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Fungal Microbiota and Inflammatory Bowel Disease

Fungálna mikrobiota a idiopatický črevný zápal

Diploma thesis

Supervisor: Mgr. Klára Kostovčíková, Ph.D.

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Declaration:

I declare that I prepared this thesis solely on my own with all information sources and literature cited. I did not use either this thesis or its substantial part as a background to obtain another or equivalent academic degree

In Prague, 07. 08. 2022

Sabína Vančíková

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Abstract

Inflammatory bowel disease (IBD) is a term for the gastrointestinal tract's chronic inflammatory disorders, evincing growing worldwide prevalence. Commonly, IBD is divided into two major subgroups, Crohn's disease (CD) and ulcerative colitis (UC). Despite intensive efforts, IBD etiology and development mechanisms are not fully understood. Gut barrier dysfunction, dysbiosis, and overreactive immune response to gut microbiota appear to have a significant impact. While bacteria are the most studied gut inhabitants, research on the role of fungal microbiota (mycobiota) in IBD lags behind.

This diploma thesis aimed to study immune responses to gut mycobiota in IBD. Levels of antifungal antibodies against gut residents and biomarkers relevant to gut barrier damage and microbial translocation were measured in the sera of IBD patients and healthy individuals. Sera of individuals diagnosed with UC, CD, and IBD associated with primary sclerosing cholangitis (PSC-IBD) were used. Since continuous intestinal macrophage activation is a feature of IBD, the effect of selected fungal mycobiota species on the reactivity of peripheral blood-derived macrophages from healthy donors was assessed *in vitro*.

The levels of serum antibodies against mycobiota components were higher in IBD patients than in controls, and the most remarkable differences were detected in CD. Serum biomarker levels were also elevated in IBD patients. The highest concentrations of markers connected to microbial translocation were shown in PSC-IBD patients' sera. Macrophages demonstrated no distinct polarization after stimulation with selected fungal species. However, increased production of pro-inflammatory cytokines was observed. Altogether, the results indicate the important role of mycobiota in IBD pathogenesis.

Key words: gut mycobiota, inflammatory bowel disease, Crohn's disease, ulcerative colitis, primary sclerosing cholangitis, macrophages, gut barrier

Abstrakt

Idiopatický črevný zápal (IČZ) je termín označujúci chronické zápalové ochorenia tráviaceho traktu preukazujúce rastúcu svetovú prevalenciu. Bežne sa IČZ delí na dve hlavné podskupiny, Crohnovu chorobu (CD) a ulceróznu kolitídu (UK). Napriek intenzívnym snahám stále nie sú plne objasnené mechanizmy etiológie a rozvoja IČZ. Zdá sa, že významný vplyv má porucha funkcie črevnej bariéry, dysbióza a nadmerná reaktivita imunitnej odpovede voči črevnej mikrobiote. Zatiaľ čo baktérie sú najviac študovanými obyvateľmi čriev, výskum roly fungálnej mikrobioty (mykobioty) v IČZ zaostáva.

Táto diplomová práca sa zameriavala na štúdium imunitných odpovedí na črevnú mykobiotu v IČZ. Hladiny anti-fungálnych protilátok proti črevným rezidentom a hladiny biomarkerov relevantných pre poškodenie črevnej bariéry a mikrobiálnu translokáciu boli merané v sérach IČZ pacientov a zdravých jedincov. Boli použité séra jedincov s diagnostikovanou UK, CD a IČZ asociovaným s primárnou sklerotizujúcou cholangitídou (PSC-IČZ). Keďže neustála aktivácia črevných makrofágov je jednou z čŕt IČZ, bol hodnotený aj *in vitro* efekt vybraných druhov črevnej mykobioty na reaktivitu makrofágov derivovaných z periférnej krvi zdravých darcov.

Hladiny sérových protilátok proti zložkám mykobioty boli vyššie u IČZ pacientov než u kontrol a najväčšie rozdiely boli detegované u CD. Hladiny sérových biomarkerov u IČZ pacientov boli taktiež zvýšené. Najvyššie koncentrácie markerov spojených s mikrobiálnou translokáciou sa ukázali v sérach PSC-IČZ pacientov. Makrofágy po stimulácii vybranými fungálnymi druhmi neprejavovali zreteľnú polarizáciu. Avšak, bola pozorovaná zvýšená produkcia prozápalových cytokínov. Celkovo výsledky naznačujú dôležitú rolu mykobioty v patogenéze IČZ.

Kľúčové slová: črevná mykobiota, idiopatický črevný zápal, Crohnova choroba, ulcerózna kolitída, primárna sklerotizujúca cholangitída, črevná bariéra

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List of abbreviations

5-ASA	5-aminosalicylic acid (mesalamine)
ASCA	anti-*Saccharomyces cerevisiae*-antibody
AU	arbitrary units
AZA	azathioprine
BMI	body mass index
BSA	bovine serum albumin
CD	Crohn's disease
ELISA	enzyme-linked immunosorbent assay
FABP	fatty acid-binding protein
FMO	Fluorescence Minus One
GALT	gut-associated lymphoid tissue
GI	gastrointestinal
HC	healthy control
HRP	horseradish peroxidase
IBD	inflammatory bowel disease
I-FABP	intestinal fatty acid-binding protein
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-1ß	interleukin 1 beta
IL-6	interleukin 6
IL-10	interleukin 10
IL-18	interleukin 18
IL-22	interleukin 22
ITS	internal transcribed spacer
LBP	lipopolysaccharide-binding protein
LPS	lipopolysaccharide
MBL	mannose-binding lectin
M-CSF	macrophage colony-stimulating factor
MMP-9	matrix metalloproteinase-9
OD	optical density

PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PRR	pattern recognition receptor
PSA	polysaccharide A
PSC	primary sclerosing cholangitis
PSC-IBD	inflammatory bowel disease associated with primary sclerosing cholangitis
rDNA	ribosomal DNA
RT	room temperature
SCFA	short-chain fatty acid
SD	standard deviation
sIgA	secretory immunoglobulin A
SMD	small molecule drug
SNP	single nucleotide polymorphism
TIMP	tissue inhibitor of metalloproteinases
TNF-α	tumor necrosis factor alpha
Treg	regulatory T cell
UC	ulcerative colitis

1 Introduction

In the context of gut microbiota research, fungi were considered a minor and unimportant component for a long time. However, with the progress in novel sequencing methods at the end of the last century, the study of fungal microbiota, termed mycobiota, started to expand. Afterwards, the significance of mycobiