated total cholesterol levels were noted in 65.4% of the patients with detectable side effects. Increased serum cholesterol levels (mean value of 270.6 mg/dL) were detected in 54.5% of the patients with hypertension. Hypertension was defined as systolic pressure at or above 140 mmHg and diastolic pressure at or above 90 mmHg.

One of the most common adverse reactions was tremor. Increased serum cholesterol levels were noted in 70% of those developing tremor (mean value of 283.4 mg/dL). Neurological side effects (tremor, sensational abnormalities) occurred in 25% of the patients.

Myalgia was also a frequent side effect; 5 of 8 patients with myalgia had a high serum lipid profile (mean total cholesterol of 252.5 mg/dL). A significant correlation was found between muscular side effects (myalgia, cramping of extremities, numbness of legs) and elevated serum triglyceride levels ($p=0.038$).

Serum triglyceride levels were elevated in 19.4% of the patients. The mean serum triglyceride levels before, during and after cyclosporine therapy were 127.4 mg/dL, 155.7 mg/dL and 112.4 mg/dL, respectively (Table 14.). No difference was found in triglyceride levels compared to the control group.

Serum creatinine levels were slightly elevated in only 3 patients during cyclosporine therapy (Table 14.). No significant correlation was shown between cyclosporine therapy, the frequency of side effects and serum creatinine levels. In addition, creatinine levels did not differ between the cyclosporine and the control group.

8. DISCUSSION

It is well-known that the infection rates in patients with IBD is greater than in the normal population; infectious agents are involved in the aetiopathogenesis, but it can also initiate a relapse of the IBD (100). Some studies suggest that the aetiology of IBD with altered intestinal microbiome is a result of innate genetic defects in the intestinal epithelial and mucosal barriers, which can contribute to bacterial translocation. In addition, the aetiological background may involve a microbial imbalance in the intestine or dysfunction in the intestinal inflammatory cascade, leading to pathologic proliferation of cytokines (101). Although an exact aetiology of IBD has not been confirmed yet, the importance of microorganisms in the intestine is obvious. Due to its significance in daily routine, infection rate during an IBD relapse and the clinical utility of faecal inflammatory markers were assessed in our study. IBD is an independent risk factor of CDP, mainly UC patients are susceptible to infection, and thus microorganisms have considerable significance in patient management (20, 102,103,104). In our study, the UC/CD ratio in the *C.difficile* cohort was approximately equal.

CDP (51.1%) and *Candida* (10%) infections were the most frequent results of faecal microbiological examinations in our relapse cohort, suggesting an imbalance of intestinal microflora and some connection between flare-ups of IBD and CDP. A differential diagnosis of a relapse and CDP is difficult in some cases; furthermore, both can occur at the same time, thus a faecal microbiological examination and tests for both Toxins A and B are recommended in the event of a flare-up, as the evidence-based consensus of European Crohn's and Colitis Organisation also suggests (20). *C. difficile* is mostly a community-acquired infection, thus it mainly develops among our outpatients; however, hospitalisation, colectomy and mortality rates are higher in a population with CDP.

Colonising microbes inhabiting the intestinal mucosa are important for normal intestinal function and homeostasis. Dysbiosis develops when the composition of intestinal microbiome is unfavourable, and this condition may lead to various diseases. Altered microbiome may be a cause or consequence of a disease, but it may develop parallel with an abnormal condition (105). The balance of intestinal homeostasis is a sensitive system of intestinal microbiota, epithelium and immune cells (106). An alteration of colonic microbiota has been suggested in IBD (107), given that intestinal flora is altered in patients who relapse, which in turn allows the unopposed

proliferation of *C.difficile* and *Candida*. The frequency of the community-acquired CDP has been increasing worldwide with incidence rates of 3 to 40% (58,108,109,110). The occurrence of CDP was remarkably high in our IBD patients (40.3%); in addition, the frequency of *Candida* infections was 10% among the enrolled subjects. Presence of *Candida* in gastrointestinal tract is commonly benign, but in susceptible individuals with low level of inflammation (eg. IBD) may promote colonisation that promotes further inflammation, thus may become a trigger factor (111). The role of *C.difficile* may be the similar.

The disease course of a *C.difficile* infection can be extremely serious, even life-threatening complications may occur, which is confirmed beyond a doubt by the mortality rate of hospitalised IBD patients with a *C.difficile* infection (112). Although, CDP was not severe in most of the cases in our study: half of the subjects showed signs of clinical remission within 8 weeks of follow-up, and serious complications did not occur during the tenure of the study. However, hospitalisation was needed in 19 of 51 cases, most of them because of a severe flare-up requiring intravenous steroid treatment. It seems that CDP is also a trigger and an additive factor; however, the isolated treatment of the infection does not affect symptomless patients in the vast majority of the cases. In some studies, antimicrobial and immunomodulator agent-exposure has been shown to increase the possibility of a *C.difficile* infection and carriage (61). Basically, any type of antibiotic may be linked with the manifestation of a *C.difficile* infection; however, the antibiotics most commonly connected to an occurrence of infection include penicillins, clindamycin, cephalosporines and fluoroquinolons (113). Regardless, antibiotic use for IBD patients in general seems to be a critical risk factor for the development of a CDP, as our results showed (where $p=0.03$). The use of immunomodulators specifically has not been confirmed in our study to be predictive of a CDP risk, however. The data available on immunomodulators is conflicting; some studies showed an increasing risk for CDP (61), although others did not confirm these results (114). The number of *C. difficile*-positive subjects taking immunosuppressants did not differ substantially compared to those patients not treated with immunosuppressants (26 vs. 21 subjects) in our study. A correlation between CDP and the use of gastric acid-suppressive agents, especially histamine blockers and proton pump inhibitors, has been suggested (115,116). However, similarly to our own findings (where $p=0.65$), other observational studies did not yield results to support the connection (117).

A strong correlation between intestinal inflammation and FC has been confirmed in IBD (118,119); however, its value can increase in enteral infections also (120,121). Enhanced levels

of MMP-9 have been noted in the intestinal tissue in the case of IBD and seem to be actively involved in the inflammatory and remodelling process (122,123). In this study, levels of FC and MMP-9 were measured both in faecal microbiology positive and negative cases; however, we did not find a significant difference between those groups regarding the two assessed faecal inflammatory parameters. We thought that if the measurement of faecal markers helps in making a prompt decision about the need of antibiotics in the treatment of a flare-up, it would be a supportive method, since the result of microbiological examinations usually takes a few days, which can cause a delay in the initiation of the targeted therapy. Although we observed higher levels of FC and faecal MMP-9 in natural relapses than in infection-induced relapses, the statistical analysis did not confirm this observation.

Laboratorial techniques have basic importance in diagnosis of infectious and other organic intestinal disorders. However, enteral infections caused by univocal microorganisms may present clinical signs and symptoms, e.g. extraintestinal manifestations that make differential diagnostic difficulty. *Blastocystis* sp. is a common intestinal parasite worldwide that often causes atypical gastrointestinal symptoms. The prevalence is higher in developing countries and is usually connected to poor standards of hygiene, exposure to animals and the consumption of contaminated food or water (124). The occurrences of *Blastocystis* sp. infections spanning the time period relevant to our study were an overall 6% in patients treated at the clinics at the University of Szeged and 5.4% among patients examined in our clinic and whose data was used in this study. Despite this parasite coming into question as a possible causative microorganism of some intestinal illnesses, its pathogenicity and clinical significance has not yet been confirmed. Some studies reference *Blastocystis* sp. as a potential pathogen (125), but the role of the protozoon in the development of certain intestinal disorders is not fully understood. However, data are available about *Blastocystis* sp. supporting a connection with IBS and IBD (126,127,128, 129). In the study of Tai et al *Blastocystis* sp. infection has been confirmed in 12.2% of relapsing ulcerative colitis patients (130). Moreover, recently published study suggests that *Blastocystis* sp. subtype 3 can trigger the proliferation of human colorectal cancer cells (131). On the other hand, this parasite is also common in healthy individuals, suggesting that it can be a component of normal intestinal microbiota (132). Molecular characterisation from symptomatic and asymptomatic carriers should help to reveal the clinical implications of this microorganism (133,134). Distribution of the 9 subtypes of *Blastocystis* sp. found in humans seems to be dependent on geographic locations. Subtype 3 is the most widespread in every continent except

the US, where subtype 1 is the most common (135). We did not assess subtypes of *Blastocystis* sp., because during the time period from that data was used it was not part of routine microbiological examinations, furthermore, it currently has no significance in patient management and therapy. However, the relevance of the subtypes is deemed worthy of further study.

Extraintestinal manifestations of *Blastocystis sp.* infection, such as urticaria and other skin lesions, and the role the infection many have in these manifestations have already been described in various publications (136,137,138,139,140). In a recent study (141), 13.7% of the patients with allergic skin symptoms were infected by *Blastocystis sp*.; furthermore, no other allergic agent was found to offer explanation why the urticaria manifested. The significance of *Blastocystis sp.* as background cause of urticaria was indicated in prospective research from Peru, which revealed a connection between symptomatology and *Blastocystis sp.* positivity; the symptoms in question were urticaria, abdominal pain and meteorism (142). Also supporting the connection was the gradual disappearance of dermatological symptoms after treatment and eradication of *Blastocystis* (143,144). It has been suggested that cutaneous lesions are immune-mediated, but the mechanism is not completely clear (145). In our study, 11.25% of infected patients manifested skin symptoms, predominantly the female patients. Disorders were mainly urticariform and dermatitis-like lesions. There was no significant difference observed depending on the amount of protozoa in the samples; therefore, just a few *Blastocystis sp.* cells were enough to induce a cutaneous manifestation. Skin symptoms improved after specific antimicrobial treatment.

The symptoms of *Blastocystis sp.* infection are usually nonspecific, and along with other symptoms, flatulence, nausea, vomiting and bloating may manifest as a result of this infection. Depending on the severity of the infection, everything from mild chronic diarrhoea to acute gastroenteritis may occur (67). Jones et al reported fatigue, skin rash and diarrhoea as the most frequent symptoms of *Blastocystis sp.*infection, and suggested connection between *Blastocystis* sp. and chronic gastrointestinal illness (146). Several variations of signs and symptoms were associated with *Blastocystis sp.* infection in our cohort, ranging from nonspecific intestinal symptoms to cutaneous disorders. No specific laboratory parameter was discovered that would be indicative of parasite infection, however, elevated CRP and white blood count was shown in *Blastocystis sp*.-associated skin manifestations. Interestingly, we did not find elevated eosinophil count in the whole cohort, regardless of whether or not the patients had accompanying skin

manifestations. Thus, we can state that eosinophilia is not an obligatory laboratory finding in protozoon infections, such as *Blastocystis sp.,* with or without skin lesions. In 18.75% of our patients another intestinal microorganism was discovered parallel to *Blastocystis sp*., e.g. *Ca. jejuni, Ca. lari, C. albicans, C. glabrata, C. difficile* and *E. histolytica*. On the other hand, skin lesions only manifested with one of them, and symptoms improved after specific antimicrobial therapy. Therefore, the causative agent of the skin lesions was presumably the *Blastocystis sp.* In addition, 40% of the enrolled individuals also suffered from additional underlying chronic intestinal abnormalities, such as colon tumours, IBD, IBS, coeliac disease, lactose intolerance or diverticulosis. Considering the great number of patients with the above mentioned diseases the vulnerability of the intestine is suggested to be a risk factor for a *Blastocystis sp.* infection. Nine percent of subjects suffered from IBD among assessed *Blastocystis sp.* positive population, although only one patient with UC had skin manifestations at the time of study period.

IBD associated infection and its management is a daily routine as our results showed as well; therefore, prevention has a significant importance. Prevention should be primary endpoint of infection control. However, questions still exist regarding passive immunization of immunosuppressed patients.

Vaccination is a conventional strategy for the prophylaxis of vaccine-preventable infections, especially in patients suffering from immune-mediated diseases (147). Our multicentre survey revealed that most of the patients are willing to be vaccinated against the seasonal influenza virus if it is recommended and administered by the gastroenterologist, although promoting prevention and prophylaxis seemed to be underutilized in the previous years. In a survey done in IBD patients, only 28% of the patients reported yearly influenza vaccination (148). Possible explanations for under-vaccination of IBD patients are unawareness of the increased infection risk and concerns about the safety and efficacy of the vaccination. In our study, every patient was recommended to be vaccinated and, after detailed consultations, two thirds of them accepted vaccination, which indicates that informing and assuring the patients may increase the willingness to take the vaccination. Patients on immunosuppression or biological therapy are supposed to be at increased risk of influenza (149), thus, annual influenza vaccination is recommended for all patients with IBD on immunomodulators (20). It is well-known that the majority of the studies examining the efficacy of influenza vaccination in IBD patients have been carried out in children. Mamula et al. (78) observed a reduced seroconversion rate and geometric mean titre after influenza vaccination in IBD patients receiving immunotherapy compared with

healthy controls. Another study found that vaccination responses were similar among children with IBD, regardless of immunosuppressive status (79). These studies revealed that vaccination against influenza in children with IBD is well tolerated and induces immune response, although anti-TNF therapy may selectively impair response to specific serotypes. deBruyn et al. also found that children with IBD were less likely to mount an immunogenic response for influenza B compared with controls (150). According to our results, the post-immunisation antibody titres of both Influenza A and B subtypes were increased in patients vaccinated with split virion vaccines vs. control subjects, although the study was not powered to detect differences between the different immunosuppressive maintenance therapies.

Some studies observed an impaired immune response after influenza vaccination in patients treated with anti-TNF agents. However, response rates to influenza vaccination in patients treated with immunomodulators and biologicals are somewhat conflicting. Andrisani et al. revealed that seroprotective titres in the patients were comparable to healthy controls. Seroconvertion rate was lower in IBD patients than in healthy controls either on anti TNF-α monotherapy or combined with immunosuppressants (80). In a study conducted on patients with rheumatoid arthritis and CD, postvaccination geometric mean antibody titres against influenza (A/H3N2 and B) were significantly lower in patients treated with anti-TNF (infliximab, adalimumab or etanercept) compared with patients not receiving anti-TNF therapy and the healthy controls (151). Our results revealed that the increase in post-immunisation titres of only Influenza B subtype was not influenced by anti TNF-α therapy – although only in case of the administration of split virion vaccines.

The results about the immune response to split virion vaccine vs. whole virion vaccine also seem to be controversial. A study evaluating both split virion and whole virion H5N1 vaccine formulations in children revealed that the whole virion vaccine may be immunogenically better than the split virion vaccine, confirming the previous findings in adults (152). However, in another study, AS03B-adjuvanted split virion or a non-adjuvanted whole virion H1N1 (2009) vaccine was used in 943 children and both vaccines proved to be safe and immunogenic (153). In Hungary, whole virion vaccines are supported by the Hungarian National Health Fund (OEP), and thus these are used routinely in the clinical practice. However, in this study, we had the opportunity to assess the efficacy of the less immunogenic split virion vaccines. Our results demonstrated significantly higher post-immunisation antibody levels in case of split virion vaccines. The frequency of the side effects was higher in case of split vaccines; however, it is

important to note that no severe adverse reactions occurred. The rate of influenza-like infections was quite low during the follow-up period and did not differ significantly in vaccinated vs. control subjects. Influenza-like symptoms were more likely to develop in patients with immunosuppressive therapy in both vaccinated and control patients.

Antibody response is an integral component of normal immunity. Successful humoral immunity depends on cellular interactions and it is not unusual to observe deficient antibody responses in immunologically compromised hosts. Age and concomitant therapy may also influence immune response after influenza vaccine – thus that it is very difficult to assess whether in vitro immunity corresponds to in vivo immunity. In a study from the ‘80s (154), 14 monkeys received a trivalent influenza vaccine and antibody response was determined based on a change in plasma antibody content before and after the vaccine. In vitro anti-influenza antibody synthesis was found to correlate well with the in vivo response. Ershler et al. also measured the antibody response in young and elderly volunteers after in vivo and in vitro immunisation with trivalent influenza vaccine. After in vivo immunisation, plasma antibody levels and in vitro synthesis capabilities significantly increased in the young subjects. The capacity for in vitro immunisation was also greater in the young, but the difference did not reach statistical significance and there was no correlation with in vitro immunisation potential and the response after in vivo vaccination (155).

It is not in question whether inactivated influenza vaccination is recommended yearly for patients with IBD. Despite the initial results, a number of studies have shown that immunosuppressive and anti-TNF therapy are safe and effective in regard to the response to annual trivalent inactivated influenza vaccination. In this cohort, split vaccines seemed to be more effective than whole virion vaccines, and split vaccines also resulted in an increase in post-immunisation titres of Influenza B subtype besides anti TNF-α therapy.

The use of immunosuppressants revolutionized the therapy of immuno-mediated and autoimmune diseases; however, this type of drugs increases not only the probability of infections, but also other side effects (156,157). For the past 30 years, corticosteroids have been the mainstay of therapy in patients with moderate to severe active IBD (158). Intravenous therapy generally produces rapid improvement of symptoms. Once improvement has been achieved, corticosteroids should be tapered gradually per week until the drug is discontinued. The mean goal of IBD therapy is to decrease the steroid-related side-effects and to minimize steroid dependency with the development of new series of anti-inflammatory glucocorticoids with

enhanced topical potency and less systemic activity such as budesonide or beclamethasone (159). Less attention is paid to the dosing of steroids, although it seems to be also important.

Although are no trials between different steroid-tapering regimens, the goal in the daily practice is to get patients off corticosteroids within 12 weeks and maintain disease remission. Alternatively, alternate-day corticosteroid therapy can also be used in patients with refractory Crohn's disease – even for longer time (160). However, some available evidence suggested that the manner of corticosteroid tapering probably did not change the long term outcome in IBD (161). Use of "bolus-administered" steroids is a novel possibility to optimize the therapy. Bolus administration is actually an untested manner that has been anecdotally recognized to be more effective than the conventional use of steroid therapy. Multiple doses of steroids were previously shown to cause more adverse effects than a single dose (162). In a single-center, double-blind trial performed by Bossa et al. patients with a severe attack of UC were scheduled to receive equal iv. doses of methylprednisolone, randomly given as either a bolus injection administered twice daily or continuous infusion (163). Methylprednisolone given as a continuous infusion was no better than bolus administration in terms of efficacy and safety. The aim of bolus steroid therapy is to get quicker and stronger anti-inflammatory effect. Giving a higher dose of methylprednisolone, an immediate profound anti-inflammatory effect is supposed to be achieved with lower toxicities and no prolonged suppressive effect on the hypothalamic-pituitary axis.

Our results did not show any significant difference according to the disease outcome between the two administration types at the end of the therapy and at follow up times for any of the clinical or laboratory parameters measured, confirming the same efficacy of bolus therapy as in case of conventional administration.

The widespread use of corticosteroids has been associated with an increased incidence of a variety of adverse effects involving the musculoskeletal, the endocrine, the metabolic system, neuropsychiatric wellbeing, the GI system, the skin, eyes, infectious risk, the cardiovascular and the hematological system (164). Dosage and duration of therapy are some of the most important factors influencing the development of the toxic effects of corticosteroids. Although no data is available on the harmful effects of short term corticosteroid therapy on lipid and bone metabolism, our results revealed that short-term use of steroids increases BMI and body fat percent and decrease bone density. Common adverse effects of short term therapy include moon face, mood changes, insomnia, GI intolerance, weakness, fluid retention, weight gain, increased

appetite, increased infections, amenorrhea, elevated blood glucose, slow wound healing, striae, and acneiform rash. Alternate-day steroid therapy may decrease hypothalamic-pituitary- adrenal axis suppression and therefore the development of certain side effects (165). However, in this study, the most common side effects occurred more frequently in patients with conventional vs. bolus-administration steroid therapy.

Osteoporosis is present in 30–45% of patients with CD, and its rate is somewhat lower in patients with UC (166, 167). Osteopenia is likely related to the chronic inflammatory process itself, and furthermore triggered by steroid use. Steroid related osteoporosis is multifactorial; decreased calcium absorption, development of secondary hyperparathyroidism, stimulating osteoclast activity, and decrease osteoblast production are only some of the potential etiological factors (168). Hyperlipidaemia is also a common side effect of steroid therapy (169). Steroids are supposed to influence lipid metabolism by redistributing body fat and facilitating of effects of lipolytic agents. Large doses of glucocorticoids lead to redistribution of fat to the upper trunk and face, with a concomitant loss of fat in the extremities (170). Our result revealed beneficial effect of bolus-administered corticosteroid therapy on bone density and body fat percent.

Side effects related to lipid metabolism are presented in patients on cyclosporin A therapy also. Cyclosporine is one of the most effective therapeutic choices for patients with severe, refractory UC (84). Cohen et al. investigated (171) the quality of life of 42 patients with severe UC and found that patients who underwent colectomy assessed their own quality of life and health status to be worse than those who had been treated with cyclosporine. However, the frequent occurrence of side effects may limit the use of CyA in the treatment of UC. Although 72.2% of our patients developed side effects, none of these proved to be life-threatening. It should be noted that CyA had to be discontinued due to intolerable severe side effects in 29.2% of the patients. 21 patients developed intolerable side effects; one achieved remission and 3 of the 21 patients had loss of response. Our findings suggest that CyA therapy may result in increased serum cholesterol levels even in the long-term, after discontinuation of the therapy (Figure 5).

Although most of the adverse effects associated with the use of CyA are dose-dependent, the study of van Assche et al. did not find any significant difference between the low-dose (2 mg/kg) and the high-dose (4 mg/kg) group with regard to cyclosporine-associated adverse effects (172). In addition, oral administration is recommended after the acute period to minimize the risk of developing side effects (173). In the acute phase of our study, CyA was administered

intravenously and in cases of good initial response, orally, with azathioprine treatment added in some cases (174). CyA was administered with a mean oral dose of 4.7 mg/kg.

Nephrotoxicity and hepatotoxicity are common and severe side effects of CyA therapy, but minor side effects also occur frequently during the therapy. Lichtiger et al. revealed (175) that the most common adverse reactions in UC patients treated with CyA were paraesthesia and hypertension. Both symptoms were also detected in our cohort; paraesthesia occurred in 4, hypertension in 11 patients. In addition, 10 of our UC patients experienced tremor, 7 had hypertrichosis and 11 noted myalgia or muscle cramping. Weber et al. reported (176) similar adverse reactions and the occurrence of infections, as well. Ten out of 19 patients developed side effects (2 of them had high serum creatinine levels, 1 had hypertension, 5 had tremor, 1 had hirsutism and 1 had gingival hyperplasia), two patients had systemic CMV infection, and one also had a herpes virus infection and oesophageal candidiasis. Although no prophylactic antimicrobial treatment was administered during the CyA therapy, the incidence of infections was low: only viral infection occurred in 3 cases (4.2%).

Nevertheless, there are limited data about the long-term effects of CyA therapy on the lipid profile. In our study, serum total cholesterol, triglyceride and creatinine levels before, during and after CyA therapy were compared in severe, steroid-refractory UC. Significant increase was found only in serum cholesterol levels during and after discontinuation of the CyA therapy. However, the control group did not experience the same results. Kuster et al. (177) reported that serum levels of CyA correlate significantly with total cholesterol levels, LDL-cholesterol levels, the apoB and cholesterol/HDL ratio, but not with triglyceride levels, suggesting that CyA may cause atherogenic dyslipidaemia. Other publications also describe similar or identical results regarding the relationship between CyA and cholesterol levels (178,179). Spinelli et al. studied patients treated with a combination of prednisolone and another immunosuppressive drug such as CyA, sirolimus, mycophenolate mofetil, or everolimus (180). The changes in lipid profiles were evaluated after 1 year of therapy, and dyslipidaemia was found to be frequent. Patients treated with CyA had worse lipid profiles than those in the other treatment groups. No data is available about the relationship between hypercholesterolaemia and other drug-related side effects.

Cyclosporine also has an effect on bile acid metabolism by blocking bile acid synthesis, hepatic uptake and secretion. These effects may lead to hyperlipidaemia. Previous trials have shown that CyA treatment affects bile salt kinetics and plasma lipid levels. However, corticosteroids

affecting the bile salt synthesis could not be excluded (181,182). In our study, we attempted to eliminate that effect with the control group treated with infliximab and streroids. Results showed higher cholesterol levels in the CyA cohort, as compared to the control group. Regarding the correlation between hypertension and increased CyA levels as a side effect, Wang et al. suggested that cyclosporine stimulates the renal sodium channel by elevating the level of cholesterol (183). Our results showed that hypertension and increased cholesterol levels occurred frequently in UC patients treated with CyA.

Other investigations suggested that the inhibition by CyA leads to dyslipidaemia by reducing apolipoprotein A1 gene expression. ApoA1 is a major component of HDL, therefore decreased levels of apoA1 result in low levels of HDL. The calcineurin pathway stimulates apoA1 gene expression; therefore, by inhibiting this pathway with cyclosporine, apoA1 levels may be reduced and as a result, HDL levels may also be reduced (184). Only total serum cholesterol levels were assessed in our study. A population-based study suggested that there are no benefits to measuring apoB or apoA1 compared with a total cholesterol and HDL cholesterol measurement (185). Furthermore, the study of Suk et al. (186) showed that calcineurin has an effect on the cAMP signalling pathway and furthermore, indirectly affects protein kinase A activation by regulating the phosphodiesterases, which play an important role in the adipokine gene transcription. In addition, calcineurin provides short- and long-term regulation to control gene expression and function.

Charco et al. (187) compared the long-term effects of tacrolimus and CyA therapies on serum cholesterol levels in liver transplant patients. The average follow-up period was 36 months, and the incidence of hypercholesterolaemia was found to be 34.6%. Higher mean cholesterol levels were found in the group treated with CyA ($p=0.01$). At the end of the study, a significant difference was found between the steroid and cyclosporine vs. tacrolimus and steroid-free groups. In our study, a control group treated with biologicals and steroids was employed to eliminate the additive effect of corticosteroids on the serum lipid profile. Results showed that cholesterol levels were higher in the CyA group than in the control group, and stayed at an increased level after therapy.

9. CONCLUSIONS AND NEW RESULTS ESTABLISHED IN THE STUDIES RELATED TO THE THESIS

1. Our data showed 40.3% of occurrences of CDP in IBD outpatients, and 51.1% of the patients in the relapse cohort were *C.difficile*-positive. We revealed a significant difference regarding CDP between IBD patients in relapse and those in remission (where $p<0.001$). Our results confirmed an association between previous antibiotic use and CDP. The occurrences of *C.difficile* and *Candida* positivity were excessively high in patients in an acute relapse, which suggests the importance of intestinal microbiota in IBD and an important role in the relapse, therefore stool analysis is recommended in flare-ups. FC and MMP-9 has no diagnostic value to differentiate between infection-induced and natural relapse.

2. We discovered that 11.25% of our enrolled patients exhibited skin manifestations associated to *Blastocystis sp.*, mainly on the females. The occurrence of *Blastocystis sp*. was 6% in symptomatic patients who required medical attendance in the time period between 2005 and 2013. We did not find significant difference in eosinophilia between patients with vs. without skin manifestations. 73.75% of the patients indicated that they had gastrointestinal symptoms: 40 patients complained of abdominal pain, 17 with blood in their stool, while other symptoms, such as meteorism (15 subjects), weigh loss (8 subjects), perianal pain or itching (6 subjects), passing stool with mucus (5 subjects), vomiting (2 subjects) and fever (2 subjects) were less frequent.

3. The third study revealed that two thirds of the patients agreed to influenza vaccination. Split virion vaccines proved to be more effective in the vaccination procedure: post-immunisation titres of both subtypes increased significantly after the administration of split virion vaccines compared with the controls and with those patients vaccinated with whole virion vaccines. The antibody titres of Influenza B also increased significantly in patients immunised with split virion vaccine and treated with anti TNF-α therapy. The high number of cases with pre-existing antibody levels can be explained with previous vaccinations and prior influenza infections, although cross-protection against influenza virus strains could also be present. Although no serious side effects were developed after influenza vaccination, influenza-like symptoms in the first weeks after immunisation occurred more frequently in patients receiving split virion vaccine.

Influenza-like symptoms did not differ significantly between vaccinated vs. control patients (8.3% vs. 7.5%). A mild relapse of the disease was observed in only 10% of the patients and was more common in vaccinated than in control subjects. In conclusion, our results suggest that IBD patients on immunosuppressive therapy are recommended to be immunised with split virion vaccines and that measuring the antibody responses is worthwhile in patients treated with immunosuppressants to determine the efficacy of influenza vaccination. Larger and more detailed studies are needed to compare the efficacy of these vaccinations and to examine the antibody response in immunocompromised patients.

4. Our results suggest that bolus tapering of equivalent doses of methylprednisolone administered in conventional daily doses has equivalent clinical efficacy, but more favourable side effect profile. As no significant difference was detected between the two administration types on the clinical and laboratory parameters of disease activity, it appears that bolus administration of corticosteroids can safely and effectively replace the conventional use of methylprednisolone for active IBD. Of course, further controlled, randomized trials are needed to confirm these results that may revolutionize steroid therapy in IBD. It should be noted, significant changes may develop in bone and lipid metabolism during even a short-term steroid therapy.

5. Serum cholesterol levels showed significant increase during CyA therapy compared to the time before use of the drug and to the control cohort of patients being treated with infliximab and corticosteroids. This elevation remained significant for a year. Serum cholesterol levels for the UC group with adverse events were significantly higher compared to patients who did not develop any side effects. Creatinine levels did not change significantly during CyA therapy. In the control group, cholesterol, triglyceride and creatinine levels did not change significantly during therapy or after its discontinuation. In conclusion, we found increased serum cholesterol levels in severe, steroid-refractory UC patients treated with CyA not only during the therapy, but also after its discontinuation, suggesting that CyA has a long-term effect on serum lipid metabolism. Furthermore, in the presence of other adverse events, cholesterol levels were significantly higher, suggesting that drug-related impairment of cholesterol biosynthesis and other side effects are rather common, therefore practically speaking, monitoring cholesterol levels during CyA therapy is recommended. Considering the high rates of hypercholesterolaemia as a side effect of cyclosporine therapy, this topic is worth to be studied further.

10. ACKNOWLEDGEMENTS

I am grateful to **Prof. Dr. Tibor Wittmann** and **Prof. Dr. György Ábrahám**, as a past and present head of First Department of Medicine, who gave me the opportunity to work at Department.

I would like to express my deep gratitude to **Prof. Dr. Tamás Molnár**, my research supervisor, for his patient guidance, enthusiastic encouragement and useful critiques of this research work.

Special thanks should be given to colorectal research team- **Prof. Dr. Ferenc Nagy, Dr. Zoltán Szepes, Dr. Klaudia Farkas, Dr. Bor Renáta** –for professional guidance and valuable support.

I am grateful to my **colleagues from the First Department of Medicine** and **co-authors** for giving me supportive advices and for outstanding help in research work. Without them this work could not be done.

I dedicate the thesis to **my parents** for all their love, never-ending support, endless patience and encouragement.

Lastly, I would like to thank to all of the people who have helped and encourage me during my doctoral study.

Our research was supported by the Hungarian National Development Agency grants (TÁMOP-4.2.2.A-11/1/KONV-2012-0035, TÁMOP-4.2.2-A-11/1/KONV-2012-0052; TÁMOP-4.2.2.A-11/1/KONV-2012-0073).

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12. ADNEXES

Annex I.

Parasitol Res (2014) 113:1585–1590
DOI 10.1007/s00436-014-3805-0

ORIGINAL PAPER

Do not forget the stool examination!—cutaneous and gastrointestinal manifestations of *Blastocystis* sp. infection

Anita Bálint · Ilona Dóczi · László Bereczki · Rolland Gyulai · Mónika Szűcs · Klaudia Farkas · Edit Urbán · Ferenc Nagy · Zoltán Szepes · Tibor Wittmann · Tamás Molnár

Received: 11 December 2013 / Accepted: 28 January 2014 / Published online: 20 February 2014

Abstract *Blastocystis* sp. is one of the most common parasites in the human intestinal tract. This infection commonly is accompanied by diarrhoea and abdominal pain, but extraintestinal symptoms, such as skin lesions, may also accompany the disease. In this study, our aim was to assess the frequency, clinical symptoms and skin manifestations of confirmed positive *Blastocystis* sp. infections. Data of 80 patients with confirmed positive *Blastocystis* sp. infections were assessed retrospectively. The average age of the patients was 46.3 years of age (with a range between 13 and 85 years of age). The number of female patients was higher than the number of males (48 vs. 32; 60 vs. 40 %). Gastrointestinal and dermatological symptoms and the results of routine biochemical and haematological blood tests of enrolled patients were collected and analyzed. The skin manifestations were analyzed using the data available (including descriptions, photos and histologies). We discovered that 11.25 % of our enrolled patients exhibited skin manifestations associated to *Blastocystis* sp., mainly on the females. The occurrence of *Blastocystis* sp. was 6 % in symptomatic patients who required medical attendance in the time period between 2005 and 2013. Of the 80 patients, 73.75 % indicated that they had gastrointestinal symptoms: 40 patients complained of abdominal pain and 17 with blood in their stool, while other symptoms, such as meteorism (15 subjects), weigh loss (8 subjects), perianal pain or itching (6 subjects), passing stool with mucus (5 subjects), vomiting (2 subjects) and fever (2 subjects) were less frequent. The prevalence of abdominal pain in the cohort without skin lesions was higher compared to those patients with skin problems ($p=0.007$). The mean value of C-reactive protein showed elevated levels, but eosinophils were within a normal range. In addition, we did not find significant difference in eosinophilia between patients with vs. without skin manifestations. Thus, we suggest that eosinophilia is not an obligatory laboratory finding in protozoon infections, such as *Blastocystis* sp. In the light of our results, we suggest a stool parasite examination for patients with skin lesions of unknown origin.

A. Bálint · K. Farkas · F. Nagy · Z. Szepes · T. Wittmann · T. Molnár (✉)
1st Department I of Medicine, University of Szeged, 8-10 Korányi fasor, 6720 Szeged, Hungary
e-mail: molnar.tamas@med.u-szeged.hu

I. Dóczi · L. Bereczki · E. Urbán
Institute of Clinical Microbiology, University of Szeged, Szeged, Hungary

R. Gyulai
Department of Dermatology, Venereology and Oncodermatology, University of Pécs, Pécs, Hungary

M. Szűcs
Department of Medical Physics and Informatics, University of Szeged, Szeged, Hungary

Introduction

Many gastrointestinal diseases are accompanied by cutaneous manifestations, and some skin disorders also may result in lesions in the intestine. Inflammatory bowel diseases of unknown origin, gluten-sensitive enteropathy, polyposis syndromes and gastrointestinal malignancies are bowel diseases which are often accompanied by special extraintestinal cutaneous manifestations (Ghevariya et al. 2013). However, a much more typical clinical scenario is a patient who has non-specific gastrointestinal symptoms and recurrent non-specific skin abnormalities. In this case, food allergy and small intestinal bacterial overgrowth are the most common causes, but testing first for a *Helicobacter pylori* infection is also a common practice, although a microbiological stool examination should be one of the first diagnostic procedures in these cases.

Blastocystis sp. is a protozoan genus with mitochondria-like organelles and at least one nucleus. It is an anaerobic microorganism; therefore, the mitochondria-like organelles presumably do not function in oxidative phosphorylation (Hotez 2000). This unicellular, obligate anaerobic protozoon, which resides mostly in the colon and the caecum, is one of the most common parasites in the human intestinal tract, and colonization has also been observed in many vertebrates. Incidence of *Blastocystis* sp. is varying worldwide. In healthy adults, the prevalence has been reported to be between 30 and 50 % in developing countries and between 1.5 and 10 % in developed countries (Sohail and Fisher 2005). *Blastocystis* sp. infection commonly causes diarrhoea and abdominal pain, but extraintestinal symptoms, such as skin lesions, may also accompany this condition (Katsarou-Katsari et al. 2008). The symptoms are usually non-specific, and along with other symptoms, flatulence, nausea, vomiting and bloating may manifest as a result of this infection. Depending on the severity of the infection, everything from mild chronic diarrhoea to acute gastroenteritis may occur. Jones et al. reported fatigue, skin rash and diarrhoea as the most frequent symptoms of *Blastocystis* infection and suggested connection between *Blastocystis* sp. and chronic gastrointestinal illness (Jones et al. 2009). On the other hand, it is not absolutely clear whether *Blastocystis* sp. is a commensal microorganism, harmless and only a cause of infection under special circumstances or a true pathogen (Basak et al. 2014; Stenzel and Boreham 1996). Several studies reported skin manifestations, particularly urticaria, connected to this protozoon (Grattan and Humphreys 2007; Tan Kevin et al. 2010). *Blastocystis* sp. and *Giardia intestinalis* (Karaman et al. 2011) are supposed to be the two most culpable protozoa on the basis of studies of the parasitological aetiology of urticaria. Since the symptoms of many of our patients at the 1st Department of Medicine support a link between chronic skin disorders and a *Blastocystis* sp. infection, it was decided that an analysis of the clinical data gathered regarding this parasitic infection would be advisable.

In this study, our aim was to retrospectively assess the attributes of patients with a confirmed positive *Blastocystis* sp. infection who came to our clinics at the University of Szeged between 2005 and 2013, primarily to examine the occurrence of clinical gastrointestinal symptoms and skin manifestations.

Patients and methods

The first part of our study determined the frequency of occurrence of a positive *Blastocystis* sp. infection among patients treated at the University of Szeged clinics between 2005 and 2013. A routine microscopic parasitological examination was performed on stool samples from 3,255 patients based on the patients' symptoms and complaints. Physicians requested stool examinations based on their assessment of the patients' clinical characteristics.

We subsequently assessed the frequency of *Blastocystis* sp. in 1,471 patients at the 1st Department of Medicine with adult gastroenterology profiles who needed a stool protozoon examination. In addition, data of 80 patients at the 1st Department of Medicine with confirmed positive *Blastocystis* sp. infections were assessed retrospectively. Every patient of our cohort complained about different gastrointestinal symptoms, such as diarrhoea, abdominal pain, bloody stool and meteorism. The results of routine biochemical and haematological blood tests of positive cases, as well as the results of any relevant examinations performed during dermatological evaluations were collected from the clinical database. Detailed data of visits to the Gastroenterology and Dermatology Department were also assessed. Clinical characteristics of the patients are detailed in Table 1. The examinations of the stool samples were performed at Department of Clinical Microbiology between January 2005 and May 2013.

The laboratorial diagnosis of *Blastocystis* sp. colonization was based on microscopic detection of trophozoites and/or cyst forms. All stool samples sent to the laboratory for routine detection of protozoa were examined directly using wet mounts both unstained and stained with Lugol's iodine solution (Garcia and Bruckner 1993a). This parasite has two main forms: various types of trophozoites and the cyst. Trophozoites generally can be found in diarrhoeal fluid. Vacuolar forms are the most common, varying in size on a scale between 6 and 40 μm. They are spherical and characterized by a large central body, which occupies approximately 90 % of the cell. Nuclei are located at the margin of the cell (Garcia and Bruckner 1993b; Leber and Novak-Weekly 2011). A culture of the organism from the stool was also performed in cases of microscopically negative direct smears. It was carried out using Boeck-Drbohlav-Locke-egg-serum medium (Garcia and

Table 1 Clinical characteristics of patients

Number of patients	80
Gender (male/female)	32/48
Age at *Blastocystis* sp. infection (years)	13–85 (mean: 46.3)
The most frequent symptoms in enrolled patients:	Number of cases:
Abdominal pain	40
Blood in stool	17
Meteorism	15
Weight loss	8
Perianal symptoms	6
Mucus in stool	5
Vomitus	2
Fever	2
Skin manifestations	8

Bruckner 1993c). The duration of the incubation was 48 h at 37 °C. After this time, a representative sample was taken on the border of liquid and solid phases and examined under a light microscope at ×400 magnification.

Symptoms, laboratory findings (elevated C-reactive protein level (CRP), increased number of eosinophils, leucocytes and lymphocytes, etc.) and accompanying chronic diseases (lactose intolerance, inflammatory bowel diseases, gastro-esophageal reflux disease, etc.) were statistically compared between patients with vs. without skin manifestations. Categorical data was analyzed using Fischer's exact test or one-sided Fischer's exact test. Continuous data was analyzed using Student's *t* test and Mann-Whitney *U* test. $P<0.05$ was considered statistically significant. For the statistical analysis, SPSS15.0 (SPSS Inc, Chicago, IL, USA) was used.

Results

Four thousand five hundred sixty-seven faecal samples from 3,255 patients from the clinics at the University of Szeged were analyzed at the Department of Clinical Microbiology, Szeged, Hungary, between January 01, 2005 and May 01, 2013. *Blastocystis* sp. infection was discovered in 275 of 4,567 faecal samples (196 of 3,255 patients)—the occurrence of *Blastocystis* sp. infection was 6 % in the symptomatic patients who required medical attendance in that period.

Eighty of 1,471 patients tested positive for *Blastocystis* sp. at our clinic during this 8-year period, representing 5.4 % of all stool samples sent during that time from our clinic to be examined for the presence of protozoa. Nine out of 80 people had accompanying skin manifestations. We did not find a statistically significant difference regarding age and gender between individuals with vs. without skin manifestations ($p=0.73$ and $p=0.34$, respectively).

Of the 80 patients, 73.75 % indicated that they had gastrointestinal symptoms: 40 patients complained of abdominal pain and 17 with blood in their stool, while other symptoms, such as meteorism (15 subjects), weight loss (8 subjects), perianal pain or itching (6 subjects), passing stool with mucus (5 subjects), vomiting (2 subjects) and fever (2 subjects) were less frequent. The prevalence of abdominal pain in the cohort without skin lesions was higher compared to those patients with skin problems ($p=0.007$). Full blood count, including thrombocytes, leucocytes (eosinophils, basophils, neutrophils, etc.), was within a normal range, except for CRP, which showed elevated levels (at a mean value of 13.89 mg/l in a range between 1 and 89.9 mg/l) at admission.

Significant differences were revealed between patients with and without skin manifestations regarding laboratory findings, namely the value of CRP ($p=0.038$), leucocytes ($p=0.024$), neutrophil granulocytes and its percentage ratio ($p=0.007$, $p=0.012$), thrombocytes ($p=0.002$) and red blood cell distribution width (RDW; $p=0.025$) were significantly higher in patients with skin manifestations. The value of lymphocytes ($p=0.011$) and monocytes ($p=0.023$, $p=0.011$) and their percentage ratios were higher in subjects without skin lesions. Interestingly, we did not find a significant difference in eosinophil counts.

Of the faecal specimens, 41.1 % contained few *Blastocystis* sp. cells, 5.5 % of specimens contained a moderate amount and 53.4 % contained a high number of the parasites. In 18.75 % of the cases, other microorganisms were also present besides *Blastocystis* sp., such as *Campylobacter jejuni*, *Campylobacter lari* and *Clostridium difficile*; among the fungi such as *Candida albicans*, *Candida glabrata* and *Geotrichum candidum*; as well as other parasites such as *Entamoeba histolytica* and *Entamoeba coli* (Table 2). Seven of those 15 patients had great number of *Blastocystis* sp. cells in stool samples, and six individuals had low numbers of this form; therefore, the number of *Blastocystis* sp. cells in co-infections did not make a difference. However, every subject had symptoms that improved after antimicrobial therapy. Only one patient with gastrointestinal co-infection (*C. glabrata*) had skin symptoms.

The diagnosed accompanying conditions (e.g. gastro-esophageal reflux disease, weight loss and hepatomegaly) of a *Blastocystis* sp. infection are summarized in Fig. 1. Weight loss, lactose intolerance and GERD were significantly more common in patients with skin lesions vs. without skin manifestations ($p=0.04$, $p=0.04$, $p=0.02$, respectively). Seven patients were treated with antibiotics previously due to other infections (prostatitis, pharyngitis, etc.) No association was found between previous antibiotic use and the occurrence of skin lesions.

Skin manifestations occurred in nine patients, predominantly with females (seven out of nine patients). Those were urticariform—itching, tiny rashes 1–2 mm in diameter—in

Table 2 Microorganisms detected in the same stool samples together with *Blastocystis* sp.

Microorganism in stool	Number of cases
Campylobacter jejuni	3
Candida albicans	3
Clostridium difficile A and/or B toxin(s)	2
Campylobacter lari	1
Non-toxin-producing *Clostridium difficile*	1
Candida glabrata	1
Geotrichum candidum	1
Entamoeba coli	1
Entamoeba histolytica and *Clostridium difficile* A and/or B toxin(s)	1
Entamoeba histolytica with *Geotrichum candidum*	1

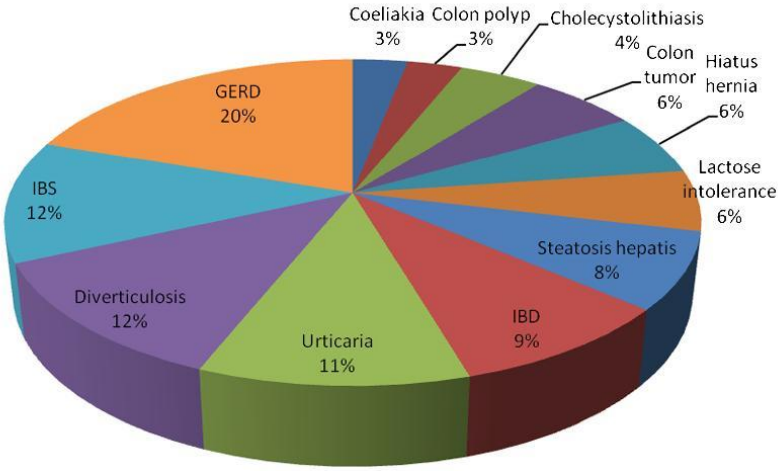

Fig. 1 Common conditions associated with *Blastocystis* sp. positivity in our patients

five patients and reddish-brown infiltrated papules, sometimes with hyperaemic and irregular borders, in three subjects (Figs. 2, 3, 4). In one patient, a nutritive allergy was found by a dermatologist.

Eight patients with cutaneous symptoms received metronidazole, although three needed an additional antimicrobial agent due to co-infection (e.g. *Borrelia burgdorferi*, *Mycoplasma pneumoniae* and *Escherichia coli* infections.) One patient had doxycycline therapy. With regard to gastrointestinal symptoms and skin manifestations, all patients became asymptomatic after antimicrobial treatment.

Forty of the patients had persistent gastrointestinal symptoms, both with and without skin lesions, justifying the initiation of antimicrobial therapy. We used metronidazole in 92.5 % of the cases, but 10 patients needed combined therapy or a switched antimicrobial therapy using mainly rifaximin or sulphametoxazole and trimethoprim due to synchronous infections and the ineffectiveness of metronidazole.

Discussion

Blastocystis sp. is a common intestinal parasite worldwide. The prevalence is higher in developing countries and is usually connected to poor standards of hygiene, exposure to animals and the consumption of contaminated food or water (Tan et al. 2008). The occurrences of *Blastocystis* sp. infections spanning the time period relevant to this study were an overall 6 % in patients treated at the clinics at the University of Szeged and 5.4 % among patients examined in our clinic and whose data was used in this study.

Half of our patients with confirmed *Blastocystis* sp. infections also experienced abdominal pain. In addition, common gastrointestinal complaints included blood visible on the surface of faeces (28.8 %) and meteorism (25.4 %). In this retrospective analysis, we found that 11.25 % of our enrolled patients with *Blastocystis* sp. also had skin manifestations, mainly female patients.

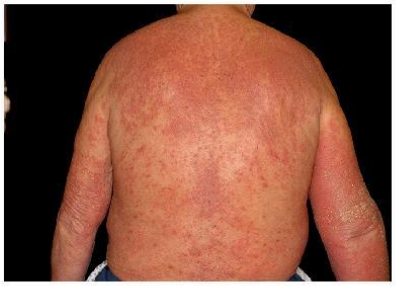

Fig. 2 Extensive papulopustular skin lesions. Eosinophil cellulitis were confirmed by histopathologic examination

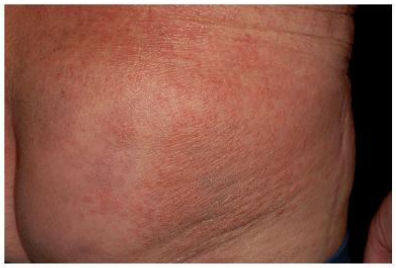

Fig. 3 Numerous 5–10-mm diameter-sized, sporadically confluent, itching, urticariform-papular skin lesion on back and gluteal region

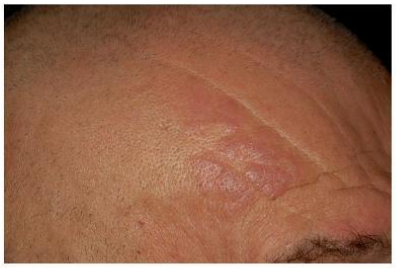

Fig. 4 Several centimeters diameter-sized erythematic, itching skin plaque on right side of the forehead

Despite *Blastocystis* sp. coming into question as a possible causative microorganism of some intestinal illnesses, its pathogenicity and clinical significance have not yet been confirmed. This parasite often causes atypical gastrointestinal symptoms. Some studies reference *Blastocystis* sp. as a potential pathogen (Dagci et al. 2002), but the role the protozoon in the development of certain intestinal disorders is not fully understood. However, data are available about *Blastocystis* sp. supporting a connection with irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) (Boroom et al. 2008; Ghoshal et al. 2010; Yamamoto-Furusho and Torijano-Carrera 2010; Ustün and Turgay 2006). In the study of Tai et al., *Blastocystis* infection has been confirmed in 12.2 % of relapsing ulcerative colitis patients (Tai et al. 2011). Moreover, a recently published study suggests that *Blastocystis* sp. subtype 3 can trigger the proliferation of human colorectal cancer cells (Kumarasamy et al. 2013). On the other hand, this parasite is also common in healthy individuals, suggesting that it can be a component of normal intestinal microbiota (Scanlan 2012). Molecular characterization from symptomatic and asymptomatic carriers should help reveal the clinical implications of this microorganism (Iguchi et al. 2007; Stensvold et al. 2008). Distribution of the nine subtypes of *Blastocystis* sp. found in humans seems to be dependent on geographic locations. Subtype 3 is the most widespread in every continent except the USA, where subtype 1 is the most common (Alfellani et al. 2013). We did not assess the subtypes of *Blastocystis* sp. because during the time period from that data was used, it was not part of routine microbiological examinations; furthermore, it currently has no significance in patient management and therapy. However, the relevance of the subtypes is deemed worthy of further study.

Extraintestinal manifestations of *Blastocystis* sp. infection, such as urticaria and other skin lesions, and the role the infection may have in these manifestations have already been described in various publications (Gupta and Parsi 2006; Hameed and Hassanin 2011; Pasqui et al. 2004; Verma and Delfanian 2013; Wedi et al. 2009). In a recent study (Zaglool et al. 2012), 13.7 % of the patients with allergic skin symptoms were infected by *Blastocystis* sp.; furthermore, no other allergic agent was found to offer explanation why urticaria was manifested. The significance of *Blastocystis* sp. as background cause of urticaria was indicated in prospective research from Peru, which revealed a connection between symptomatology and *Blastocystis* sp. positivity; the symptoms in question were urticaria, abdominal pain and meteorism (Barahona Rodón et al. 2003). Also, supporting the connection was the gradual disappearance of dermatological symptoms after treatment and eradication of *Blastocystis* (Kick et al. 2002; Valsecchi et al. 2004). It has been suggested that cutaneous lesions are immune-mediated, but the mechanism is not completely clear (Dilek et al. 2012)).

In our study, 11.25 % of patients manifested skin symptoms, predominantly the female patients (seven females vs. two males). Disorders were mainly urticariform and dermatitis-like lesions. There was no significant difference observed depending on the amount of protozoa in the samples; therefore, just a few *Blastocystis* sp. cells were enough to induce a cutaneous manifestation. Skin symptoms improved after specific antimicrobial treatment.

Several variations of signs and symptoms were associated with *Blastocystis* sp. infection in our cohort, ranging from non-specific intestinal symptoms to cutaneous disorders. No specific laboratory parameter was discovered that would be indicative of parasite infection; however, elevated CRP and white blood count were shown in *Blastocystis* sp.-associated skin manifestations. Interestingly, we did not find elevated eosinophil count in the whole cohort, regardless of whether or not the patients had accompanying skin manifestations. Thus, we can state that eosinophilia is not an obligatory laboratory finding in protozoon infections, such as *Blastocystis*, with or without skin lesions. In 18.75 % of our patients, another intestinal microorganism was discovered parallel to *Blastocystis* sp., e.g. *C. jejuni*, *C. lari*, *C. albicans*, *C. glabrata*, *Cl. difficile* and *E. histolytica*. On the other hand, skin lesions only manifested with one of them, and symptoms improved after specific antimicrobial therapy. Therefore, the causative agent of the skin lesions was presumably the *Blastocystis* sp. In addition, 40 % of the enrolled individuals also suffered from additional underlying chronic intestinal abnormalities, such as colon tumours, IBD, IBS, coeliac disease, lactose intolerance or diverticulosis. Considering the great number of patients with the abovementioned diseases, the vulnerability of the intestine is suggested to be a risk factor for a *Blastocystis* sp. infection.

In light of our results, we suggest that in cases of non-specific gastrointestinal symptoms (with or without extraintestinal manifestations) or in cases of skin disorders of unknown origin, we should consider protozoan infections—including *Blastocystis* sp. infection—and perform a stool examination to screen for parasites.

Acknowledgments This work was supported by OTKA Research Proposal PD 105948 (PI: Klaudia Farkas) and TÁMOP-4.2.2.A-11/1/KONV-2012-0035, TÁMOP-4.2.2-A-11/1/KONV-2012 0052 TÁMOP-4.2.2.A-11/1/KONV-2012-0073 and TÁMOP-4.2.2.A-11/1/KONV-2012-0052.

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Annex II.

Scandinavian Journal of Gastroenterology. 2014; Early Online, 1–8

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healthcare

ORIGINAL ARTICLE

Antibody and cell-mediated immune response to whole virion and split virion influenza vaccine in patients with inflammatory bowel disease on maintenance immunosuppressive and biological therapy

ANITA BÁLINT[1*], KLAUDIA FARKAS[1*], PALLAGI-KUNSTÁR ÉVA[1], GABRIELLA TERHES[2], EDIT URBÁN[2], MÓNIKA SZŰCS[3], TIBOR NYÁRI[3], ZSUZSANNA BATA[4], FERENC NAGY[1], ZOLTÁN SZEPES[1], PÁL MIHELLER[5], KATALIN LŐRINCZY[5], PÉTER LÁSZLÓ LAKATOS[6], BARBARA LOVÁSZ[6], SZAMOSI TAMÁS[7], ANDREA KULCSÁR[8], ANNA BERÉNYI[1], DALMA TÖRŐCSIK[1], TÍMEA DARÓCZI[1], ZOLTÁN SARÓDI[1], TIBOR WITTMANN[1] & TAMÁS MOLNÁR[1]

[1]*First Department of Medicine, University of Szeged, Szeged, Hungary,* [2]*Institute of Clinical Microbiology, University of Szeged, Szeged, Hungary,* [3]*Department of Medical Physics and Informatics, University of Szeged, Szeged, Hungary,* [4]*Department of Dermatology and Allergology, University of Szeged, Szeged, Hungary,* [5]*Second Department of Medicine, Semmelweis University, Budapest, Hungary,* [6]*First Department of Medicine, Semmelweis University, Budapest, Hungary,* [7]*Military Hospital, Budapest, Hungary, and* [8]*Integrated Szent Istvan and Szent László Hospital, Budapest, Hungary*

Abstract

Objective. Influenza vaccination is recommended for inflammatory bowel disease (IBD) patients on immunosuppressive therapy. The objective was to evaluate the antibody and cell-mediated immune response to the split and whole virion influenza vaccine in patients with IBD treated with anti-TNF-α and immunosuppressive therapy. ***Patients and methods.*** One hundred and fifty-six immunocompromised IBD patients were vaccinated. Fifty-three patients (control group) refused vaccination. Split virion vaccine and whole virion vaccine were used. Serum samples were obtained for pre- and postimmunization antibody titers to influenza vaccine (A/California/7/2009 [H1N1], A/Victoria/361/2011 [H3N2], B/Wisconsin/1/2010–like B/Hubei-Wujiagang/158/2009). Cell-mediated response was evaluated using an interferon (INF)-γ, interleukine (IL)-2 and tumor necrosis factor (TNF)-α ELISA. ***Results.*** Postimmunization titers of both influenza subtypes increased significantly after the administration of split virion vaccines compared to the controls and to those who received whole virion vaccine. The antibody titers of Influenza B also increased significantly in patients immunized with split vaccine and treated with anti-TNF-α therapy. After influenza vaccination, the level of serum IL-2 significantly decreased. No serious side effects developed occurred after influenza vaccination, and the influenza-like symptoms did not differ significantly between vaccinated versus control patients. The relapse of the disease was observed in only 10% of the patients and was more common in vaccinated than in control subjects. ***Conclusion.*** Split virion vaccines seem to be more effective than whole virion vaccines. Measuring the antibody responses is worthwhile in patients treated with immunosuppressants to determine the efficacy of influenza vaccination.

Key Words: *anti-TNF therapy, cellular immune response, immunosuppression, inflammatory bowel diseases, influenza, vaccination*

Correspondence: Tamás Molnár, MD, First Department of Medicine, University of Szeged, 8-10 Koranyi fasor, H6720 Szeged, Hungary. Tel: +36 62 545 186. Fax: +36 62 545 185. E-mail: molnar.tamas@med.u.-szeged.hu
*These authors contributed equally.

(Received 19 March 2014; revised 4 May 2014; accepted 20 May 2014)

ISSN 0036-5521 print/ISSN 1502-7708 online © 2014 Informa Healthcare
DOI: 10.3109/00365521.2014.928902

Introduction

Patients with inflammatory bowel diseases (IBD – Crohn's disease [CD], ulcerative colitis [UC]) on immunosuppressive therapy are at increased risk for infectious diseases, some of which can be prevented with immunization. Influenza is one of the most common vaccine-preventable illnesses in adults and, according to the latest statement of the European Crohn's and Colitis Organisation [1] on the prevention of opportunistic infections in IBD, influenza vaccination is recommended for all patients with IBD on immunomodulators evidence level 2 (EL2), recommendation graded B (RG B).

The rising use of combined immunosuppressive and biological therapy both in CD and UC may potentially increase the risk of life-threatening infections, thus highlighting the importance of vaccinations. Although several guidelines exist for the vaccination of patients with IBD, it still seems to be underused, and the results of the studies on the immune response after vaccination are also conflicting [2].

One of the studies conducted in pediatric IBD patients [3] revealed that children receiving both infliximab and immunomodulators had a lower response to two influenza vaccine antigens (A/New Caledonia/20/99 and B/Hong Kong/330/2001) when compared with healthy controls. The prospective study of Lu et al. revealed a high prevalence of seroprotection in children and young adults with IBD, particularly against A strains [4]. They did not detect a difference between non-immunosuppressed and immunosuppressed patients. However, in adults, the types of immunosuppressive and biological therapies seem to influence the immune response to vaccinations [5]. Additionally, the majority of the studies aims to assess only the rate of seroprotection and do not consider the importance of the frequency of infections in vaccinated versus non-vaccinated patients.

The primary objectives of this study were to assess the antibody response to the seasonal influenza vaccine in patients with IBD treated with anti-TNF-α alone or combined with immunosuppressive therapy and to compare them with patients receiving non-immunosuppressive therapy. The secondary goal was to compare the antibody response and the safety of whole virion and split influenza vaccines. The last objective was to determine the cellular immune response to the influenza vaccinations and to evaluate the effects of the vaccines on IBD clinical activity. Last but not least, this study also aimed to evaluate the acceptance of the vaccination in our patients and to assess the frequency of influenza infection in vaccinated versus non-vaccinated patients.

Patients and methods

Study population and study design

We conducted a multicenter, prospective cohort study between September 2012 and May 2013 at four Hungarian IBD centers. Patients with IBD were recruited during outpatient visits at the centers. Inclusion criteria included an age ≥18 years, diagnosis of IBD stable for more than 3 months, no signs of activity (biological and clinical) and not requiring any treatment modification for the disease at inclusion. Patients with active IBD were excluded. At inclusion, influenza vaccination was offered to every patient attending the involved centers. Patients were randomized to two groups on the basis of the acceptance of the vaccination. Patients refusing the vaccination served as control subjects. Patients and control subjects were followed up for 4 months to determine the clinical activity and the frequency of influenza infections. Clinical data included age at diagnosis, disease duration, gender, IBD phenotype according to the Montreal classification [6], types of concomitant therapies, and types and dosages of immunomodulator and biological therapy. Immunization history for the previous 5 years was also obtained.

Patients who received vaccination were divided into two further groups: patients treated with aminosalycilates without immunosuppressive therapy and patients treated with immunomodulator and/or biological therapy for at least 3 months before the vaccination. Control subjects had received maintenance therapy with immunomodulator and/or biological therapy for at least 3 months before the vaccination.

The type of vaccine (whole virion or split virion vaccine) was randomly selected. Validated clinical activity indices – Crohn's disease activity index (CDAI) [7] and Partial Mayo Score (pMayo score) [8] were used for CD and UC to assess disease activity. The patients were scored and blood samples were also taken before and after the vaccination. The patients were contacted by phone every week for 16 weeks. During the phone calls, data from each patient were collected using a standardized questionnaire. The patients were asked about any change in clinical activity and the development of local and systemic adverse reactions. Ethical approvals for the study had been obtained from the Scientific and Research Ethics Committee of Hungary. Written informed consent was obtained from each subject.

Vaccines

Two non-live vaccines directed against the seasonal influenza virus A/California/7/2009 (H1N1), A/Victoria/361/2011 (H3N2), B/Wisconsin/1/2010–like B/

Hubei-Wujiagang/158/2009 were used in the study. Inactivated, split virion vaccine (IDFlu9) and inactivated, whole virion vaccine (Fluval AB) were administrated depending on a random selection. The experimental use of the whole virion vaccine, which is the first inactivated vaccine formulation, dates back to the 1940s. Split virion is derived by disrupting whole virus particles with detergents and is thus less immunogenic than whole virion vaccines [9]. Whole virion vaccine was administered intramuscularly; split virion vaccine was administered intradermally.

Serological evaluation, measurement of cellular immune response to vaccination and evaluation of leukocyte and lymphocyte levels

Serum was collected at baseline (pre-vaccination) and 5–6 weeks after vaccination and it was stored at −20°C until use. From the collected serum samples, anti-influenza A virus IgG enzyme-linked immunosorbent assay (ELISA – Euroimmun, Germany), containing the "Texas" (H3N2), "Singapore" (H1N1) and "California" (H1N1) strains of influenza A virus as antigens, was performed according to the manufacturer's recommendation. The results were evaluated quantitatively using calibration curve. From the above-mentioned serum specimens, anti-influenza B virus IgG ELISA (Euroimmun, Germany) was also set up. In this case, the antigen source was the "Hongkong 5/72" strain of Influenza B virus. The results were also interpreted quantitatively.

We compared the antibody titers of influenza A and B subtypes between vaccinated and control patients, between vaccinated patients receiving immunosuppressive (thiopurine, biological therapy) and non-immunosuppressive therapy (aminosalycilates), between vaccinated and control patients receiving immunosuppressive maintenance therapy and between patients vaccinated with whole virion and split virion vaccine receiving immunosuppressive therapy.

We assessed cell-mediated immune response after vaccination and also compared it between patients treated with and without immunosuppressants. The cell-mediated response to influenza A and B vaccines was evaluated using an interferon (INF)-γ, interleukine (IL)-2 and tumor necrosis factor (TNF)-α ELISA. Human TNF-α, IFN-γ and IL-2 ELISA kits were obtained from Life Technologies (Hungary). Serum was also obtained to assess leukocyte and lymphocyte levels quantitatively.

Statistical analysis

Data were analyzed using SPSS version 21 software (SPSS, Chicago, IL). $p < 0.05$ was considered significant. Categorical data were analyzed using Pearson's chi-square test and Fisher's exact test. The effects of the vaccination on the antibody and cell-mediated immune response were examined with multivariate analysis of variance (MANOVA) models with time as repeated measures (within-subject) factor and the types of the vaccines, the immunosuppressive status, the vaccinated status, the different therapies and the development of side effects and influenza-like symptoms as between-subject factors. Pairwise comparisons were performed on estimated marginal means by considering the presence or absence of interaction; *p*-Values were corrected with the Holm-Sidak method.

Results

Patient characteristics

Two hundred and nine IBD patients (127 with CD, 82 with UC) were eligible and enrolled in the study. One hundred and fifty-six patients received influenza vaccination, whereas 53 patients (control group) refused the vaccine – the acceptance rate of vaccination was 66.3%. Whole virion vaccine was given to 57; split virion vaccine was given to 99 patients. The mean age of the vaccinated patients was 27.9 years; 84 were women and 72 were men. In the control group, the mean age was 30.7 years; 29 were women and 24 were men. Out of the 156 vaccinated patients, 98 had CD and 58 had UC. Median disease duration was 9 years for CD (interquartile range [IQR] 5–13) and 9 years for UC (IQR 4–15.8). Of the control subjects, 29 had CD and 24 had UC. Median disease duration was 7 years for both CD (IQR 5–14) and UC (IQR 4.5–12).

Of the 156 vaccinated patients, 115 received immunosuppressive therapy. The non-immunosuppressive group of vaccinated subjects was composed of 41 patients. Out of the 53 control subjects, 32 received immunosuppressive therapy. Twenty-one patients were free of immunosuppressive therapy. Of the patients, 8.3% were regularly vaccinated against seasonal influenza virus. Thirty-nine patients (21.5%) had received the last vaccination within 1 year, 25 patients (13.8%) within 3 years and 3 patients (1.7%) within 5 years. Of the patients, 63% had received the last vaccination more than 5 years earlier. Demographic and clinical characteristics, disease activity at the time of the vaccination and treatment types are summarized in Table I.

Antibody titers for Influenza A and B subtypes

The values of pre-immunization antibody levels of influenza A and B titers varied between 33.8–

Table I. Demographic, clinical characteristics, disease activities and treatment types of patients enrolled in the study.

	All patients (n = 209)	Fluval AB vaccinated (n =57)	IDFlu9 vaccinated (n = 99)	Controls (n = 53)
Gender				
Male	96	30	42	24
Female	113	27	57	29
Mean age at diagnosis (years)	28.6	27.2	28.3	30.7
Median disease duration (years)	9	9	9	7
Crohn's disease	127	46	52	29
Ulcerative colitis	82	11	47	24
Age of diagnosis				
A1	22	4	11	7
A2	158	47	74	37
A3	29	6	14	9
Disease location				
L1	25	10	10	5
L2	46	14	20	12
L3	54	21	22	11
L4	2	1	0	1
Disease behavior				
B1	41	14	19	8
B2	24	9	12	3
B3	62	23	21	18
Extent of UC				
E1	21	3	11	7
E2	28	5	16	7
E3	33	3	20	10
Therapy				
Aminosalycilates	21	5	6	10
Thiopurines	27	7	14	6
Anti-TNF-α	26	10	12	4
Combined thiopurines and anti-TNF-α	62	20	20	22
Mean CDAI	120.3	108.4	133.2	137.5
Mean pMayo score	1.8	1.7	1.6	2.1

341.3 RU/ml and 45.8–248.7 RU/ml, respectively – every patient had pre-existing protective levels of antibody to the influenza viruses. The antibody values of the post-immunization levels of influenza A and B titers varied between 43.9–301.5 RU/ml and 58.2–216.9 RU/ml. The post-immunization antibody titers of Influenza A and B subtypes significantly increased in patients immunized with split virion vaccines (mean increase in antibody levels was 13.8 RU/ml for Influenza A and 17.4 RU/ml for Influenza B), compared with control subjects (mean increase in antibody levels was 9.4 RU/ml for Influenza A and 7.3 RU/ml for Influenza B) ($p = 0.045$ for Influenza A and $p = 0.03$ for Influenza B). Although a trend of increase in the post-immunization titers was also observed in control patients, it was not significant.

The antibody titers of Influenza A and B significantly increased after the administration of split virion vaccine compared with whole virion vaccines ($p = 0.03$ for Influenza A and $p < 0.001$ for Influenza B). The post-immunization antibody titers of Influenza B also increased significantly after administrating split versus whole virion vaccine in patients treated with anti-TNF-α ($p = 0.002$). However, the study was not powered to directly compare the changes in the post-immunization antibody titers in vaccinated and control patients treated with thiopurines and anti-TNF-α alone or in combination.

Cellular immune response to influenza vaccination

Leukocyte and lymphocyte levels varied between 2.78–17.6 g/l and 0.38–20.9 g/l before and between 2.41–20.54 g/l and 7.9–43.8 g/l after the vaccination. Leukocyte and lymphocyte levels did not differ significantly after vaccination. The level of INF-γ varied between 9.9 and 39.1 pg/ml before and between 11.7 and 39.1 pg/ml after vaccination. IL-2 levels varied between 14.7 and 152.6 pg/ml before and between 13.8 and 152.3 pg/ml after the vaccination. The levels of TNF-α varied as: 11.6–360.4 pg/ml before and 8.5–216.9 pg/ml after the administration of the vaccine. Neither TNF-α nor INF-γ levels changed significantly after influenza vaccination; however, a significant decrease was observed in the

level of IL-2 after vaccination with split versus whole virion vaccine ($p = 0.004$).

Effect of vaccination on IBD activity

During the 4-month follow-up period, 1 of the control subjects and 21 of the vaccinated patients (8 CD, 13 UC) developed a flare up with an increased diarrhea or bloody stool. The mean CDAI at the flare up was 273, the mean pMayo score was 4. This was observed with both types of the vaccine; however, out of the 21 patients with flare up, 15 received IDFlu9 split virion vaccine. The flare up was developed a mean of 6 weeks after the vaccination. These 21 patients were followed up for four additional months. Absence of flare was observed in 3 weeks. Disease activity resolved spontaneously in 11 patients, two patients needed corticosteroids, five antibiotics. Three patients remitted after the subsequent biological therapy.

Side effects and development of influenza-like symptoms

Twenty-six patients developed local reaction (pain, redness, warmth, swelling) after vaccination, 32 had systemic symptoms (shivering, subfebrility, fever, fatigue, malaise, headache, muscle pain) during the first week. The most common local reaction after any type of vaccine was redness at the injection site, reported by 11%. The local reactions disappeared within 48 h in every case. The most common systemic reactions were common cold and sore throat in 39.7 and 28.8% of the patients. Systemic reactions resolved within a week.

Table II. Local and systemic side effects after the influenza vaccination.

Effect	Fluval AB ($n = 57$)	IDFlu9 ($n = 99$)
Local reaction		
Any reaction, *n* (%)	2 (3.5)	24 (24.2)
Pain, *n* (%)	1 (1.8)	11 (11.1)
Redness, *n* (%)	1 (1.8)	16 (16.2)
Swelling, *n* (%)	1 (1.8)	14 (14.1)
Itching, *n* (%)		6 (6.1)
Systemic reaction		
Any reaction, *n* (%)	28 (59.1)	70 (70.7)
Shivering, *n* (%)	0	3 (3.0)
Fever ≥38°C, *n* (%)	0	16 (16.2)
Unusual fatigue, *n* (%)	1 (1.8)	2 (2.0)
Malaise, *n* (%)	2 (3.5)	13 (13.1)
Headache, *n* (%)	4 (7.0)	18 (18.1)
Muscle pain, *n* (%)	5 (8.8)	11 (11.1)
Arthralgia, *n* (%)	1 (1.8)	7 (7.0)
Sore throat, *n* (%)	15 (26.3)	30 (30.3)
Common cold, *n* (%)	15 (26.3)	47 (47.5)
Sneeze, *n* (%)	12 (21.1)	22 (22.2)
Coughing, *n* (%)	6 (10.5)	21 (21.2)
Subfebrility, *n* (%)	10 (17.5)	8 (8.0)
Vertigo, *n* (%)	0	4 (4.0)

Upper respiratory tract infection like symptoms occurred more frequently within the first week in vaccinated versus control patients (31.4% vs. 9.4%; $p = 0.002$). During the further follow-up, no difference was found between the two groups. Influenza-like symptoms occurred in seven of the vaccinated and one of the control patients in the first 4 weeks, and six of the vaccinated and three of the controls between weeks 5 and 16. These influenza-like symptoms were developed by 12 CD and 5 UC patients, and all but one of them was on immunosuppressive therapy in both the vaccinated and the control groups. Local and systemic reactions were more common in patients vaccinated with IDFlu9 split virion vaccine versus Fluval AB ($p < 0.001$). The local and systemic side effects developed after the influenza vaccination are summarized in Table II.

Discussion

In this prospective, multicenter study conducted between September 2012 and May 2013, 156 IBD patients – the majority treated with combined immunosuppressive and biological therapy – received vaccination against influenza virus A/California/7/2009 (H1N1), A/Victoria/361/2011 (H3N2), B/Wisconsin/1/2010–like B/Hubei-Wujiagang/158/2009. The study revealed that two thirds of the patients agreed to influenza vaccination. Split virion vaccine was administered to almost twice as many patients as whole virion vaccine in this study. Split virion vaccines proved to be more effective in the vaccination procedure: post-immunization titers of both subtypes increased significantly after the administration of split virion vaccines compared with the controls and with those patients vaccinated with whole virion vaccines. The antibody titers of Influenza B also increased significantly in patients immunized with split virion vaccine and treated with anti-TNF-α therapy. The high number of cases with pre-existing antibody levels can be explained with previous vaccinations and prior influenza infections, although cross-protection against influenza virus strains could also be present. Influenza vaccination did not have any effect on the level of TNF-α and INF-γ; however, a significant decrease was observed in the level of IL-2 after vaccination. Although no serious side effects were developed after influenza vaccination, influenza-like symptoms in the first weeks after immunization occurred more frequently in patients receiving split virion vaccine. Influenza-like symptoms did not differ significantly between vaccinated versus control patients (8.3% vs. 7.5%). A relapse of the disease

was observed in only 10% of the patients and was more common in vaccinated than in control subjects.

Vaccination is a conventional strategy for the prophylaxis of vaccine-preventable infections, especially in patients suffering from immune-mediated diseases [10]. Our multicenter survey revealed that most of the patients are willing to be vaccinated against the seasonal influenza virus if it is recommended and administered by the gastroenterologist, although promoting prevention and prophylaxis seemed to be underutilized in the previous years. In a survey done in IBD patients, only 28% of the patients reported yearly influenza vaccination [11]. Possible explanations for under-vaccination of IBD patients are unawareness of the increased infection risk and concerns about the safety and efficacy of the vaccination. In our study, every patient was recommended to be vaccinated and, after detailed consultations, two-thirds of them accepted vaccination, which indicates that informing and assuring the patients may increase the willingness to take the vaccination. Patients on immunosuppression or biological therapy are supposed to be at increased risk of influenza [12], and thus, annual influenza vaccination is recommended for all patients with IBD on immunomodulators (EL2, RG B) [1]. It is well-known that the majority of the studies examining the efficacy of influenza vaccination in IBD patients have been carried out in children. Mamula et al. [3] observed a reduced seroconversion rate and geometric mean titer after influenza vaccination in IBD patients receiving immunotherapy compared with healthy controls. Another study found that vaccination responses were similar among children with IBD, regardless of immunosuppressive status [4]. These studies revealed that vaccination against influenza in children with IBD is well tolerated and induces immune response, although anti-TNF therapy may selectively impair response to specific serotypes. deBruyn et al. also found that children with IBD were less likely to mount an immunogenic response for influenza B compared with controls [13]. According to our results, the post-immunization antibody titers of both Influenza A and B subtypes were increased in patients vaccinated with split virion vaccines versus control subjects, although the study was not powered to detect differences between the different immunosuppressive maintenance therapies.

Some studies observed an impaired immune response after influenza vaccination in patients treated with anti-TNF agents. However, response rates to influenza vaccination in patients treated with immunomodulators and biologicals are somewhat conflicting. Andrisani et al. revealed that seroprotective titers in the patients were comparable to healthy controls. Seroconvertion rate was lower in IBD patients than in healthy controls either on anti-TNF-α monotherapy or combined with immunosuppressants [5]. In a study conducted on patients with rheumatoid arthritis and CD, postvaccination geometric mean antibody titers against influenza (A/H3N2 and B) were significantly lower in patients treated with anti-TNF (infliximab, adalimumab or etanercept) compared with patients not receiving anti-TNF therapy and the healthy controls [14]. Our results revealed that the increase in post-immunization titers of only Influenza B subtype was not influenced by anti-TNF-α therapy – although only in case of the administration of split virion vaccines.

The results about the immune response to split virion vaccine versus whole virion vaccine also seem to be controversial. A study evaluating both split virion and whole virion H5N1 vaccine formulations in children revealed that the whole virion vaccine may be immunogenically better than the split virion vaccine, confirming the previous findings in adults [15]. However, in another study, AS03B-adjuvanted split virion or a non-adjuvanted whole virion H1N1 (2009) vaccine was used in 943 children and both vaccines proved to be safe and immunogenic [16]. In Hungary, whole virion vaccines are supported by the Hungarian National Health Fund (OEP), and thus these are used routinely in the clinical practice. However, in this study, we had the opportunity to assess the efficacy of the less immunogenic split virion vaccines. Our results demonstrated significantly higher post-immunization antibody levels in case of split virion vaccines. The frequency of the side effects was higher in case of split vaccines; however, it is important to note that no severe adverse reactions occurred. The rate of influenza-like infections was quite low during the follow-up period and did not differ significantly in vaccinated versus control subjects. Influenza-like symptoms were more likely to develop in patients with immunosuppressive therapy in both vaccinated and control patients.

Immunotherapy is known to predominantly impair cellular immunity, leaving the humoral immune response more or less intact [10]. In our study, we examined whether influenza vaccination has an effect on the cell-mediated immune response by measuring the pre- and post-immunization levels of INF-γ, IL-2 and TNF-α. Interestingly, only the level of IL-2 decreased significantly after vaccination. The study by Holvast et al. assessed cell-mediated responses to influenza vaccination in patients with systemic lupus erythematosus (SLE). They found that the frequencies of $CD4^+$ T cells producing TNF and IL-2 were lower in patients after vaccination compared with healthy control subjects. They also found that this diminished cell-mediated response may

reflect the effects of concomitant use of immunosuppressive drugs [17]. The study of Long et al. also found a diminished humoral and cell-mediated immune response to monovalent 2009 pandemic influenza A (H1N1/2009) and seasonal trivalent influenza vaccines in subjects with SLE but not with sickle cell disease or asthma, presumably due to the different immunocompromised status of these children [18].

Antibody response is an integral component of normal immunity. Successful humoral immunity depends on cellular interactions, and it is not unusual to observe deficient antibody responses in immunologically compromised hosts. Age and concomitant therapy may also influence immune response after influenza vaccine – thus that it is very difficult to assess whether *in vitro* immunity corresponds to *in vivo* immunity. In a study from the 80s [19], 14 monkeys received a trivalent influenza vaccine and antibody response was determined based on a change in plasma antibody content before and after the vaccine. *In vitro* anti-influenza antibody synthesis was found to correlate well with the *in vivo* response. Ershler et al. also measured the antibody response in young and elderly volunteers after *in vivo* and *in vitro* immunization with trivalent influenza vaccine. After *in vivo* immunization, plasma antibody levels and *in vitro* synthesis capabilities significantly increased in the young subjects. The capacity for *in vitro* immunization was also greater in the young, but the difference did not reach statistical significance and there was no correlation with *in vitro* immunization potential and the response after *in vivo* vaccination [20].

The authors are aware of some limitations of this study. First, we did not examine the effect of the influenza vaccination received in the previous year on the antibody responses to the current influenza vaccination; then again, only a minority of the patients had been vaccinated within a year. Second, the division of the whole virion and split virion vaccines was unequal. Third, the proportion of patients on the basis of the type of maintenance therapy was not high enough to statistically differentiate between the alterations of antibody responses after the vaccinations. Lastly, we did not analyze the correlation between cell-mediated responses and antibody responses to influenza vaccination, although these results presumably do not have importance in the clinical practice in accordance with the vaccination strategies.

It is not in question whether inactivated influenza vaccination is recommended yearly for patients with IBD. Despite the initial results, a number of studies have shown that immunosuppressive and anti-TNF therapy are safe and effective in regard to the response to annual trivalent inactivated influenza vaccination. In this cohort, split vaccines seemed to be more effective than whole virion vaccines, and split vaccines also resulted in an increase in post-immunization titers of Influenza B subtype besides anti-TNF-α therapy. Larger and more detailed studies are needed to compare the efficacy of these vaccinations and to examine the antibody and cell-mediated response in immunocompromised patients. In conclusion, our results suggest that IBD patients on immunosuppressive therapy are recommended to be immunized with split virion vaccines and that measuring the antibody responses is worthwhile in patients treated with immunosuppressants to determine the efficacy of influenza vaccination.

Acknowledgment

This work was supported by TAMOP-4.2.2.A-11/1/KONV-2012-0035, TAMOP-4.2.2-A-11/1/KONV-2012-0052 TAMOP-4.2.2.A-11/1/KONV-2012-0073 and OTKA PD 105948 (PI: Dr. Klaudia Farkas).

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Annex III.

Journal of Crohn's and Colitis (2014) 8, 992–997

Available online at www.sciencedirect.com

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Bolus administration of steroid therapy is more favorable than the conventional use in preventing decrease of bone density and the increase of body fat percentage in patients with inflammatory bowel disease☆

Klaudia Farkas[a,*,1], Anita Bálint[a,1], Zsuzsanna Valkusz[a], Zoltán Szepes[a], Ferenc Nagy[a], Mónika Szűcs[b], Renáta Bor[a], Tibor Wittmann[a], Tamás Molnár[a]

[a] First Department of Medicine, University of Szeged, Szeged, Hungary
[b] Department of Medical Physics and Informatics, University of Szeged, Szeged, Hungary

Received 17 November 2013; received in revised form 22 December 2013; accepted 29 January 2014

KEYWORDS
Steroid therapy;
Inflammatory bowel disease;
Bolus administration;
Metabolism

Abstract

Introduction: The effects of short course of corticosteroids on the metabolic processes and bone formation has not been well studied. Our aim was to compare the efficacy, the side effects and the bone and lipid metabolisms in IBD patients using bolus or conventional tapering of methylprednisolone for 12 weeks.
Patients and methods: Nineteen IBD patients received intravenous methylprednisolone of 1 mg/kg for 5 days tapered by 4 mg per week. Patients were prospectively randomized in two groups. In "conventional" group (I) steroids were given daily. In "pulse" group (II) weekly doses of steroids were given on special days of the week. The body mass index (BMI) was measured before and after the corticosteroid therapy. Blood samples were collected to assess glucose level, electrolytes, cholesterol and triglyceride levels, inflammatory parameters, cortisol, osteocalcin and crosslaps values. Total body composition analysis was performed at the beginning and at the end of the steroid therapy.

☆ **Specific author contributions**: Study design, data collection, supervision of patient selection and manuscript preparation: Klaudia Farkas, Anita Bálint, Tamás Molnár; study design, data collection, statistical analysis and manuscript preparation: Tamas Molnar, Klaudia Farkas, Mónika Szűcs; data collection and manuscript preparation: Klaudia Farkas, Anita Bálint, Ferenc Nagy, Zoltán Szepes, Renáta Bor, Zsuzsanna Valkusz; supervision of the patient selection and manuscript preparation: Tamás Molnár, Tibor Wittmann. All authors have approved the final draft submitted.

* Corresponding author at: First Department of Medicine, University of Szeged. 8-10 Koranyi fasor, Szeged H6720, Hungary.
E-mail address: farkas.klaudia@gmail.com (K. Farkas).

[1] The authors contributed equally.

1873-9946/$ - see front matter
http://dx.doi.org/10.1016/j.crohns.2014.01.026

Results: In Group I, BMI increased, total body bone density decreased significantly at the end of the steroid therapy. Body fat percent showed a tendency to be higher at the end of steroid therapy in Group I. Cholesterol level increased significantly in Group I patients. The decrease in serum cortisol level was more remarkable in Group I vs. Group II after steroid therapy. Less side-effect occurred in Group II vs. Group I.
Discussion: Our results suggest that bolus tapering of corticosteroids may have more favorable short term outcome than conventional tapering that may revolutionize steroid therapy in IBD.

1. Introduction

Corticosteroids still have an important role in the management of acute episodes of inflammatory bowel disease (IBD-Crohn's disease [CD], ulcerative colitis [UC]). Parenteral corticosteroids are usually the first treatment of choice for hospitalized patients with severe UC and CD.[1] However, use of steroids is associated with some well-known potential harmful side-effects; therefore oral steroids are recommended to be gradually tapered off and discontinued after 12 weeks in case of appropriate response to the parenteral therapy. Corticosteroid therapy is known to contribute to changes in body composition with the alteration of protein synthesis and degradation in skeletal muscle, resulting in decreased muscle mass and reduced fat-free mass. Steroids also lead to a reduction in the total body bone mineral density (BMD).[2] Therefore total body composition analysis is a useful method for quantification of multiple whole body and regional components, including bone mineral, fat, and lean soft tissue in patients treated with steroids. It gives a direct measurement of the percent body fat, muscle and bone (in grams) for the entire body and sub regions like the arm, leg, and trunk.

The optimal dose response for parenteral steroids in the treatment of severe attacks has not been clarified yet; dosages of methylprednisolone 40–60 mg or 1 mg/kg per day orally are the most frequently used regimen[1,3] for flare up. Furthermore, no randomized trials have studied and even no guidelines have been developed by the European Crohn's and Colitis Organisation on taper schedules. After the induction of remission, methylprednisolone is usually tapered 8–16 mg weekly until a daily dose of 32 mg is reached followed by a tapering of 4 mg/week. Tapering steroid regimen is most frequently carried out by administrating the drug daily, although alternate-day steroid management (given every other day) has also been a widely employed and effective mode of therapy for ages associating with fewer unpleasant side effects.[4] The efficacy of "bolus-administered" corticosteroids when weekly dose of steroid regimen is given on special days has not been previously examined in patients with IBD. The effect of a "short-term" 12-week course of corticosteroids on the metabolic processes and bone formation has not been well studied too; although these are some of the most important side effects should be considered.

The aim of the present pilot study was to compare the efficacy, the frequency of side effects and the changes in bone and lipid metabolism in IBD patients using bolus or conventional tapering of methylprednisolone for 12 weeks.

2. Patients and methods

2.1. Study design and patients

This single-center, prospective, randomized trial was carried out from November 2011 to February 2013 on consecutive patients with acute exacerbation of IBD and not being on steroid therapy admitted to our clinic. Diagnosis was based on the Lennard-Jones criteria.[5] Crohn's disease phenotype was determined according to the Montreal classification.[6] Clinical activities were determined by Crohn's Disease Activity Index (CDAI)[7] in CD and by partial Mayo score[8] in UC. Twenty patients were enrolled in the study. The median CDAI and partial Mayo score were 184 and 6 in CD and UC at the time of the enrolment. None of the patients received oral corticosteroid at the time or at least 6 months before the enrolment. On admission a complete blood chemistry including erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), serum glucose, electrolytes, liver and renal function, cholesterol, triglycerides, blood count, serum cortisol, calcium, dehydroepiandrosterone (DHEA), thyroid stimulating hormone (TSH), parathyroid hormone (PTH), β-crosslaps and osteocalcin levels were performed before starting steroid therapy. The mean value of CRP, ESR, leukocytes and thrombocytes before steroid therapy were 16 mg/l, 21.5 mm/h, 8.828 G/l and 322 G/l. Flexible colonoscopy or sigmoidoscopy with biopsies was carried out only in patient with relevance to therapy, however, it was not essential for inclusion into the trial. Thus, 3 CD and 13 UC patients underwent colonoscopy at inclusion. DXA total body composition analysis was also performed at the beginning of the study to determine the fat and fat-free component of the body.

Patients eligible for iv. steroid therapy received methylprednisolone dosage 1 mg/kg for 5 days. After iv. therapy, patients were prospectively randomized in two groups. In "conventional" group (Group I) methylprednisolone was given daily while in "bolus-administered" group (Group II) weekly dose of steroids was given on special days of the week. 64 mg/day methylprednisolone dose at the first week in Group I was equal to 150 mg/day given in the first 3 days in Group II. Finally, both groups received the same methylprednisolone dose and it was tapered by 4 mg per week in both groups. The two different types of methylprednisolone dosages are detailed in Table 1. Follow-up appointments were done every two weeks. These visits involved the assessment of the clinical activities by the determination of CDAI and pMayo scores. Patients were asked about side effects, the body mass index was determined and the waist and hip circumferences were also measured. Laboratory assessment

Table 1 Methylprednisolone dosages in the conventional and the bolus administration groups.

Week/day	1	2	3	4	5	6	7	Total dose
Conventional administration								
1	64	64	64	64	64	64	64	448
2	48	48	48	48	48	48	48	336
3	32	32	32	32	32	32	32	224
4	28	28	28	28	28	28	28	196
5	24	24	24	24	24	24	24	168
6	20	20	20	20	20	20	20	140
7	16	16	16	16	16	16	16	112
8	12	12	12	12	12	12	12	84
9	8	8	8	8	8	8	8	56
10	4	4	4	4	4	4	4	28
11	2	2	2	2	2	2	2	14
12	0	0	0	0	0	0	0	0
Bolus administration								
1	150	0	150	0	150	0	0	450
2	112	0	112	0	112	0	0	336
3	75	0	75	0	75	0	0	225
4	98	0	98	0	0	0	0	196
5	84	0	84	0	0	0	0	168
6	70	0	70	0	0	0	0	140
7	112	0	0	0	0	0	0	112
8	84	0	0	0	0	0	0	84
9	56	0	0	0	0	0	0	56
10	28	0	0	0	0	0	0	28
11	12	0	0	0	0	0	0	12
12	0	0	0	0	0	0	0	0

(including inflammatory parameters, electrolytes, glucose level, liver and renal function, and blood count) was carried out every four weeks. Detailed laboratory parameters (DHEA, TSH, PTH, serum cortisol, serum β-crosslaps and osteocalcin levels) and DXA for total body composition analysis were performed at week 0 and week 12. Clinical remission was defined as a CDAI of <150 points and a Mayo score of <2 points. The study was approved by the Regional and Institutional Human Medical Biological Research Ethics Committee of the University of Szeged (Number: 74/2011).

2.2. End points

The primary end point of the study was the comparison of the efficacy of the conventional and the bolus-administered corticosteroid therapy and also the assessment of their effects on the adrenal glands hormone secretion and on the lipid and bone metabolisms. Secondary end points were the frequency of steroid-related side effects in the two groups.

2.3. Statistical analysis

Student's *t*-test was employed to compare continuous variables. Multivariate analysis with stepwise logistic regression by SPSS software was performed to investigate the parameters with a possible influence on clinical outcome, such as age, gender, location of disease, duration of disease, concomitant immunosuppressive therapy. The differences between the two groups were performed by mixed effects ANOVA model for repeated measures. The results were corrected using a Bonferroni–Holm method for multiple testing. A p value less than 0.05 was considered statistically significant.

3. Results

3.1. Patient characteristics, clinical response

During the study period, 20 patients with IBD (5 with CD, 15 with UC) were enrolled. At day 5, all but one patient achieved clinical remission. One patient proved to be refractory to intravenous steroid and needed rescue therapy therefore she was excluded because of treatment failure. Methylprednisolone was tapered weekly and stopped at week 12 in these 19 patients who could complete the study. The clinical characteristics of the participated 19 patients are presented in Table 2. Ten patients had already been diagnosed with IBD, while the remaining 9 patients were at disease onset. Although the male/female ratio was higher in Group II, baseline clinical characteristics of patients did not differ significantly between the two treatment groups. CDAI and pMayo score showed decreasing pattern in both groups during the steroid therapy. CDAI and pMayo score decreased to a median value of 21 and 0 (median CDAI 35, median partial Mayo score 0 in Group I and 12 and 0 in Group II) at the end of the steroid therapy. Median CDAI and pMayo scores during the "conventional" and the "bolus" methylprednisolone treatment periods are indicated in Table 3. The mean values of CRP, ESR, leukocytes and thrombocytes after steroid therapy were 7.4 mg/l, 9.7 mm/h, 7.935 G/l and 238 G/l. The effects of bolus therapy on the clinical and laboratory parameters of disease activity did not differ from the conventional administration. The patients in both groups had not relapsed at the discontinuation of steroid therapy.

Table 2 Clinical characteristics of the enrolled patients.

	Group I (n = 9)	Group II (n = 10)
Mean age at the diagnosis (years)	34.3	30.3
Mean disease duration (years)	5.2	6.2
CD/UC	3/6	3/7
Female/male	4/5	2/8
Location/extension		
– Ileal	1	2
– Colonic	2	1
– Ileocolonic	–	–
– Extensive colitis	3	5
– Left-sided colitis	3	2
– Proctitis	–	–
Concomitant therapy		
– 5-ASA	4	6
– Budesonide	0	2
– Azathioprine	3	4
– Metronidazole	2	1

Table 3 Median CDAI and pMayo scores during the "conventional" and the "bolus" methylprednisolone treatment periods.

Weeks	Group I		Group II	
	Median CDAI	Median pMayo	Median CDAI	Median pMayo
0	204	6.2	164	5.1
2	184	5	152	4
4	146	3.4	126	2
6	86	1	90	0
8	72	0	64	0
10	68	0	48	0
12	35	0	12	0

3.2. Changes in adrenal glands hormone secretion, in the lipid and bone metabolism after methylprednisolone therapy

In Group I, BMI increased significantly at the end of the steroid therapy ($p = 0.008$). In Group II, no difference was observed in BMI before and after the steroid therapy. Total body composition analysis showed significant decrease in bone density in Group I ($p = 0.032$). Body fat percent showed a tendency to be higher at the end of steroid therapy in Group I, although the difference was not significant.

Considering the laboratory parameters, serum cholesterol level increased significantly in Group I patients after steroid therapy ($p = 0.028$). The decrease in serum cortisol level was more remarkable in Group I vs. Group II after steroid therapy ($p = 0.02$ and $p = 0.055$). Fig. 1 summarizes the significant changes in the examined parameters.

No changes were detected in the waist and hip circumference, T and Z scores, electrolytes, liver and renal function, serum glucose, serum calcium, triglyceride, DHEA, TSH, PTH and β-crosslaps before and after the steroid therapy neither in Group I, nor in Group II.

3.3. Steroid-related side effects

The most common side effects occurring during the therapy were the Cushingoid appearance, development of acne, fatigue, gastrointestinal complaints. Side effects were presented in 5/9 (55.6%) vs. 4/10 (40%) of the patient in Groups I and II. Cushingoid appearance did not occur in Group II. The side effects are summarized in Table 4.

4. Discussion

This prospective study revealed that "bolus-administered" corticosteroid therapy was as effective as the conventional administration of the drug, but had less harmful effects on bone and lipid metabolims and was associated with fewer side effects than the previous one. Significant increase in BMI and serum cholesterol level and decrease in body density were shown when methylprednisolone was tapered conventionally, compared to the bolus administration of the drug. Body fat percent also showed a tendency to be higher at the end of steroid therapy in patients using the conventional tapering regimens. Cushingoid appearance also occurred only in patients on conventional administration.

For the past 30 years, corticosteroids have been the mainstay of therapy in patients with moderate to severe active IBD.[9] Intravenous therapy generally produces rapid improvement of symptoms. Once improvement has been achieved, corticosteroids should be tapered gradually per week until the drug is discontinued. The mean goal of IBD therapy is to decrease the steroid-related side-effects and to minimize steroid dependency with the development of new series of anti-inflammatory glucocorticoids with

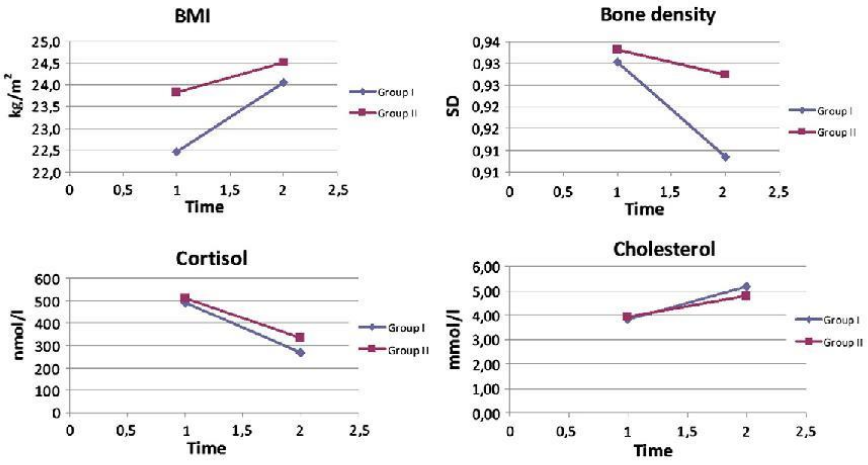

Figure 1 Significant changes in the examined parameters after 12-week methylprednisolone therapy in Groups I and II.

Table 4 Side effects developed in patients in Groups I and II.

Patients	Side effects
Group I	
Patient 1	Cushingoid appearance
Patient 2	Cushingoid appearance
Patient 3	Acnes
Patient 4	Acnes
	Arthralgia
Patient 5	Cushingoid appearance
	Arthralgia
	Fatigue
	Nausea
	Stomatitis
Group II	
Patient 1	Acnes
Patient 2	Acnes
Patient 3	Arthralgia
Patient 4	Fatigue
	Hypertension

enhanced topical potency and less systemic activity such as budesonide or beclamethasone.[10] Less attention is paid to the dosing of steroids, although it seems to be also important.

Although there are no trials between different steroid-tapering regimens, the goal in the daily practice is to get patients off corticosteroids within 12 weeks and maintain disease remission. Alternatively, alternate-day corticosteroid therapy can also be used in patients with refractory Crohn's disease — even for longer time.[11] However, some available evidence suggested that the manner of corticosteroid tapering probably did not change the long term outcome in IBD.[12] Use of "bolus-administered" steroids is a novel possibility to optimize the therapy. Bolus administration is actually an untested manner that has been anecdotally recognized to be more effective than the conventional use of steroid therapy. Multiple doses of steroids were previously shown to cause more adverse effects than a single dose.[13] In a single-center, double-blind trial performed by Bossa et al., patients with a severe attack of UC were scheduled to receive equal iv. doses of methylprednisolone, randomly given as either a bolus injection administered twice daily or continuous infusion.[14] Methylprednisolone given as a continuous infusion was no better than bolus administration in terms of efficacy and safety. The aim of bolus steroid therapy is to get quicker and stronger anti-inflammatory effect. Giving a higher dose of methylprednisolone, an immediate profound anti-inflammatory effect is supposed to be achieved with lower toxicities and no prolonged suppressive effect on the hypothalamic–pituitary axis.

Our results did not show any significant difference according to the disease outcome between the two administration types at the end of the therapy and at follow up times for any of the clinical or laboratory parameters measured, confirming the same efficacy of bolus therapy as in case of conventional administration.

The widespread use of corticosteroids has been associated with an increased incidence of a variety of adverse effects involving the musculoskeletal, the endocrine, the metabolic system, the neuropsychiatric wellbeing, the GI system, the skin, the eyes, the infectious risk, the cardiovascular and the hematological system.[12] Dosage and duration of therapy are some of the most important factors influencing the development of the toxic effects of corticosteroids. Although no data is available on the harmful effects of short term corticosteroid therapy on lipid and bone metabolism, our results revealed that short-term use of steroids increases BMI and body fat percent and decrease bone density. Common adverse effects of short term therapy include moon face, mood changes, insomnia, GI intolerance, weakness, fluid retention, weight gain, increased appetite, increased infections, amenorrhea, elevated blood glucose, slow wound healing, striae, and acneiform rash. Alternate-day steroid therapy may decrease hypothalamic–pituitary–adrenal axis suppression and therefore the development of certain side effects.[15] However, in this study, the most common side effects occurred more frequently in patients with conventional vs. bolus-administration steroid therapy.

Osteoporosis is present in 30–45% of patients with CD, and its rate is somewhat lower in patients with UC.[15,16] Osteopenia is likely related to the chronic inflammatory process itself, and furthermore triggered by steroid use. Steroid related osteoporosis is multifactorial; decreased calcium absorption, development of secondary hyperparathyroidism, stimulating osteoclast activity, and decrease osteoblast production are only some of the potential etiological factors.[17] Hyperlipidemia is also a common side effect of steroid therapy.[18] Steroids are supposed to influence lipid metabolism by redistributing body fat and facilitating of effects of lipolytic agents. Large doses of glucocorticoids lead to redistribution of fat to the upper trunk and face, with a concomitant loss of fat in the extremities.[19] Our result revealed beneficial effect of bolus-administered corticosteroid therapy on bone density and body fat percent.

The main limitation of this pilot study is the low number of participating patients. This is mainly due to the relatively high costs of total body composition analysis, which is used for the accurate determination of the various body weight components: fat mass, fat-free mass, total body water and bone mass.[20] However, use of total body composition analysis gives a valuable part of this prospective randomized study, since the alterations of bone and lipid metabolisms could be examined in a parallel way.

In conclusion, this single-center study suggests that bolus tapering of equivalent doses of methylprednisolone administered in conventional daily doses has equivalent clinical efficacy, but more favorable side effect profile. As no significant difference was detected between the two administration types on the clinical and laboratory parameters of disease activity, it appears that bolus administration of corticosteroids can safely and effectively replace the conventional use of methylprednisolone for active IBD. Of course, further controlled, randomized trials are needed to confirm these results that may revolutionize steroid therapy in IBD.

Conflict of interest

The authors have declared that they have no conflict of interest.

Acknowledgment

This work was supported by OTKA (Research Proposal PD 105948; PI: Klaudia Farkas) and TÁMOP (4.2.2.A-11/1/KONV-2012-0035, 4.2.2-A-11/1/KONV-2012 0052, 4.2.2.A-11/1/KONV-2012-0073).

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Annex IV.

Scandinavian Journal of Gastroenterology. 2014; 49: 59–65

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healthcare

ORIGINAL ARTICLE

Long-term increase in serum cholesterol levels in ulcerative colitis patients treated with cyclosporine: an underdiagnosed side effect frequently associated with other drug-related complications

ANITA BÁLINT[1], KLAUDIA FARKAS[1], MÓNIKA SZŰCS[2], ZOLTÁN SZEPES[1], FERENC NAGY[1], TIBOR WITTMANN[1] & TAMÁS MOLNÁR[1]

[1]*Department I of Medicine, University of Szeged, Szeged, Hungary, and* [2]*Department of Medical Physics and Information Technology, University of Szeged, Szeged, Hungary*

Abstract

Introduction. Several serious side effects may limit the use of cyclosporine. Cyclosporine has been reported to increase the total cholesterol level; however, the change in serum cholesterol levels before and after cyclosporine therapy has not been examined in ulcerative colitis (UC) patients. The purpose of this article was to compare serum cholesterol levels before and after cyclosporine therapy in patients with refractory UC and to examine the relationship between serum cholesterol levels and other common side effects. ***Patients and methods.*** We prospectively assessed serum cholesterol levels in UC patients who had been treated with cyclosporine. Data of 72 patients were analyzed and compared to a control group treated with Infliximab. ***Results.*** The average duration of cyclosporine therapy was 9.6 months, and side effects developed in 52 patients. Elevated cholesterol levels were detected in 47.2% of the patients. Serum cholesterol levels were significantly increased during and after discontinuation of cyclosporine therapy compared to the time before use of the drug. However, cholesterol levels measured during cyclosporine therapy were significantly higher compared to the time after its discontinuation ($p < 0.001$). Patients with drug-related side effects showed higher cholesterol levels after discontinuation of the therapy compared to those who did not experience any adverse events. ***Conclusions.*** Our findings suggest that cyclosporine therapy may result in increased serum cholesterol levels even in the long-term, after discontinuation of the therapy. Considering that significantly higher post-therapy cholesterol levels were more common in patients who developed drug-related complications, routine measurement of serum cholesterol may increase the safety of the drug.

Key Words: *cholesterol, cyclosporine, ulcerative colitis*

Introduction

Ulcerative colitis (UC) is a chronic, immune-mediated disease with periods of remissions and relapses. Flare-ups can be very severe, requiring hospitalization, and they can have life-threatening complications, such as toxic megacolon, perforation, and severe bleeding in 15% of the cases [1]. Intravenous corticosteroid therapy is the recommended treatment option for these cases, although approximately one-third of the patients seem to be refractory to steroid therapy. In these severe clinical situations, possible alternatives to total proctocolectomy are Cyclosporine A (CyA) and Infliximab [2]. Meta-analyses of the relatively limited amount of published data available have revealed a similar effectiveness profile for CyA and infliximab therapies [3], although the lower cost of CyA is undeniable [4]. Since the short-term bowel-saving capacity of CyA is excellent, the remaining major issues are presented by long-term outcomes and the safety of CyA [5]. Recently published studies have proven that the longer duration of CyA therapy and the concomitant use of azathioprine are associated with a significantly lower colectomy

Correspondence: Tamás Molnár, MD, Department I of Medicine, University of Szeged, 8-10 Koranyi fasor, Szeged, H6720, Hungary. E-mail: molnar.tamas@med.u-szeged.hu

(Received 9 September 2013; revised 19 September 2013; accepted 19 September 2013)

ISSN 0036-5521 print/ISSN 1502-7708 online © 2014 Informa Healthcare
DOI: 10.3109/00365521.2013.848231

rate than expected, although the development of side effects still remains a problem [5,6].

The most well-known major adverse effects of CyA are nephrotoxicity and hepatotoxicity. The incidence of minor adverse reactions such as tremor, paresthesia, malaise, headache, gingival hypertrophy, and hypertrichosis varies between 31% and 51%, respectively [6]. The manifestation of these side effects may lead to the discontinuation of the CyA therapy in a significant proportion of patients, although the majority of the patients under regular monitoring of CyA blood levels are able to continue the therapy for >1 year for cases of psoriasis [8]. An increase in cholesterol levels is a known side effect of CyA in transplant patients; however, we have limited data about the long-term influence on lipid metabolism in UC. Ballantyne et al. [9] measured lipoprotein levels in cyclosporine-treated patients undergoing heart or kidney transplantation. Significant increases in total (21%) and low-density lipoprotein cholesterol (31%) occurred only in the cyclosporine group of patients. Cyclosporine therapy by itself was found to affect plasma lipoprotein levels adversely by increasing total cholesterol levels.

The objective of this study was to prospectively assess serum cholesterol, triglyceride and creatinine levels before, during, and after CyA therapy in patients with severe, refractory UC and to examine the correlation between plasma lipoprotein levels and other side effects.

Patients and methods

Seventy-two patients (39 females and 33 males) suffering from severe, steroid-refractory UC were enrolled in our study from our tertiary clinic between January 1998 and June 2009. In the acute phase, intravenous cyclosporine was administered at the initial dose of 5 mg/kg for 7 days; then, depending on serum levels of the drug, the dosage was modified (to 2–4 mg/kg). Subsequently, oral treatment was administered at a mean dose of 4.7 mg/kg, adjusted according to serum levels of the drug, and coadministration of azathioprine, at a dose of 2 mg/kg, was also started in naïve patients. Patient data were collected prospectively. The mean age value for patients at the start of the cyclosporine therapy was 40.3 years. The mean age value at diagnosis was 31.8 years, and the average disease duration at the beginning of the CyA therapy was 8.6 years. The mean body mass index (BMI) at the time of starting cyclosporine was 23.6 kg/m^2 (range: 15.2–38.3 kg/m^2). Patient demographic data are detailed in Table I. 35.6% of the patients had already been treated with azathioprine by the time cyclosporine was introduced. Cyclosporine serum levels were regularly monitored during application of the therapy. The normal range of fasting levels and of 2-h-postdose levels were determined, based on transplant data, to be between 100 and 200 μg/L and between 800 and 1,400 μg/L, respectively. Our UC patients had received concomitant steroid therapy, but this was tapered off within 3 months after starting cyclosporine therapy.

Table I. The demographics of the patients treated with cyclosporine.

Gender(female/male)	39/33
BMI (kg/m^2)	23.6 (15.2–38.3)
Mean age at diagnosis (years)	31.8 (14–69)
Mean disease duration (years)	13.5 (3–42)
Left-sided colitis/extensive colitis	27/45
Previous corticosteroid therapy	39
Concomitant azathioprine	26
Mean age at start of cyclosporine therapy (years)	40.3 (15–72)
Mean disease duration at the beginning of cyclosporine therapy (years)	8.6 (0–40)
Mean duration of cyclosporine therapy (months)	9.6(0.1–60)
Mean dose of cyclosporine/bwkg (mg)	4.7
Mean trough level of cyclosporine (μg/l)	193.18
Mean peak level of cyclosporine (μg/l)	866.04

Total cholesterol, triglyceride and creatinine levels had been collected prospectively before, during, and after administration of the cyclosporine therapy (Table II). The normal total cholesterol level was defined as less than 200 mg/dL, normal triglyceride level as <150 mg/dL and normal serum creatinine in the range of 0.6–1 mg/dL. Cholesterol and triglyceride levels were measured at months 3, 6, and 12, respectively, after discontinuation of the cyclosporine therapy.

All patients were regularly monitored: in monthly follow-up visits during the CyA therapy and every second month after discontinuation. The activity of UC and the presence of adverse events were carefully assessed at each visit, and laboratory findings were entered into the clinical record. The side effects that led to discontinuation of the therapy were categorized as major, while others were categorized as minor.

To compare results and eliminate additive effects of corticosteroids on the lipid profile, we used control patients treated with infliximab and concomitant steroids. There were 24 patients in the control group, with similar clinical characteristics to the cyclosporine-treated group (Table III)

The collected data were analyzed statistically. Categorical data were analyzed using Pearson's chi-square test, Fischer's exact test, or one-sided Fischer's exact test. The effects of cyclosporine therapy on serum cholesterol, triglyceride, and creatinine levels were examined with mixed-design variance of

Table II. Mean levels of serum total cholesterol, triglyceride and creatinine.

	Before cyclosporine therapy	During cyclosporine therapy	After cyclosporine therapy
Mean level of serum total cholesterol (mmol/l)	172.9	235.5	196.2
Mean level of serum total triglyceride (mmol/l)	127.4	155.7	112.4
Mean level of serum creatinine (μmol/l)	0.81	0.83	0.78

Table III. The demographics of the control group.

Gender (female/male)	14/11
Mean age at diagnosis (years)	30.8 (15–52)
Left-sided colitis/extensive colitis	10/15
Concomitant azathioprine	16
Mean age at start of cyclosporine therapy (years)	39.2 (19–67)
Mean disease duration at the beginning of cyclosporine therapy (years)	9.2 (1–32)
Mean duration of biological therapy (months)	12

analysis (ANOVA) models with time as repeated measures (within-subject) factor and both group and side effects as between-subject factors. $p < 0.05$ was considered statistically significant. Pairwise comparisons were performed on estimated marginal means by considering the presence or absence of interaction; p-Values were corrected by the Holm–Sidak method. For the statistical analysis, SPSS 20.0 (SPSS Inc, Chicago, IL, USA) was used.

Results

The average duration of cyclosporine therapy was 9.6 months. The mean oral dose of cyclosporine was 4.7 mg/kg, the mean fasting level of cyclosporine was 193.18 μg/L, and the mean 2 h-postdose level was 866.04 μg/L.

Side effects occurred in 52 patients (72.2%) during the therapy (Figure 1). The most frequent side effects were hypertension (15.23%), tremor (13.8%), hypertrichosis (9.72%), myalgia and muscle cramping (11.1% and 4.16%, respectively), and numbness of legs (5.5%). Nephrotoxicity or hepatotoxicity occurred in 6 patients (8.33%). Increased serum cholesterol and triglyceride levels were detected in 47.2% and 19.4% of the patients, respectively. Major side effects resulting in discontinuation of the cyclosporine therapy occurred in 21 patients. The most frequent major side effects were adverse muscle reactions (47.6%) and hypertension (38.1%), followed by gastrointestinal side effects including liver enzyme abnormalities (23.8%) and skin side effects (14.3%). Major side effects are detailed in Figure 2.

The mean value of serum cholesterol levels measured before starting the cyclosporine therapy was 172.9 mg/dL (range: 84.9–331.6 mg/dL) and in the control group, it was 200 mg/dL (115.8–370.6 mg/dL). During the therapy, serum cholesterol levels increased to the mean value of 235.5 mg/dL (range: 134.4–370.6 mg/dL), while this value remained within normal range in the control group (200 mg/dL, range: 137.8–301.5 mg/dL). After discontinuation of the cyclosporine therapy, serum cholesterol levels decreased to the mean value of 196 mg/dL (range: 88–323.2 mg/dL) (Figure 3). Decreased levels of

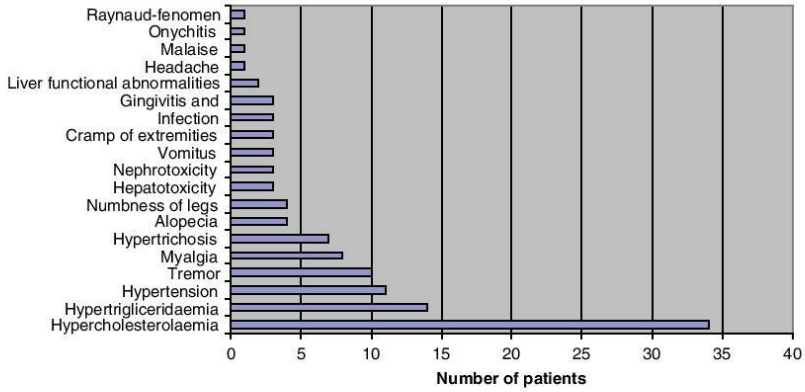

Figure 1. Side effects occurred in our enrolled patients.

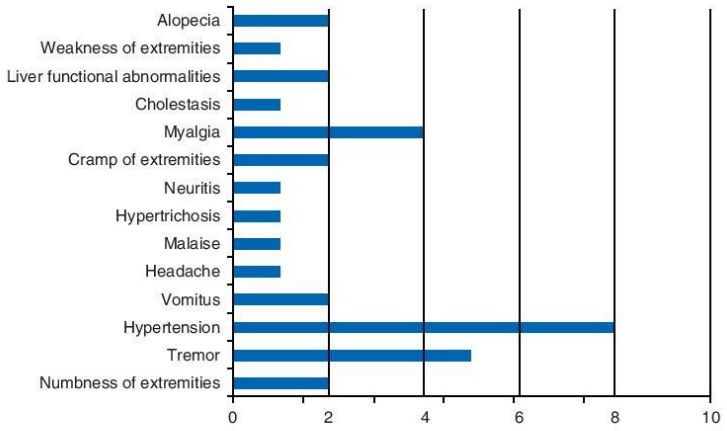

Figure 2. The major side effects during cyclosporine therapy.

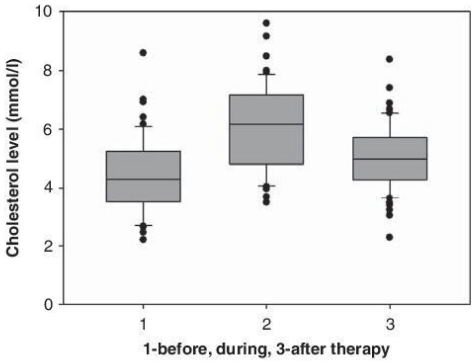

Figure 3. Cholesterol levels before, during, and after cyclosporine therapy.

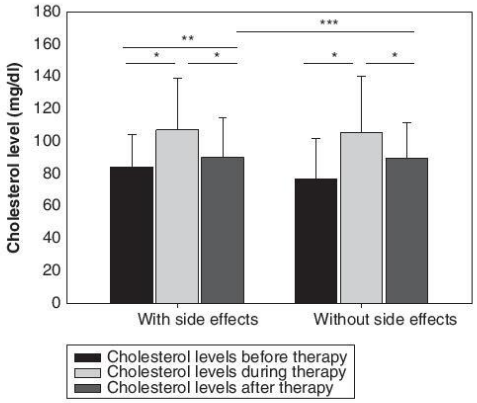

Figure 4. Cholesterol levels after cyclosporine therapy were significantly higher in patients with an adverse reaction versus patients without adverse reactions.
*: p <0.001
**: p = 0.015
***: p = 0.045

serum cholesterol were detected in patients treated with combined infliximab-steroid group after cyclosporine therapy (190.3 mg/dL; range: 110.8–277.6 mg/dL). Statistically, serum cholesterol levels increased significantly during the therapy and remained higher for one year after the discontinuation of CyA ($p < 0.001$, $p < 0.001$). Further, serum cholesterol levels measured during the cyclosporine therapy were significantly higher than those after the discontinuation of the drug ($p < 0.001$). In the cyclosporine group, cholesterol levels were considerably higher during therapy than in the control group ($p < 0.001$).

Cholesterol levels measured after cyclosporine therapy were found to be significantly higher in patients with an adverse reaction ($p = 0.045$) versus patients without adverse reactions. In both groups, serum cholesterol levels showed a significant elevation at the time of the cyclosporine therapy ($p < 0.001$) and afterward ($p < 0.001$; Figure 4). No difference was found between patients who had only minor side effects during therapy and those who had none, but those patients who had major adverse effects had higher cholesterol levels after therapy ($p = 0.013$).

Elevated total cholesterol levels were noted in 65.4% of the patients with detectable side effects. Increased serum cholesterol levels (mean value of

270.6 mg/dL) were detected in 54.5% of the patients with hypertension. Hypertension was defined as systolic pressure at or above 140 mmHg and diastolic pressure at or above 90 mmHg.

One of the most common adverse reactions was tremor. Increased serum cholesterol levels were noted in 70% of those developing tremor (mean value of 283.4 mg/dL). Neurological side effects (tremor, sensational abnormalities) occurred in 25% of the patients.

Myalgia was also a frequent side effect; 5 of 8 patients with myalgia had a high serum lipid profile (mean total cholesterol of 252.5 mg/dL). A significant correlation was found between muscular side effects (myalgia, cramping of extremities, numbness of legs) and elevated serum triglyceride levels ($p = 0.038$).

Serum triglyceride levels were elevated in 19.4% of the patients. The mean serum triglyceride levels before, during, and after cyclosporine therapy were 127.4 mg/dL, 155.7 mg/dL and 112.4 mg/dL, respectively. No difference was found in triglyceride levels compared to the control group.

Serum creatinine levels were slightly elevated only in three patients during cyclosporine therapy. No significant correlation was shown between cyclosporine therapy, the frequency of side effects, and serum creatinine levels. In addition, creatinine levels did not differ between the cyclosporine and the control group.

Discussion

This prospective study showed a significant increase in serum cholesterol levels during cyclosporine therapy compared to the time before use of the drug and to the control cohort of patients being treated with infliximab and corticosteroids. This elevation remained significant for a year. Serum cholesterol levels for the UC group with adverse events were significantly higher compared to patients who did not develop any side effects. Creatinine levels did not change significantly during cyclosporine therapy. In the control group, cholesterol, triglyceride and creatinine levels did not change significantly during therapy or after its discontinuation.

Cyclosporine is one of the most effective therapeutic choices for patients with severe, refractory UC [8]. Cohen et al. investigated [10] the quality of life of 42 patients with severe UC and found that patients who underwent colectomy assessed their own quality of life and health status to be worse than those who had been treated with cyclosporine. However, the frequent occurrence of side effects may limit the use of cyclosporine in the treatment of UC. Although 72.2% of our patients developed side effects, none of these proved to be life-threatening. It should be noted that cyclosporine had to be discontinued due to intolerable severe side effects in 29.2% of the patients. Twenty-one patients developed intolerable side effects; one achieved remission and 3 of the 21 patients had loss of response.

Although most of the adverse effects associated with the use of cyclosporine are dose-dependent, the study of van Assche et al. did not find any significant difference between the low-dose (2 mg/kg) and the high-dose (4 mg/kg) group with regard to cyclosporine-associated adverse effects [11]. In addition, oral administration is recommended after the acute period to minimize the risk of developing side effects [12]. In the acute phase of our study, cyclosporine was administered intravenously and in cases of good initial response, orally, with azathioprine treatment added in some cases [13]. Cyclosporine was administered with a mean oral dose of 4.7 mg/kg.

Nephrotoxicity and hepatotoxicity are common and severe side effects of cyclosporine therapy, but minor side effects also occur frequently during the therapy. Lichtiger et al. revealed [14] that the most common adverse reactions in UC patients treated with cyclosporine were paraesthesia and hypertension. Both symptoms were also detected in our cohort; paresthesia occurred in 4, hypertension in 11 patients. In addition, 10 of our UC patients experienced tremor, 7 had hypertrichosis and 11 noted myalgia or muscle cramping. Weber et al. reported [15] similar adverse reactions and the occurrence of infections, as well. Ten out of 19 patients developed side effects (two of them had high serum creatinine levels, one had hypertension, five had tremor, one had hirsutism, and one had gingival hyperplasia), two patients had systemic CMV infection, and one also had a herpes virus infection and esophageal candidiasis. Although no prophylactic antimicrobial treatment was administered during the cyclosporine therapy, the incidence of infections was low: only viral infection occurred in three cases (4.2%).

Nevertheless, there are limited data about the long-term effects of cyclosporine therapy on the lipid profile. In our study, serum total cholesterol, triglyceride, and creatinine levels before, during, and after cyclosporine therapy were compared in severe, steroid-refractory UC. Significant increase was found only in serum cholesterol levels during and after discontinuation of the cyclosporine therapy. However, the control group did not experience the same results. Kuster et al. [16] reported that serum levels of cyclosporine correlate significantly with total cholesterol levels, LDL-cholesterol levels, the apoB and cholesterol/HDL ratio, but not with triglyceride levels, suggesting that cyclosporine may cause atherogenic dyslipidemia. Other publications also describe similar or identical results

regarding the relationship between cyclosporine and cholesterol levels [17,18]. Spinelli et al. studied patients treated with a combination of prednisolone and another immunosuppressive drug such as cyclosporine, sirolimus, mycophenolate mofetil, or everolimus [19]. The changes in lipid profiles were evaluated after 1 year of therapy, and dyslipidemia was found to be frequent. Patients treated with cyclosporine had worse lipid profiles than those in the other treatment groups. No data are available about the relationship between hypercholesterolemia and other drug-related side effects.

Cyclosporine also has an effect on bile acid metabolism by blocking bile acid synthesis, hepatic uptake and secretion. These effects may lead to hyperlipidemia. Previous trials have shown that cyclosporine treatment affects bile salt kinetics and plasma lipid levels. However, corticosteroids affecting the bile salt synthesis could not be excluded [20,21]. In our study, we attempted to eliminate that effect with the control group treated with infliximab and streroids. Results showed higher cholesterol levels in the cyclosporine cohort, as compared to the control group. Regarding the correlation between hypertension and increased cholesterol levels as a side effect, Wang et al. suggested that cyclosporine stimulates the renal sodium channel by elevating the level of cholesterol [22]. Our results showed that hypertension and increased cholesterol levels occurred frequently in UC patients treated with cyclosporine.

Other investigations suggested that the inhibition by cyclosporine leads to dyslipidaemia by reducing apolipoprotein A1 gene expression. ApoA1 is a major component of HDL, therefore decreased levels of apoA1 result in low levels of HDL. The calcineurin pathway stimulates apoA1 gene expression; therefore, by inhibiting this pathway with cyclosporine, apoA1 levels may be reduced and as a result, HDL levels may also be reduced [23]. Only total serum cholesterol levels were assessed in our study. A - population-based study suggested that there are no benefits to measuring apoB or apoA1 compared with a total cholesterol and HDL cholesterol measurement [24]. Further, the study of Suk et al. [25] showed that calcineurin has an effect on the cAMP signaling pathway and furthermore, indirectly affects protein kinase A activation by regulating the phosphodiesterases, which play an important role in the adipokine gene transcription. In addition, calcineurin provides short- and long-term regulation to control gene expression and function.

Charco et al. [26] compared the long-term effects of tacrolimus and cyclosporine therapies on serum cholesterol levels in liver transplant patients. The average follow-up period was 36 months, and the incidence of hypercholesterolemia was found to be 34.6%. Higher mean cholesterol levels were found in the group treated with cyclosporine ($p = 0.01$). At the end of the study, a significant difference was found between the steroid and cyclosporine versus tacrolimus and steroid-free groups. In our study, a control group treated with biologicals and steroids was employed to eliminate the additive effect of corticosteroids on the serum lipid profile. Results showed that cholesterol levels were higher in the cyclosporine group than in the control group, and stayed at an increased level after therapy.

In conclusion, we found increased serum cholesterol levels in severe, steroid-refractory UC patients treated with cyclosporine not only during the therapy, but also after its discontinuation, suggesting that cyclosporine has a long-term effect on serum lipid metabolism. Further, in the presence of other adverse events, cholesterol levels were significantly higher, suggesting that drug-related impairment of cholesterol biosynthesis and other side effects are rather common, therefore practically speaking, monitoring cholesterol levels during CyA therapy is recommended. Considering the high rates of hypercholesterolemia as a side effect of cyclosporine therapy, this topic is worth to be studied further.

Acknowledgments

This work was supported by OTKA Research Proposal PD 105948 (PI: Klaudia Farkas) and TÁMOP-4.2.2.A-11/1/KONV-2012-0035, TÁMOP-4.2.2-A-11/1/KONV-2012 0052 TÁMOP-4.2.2.A-11/1/KONV-2012-0073.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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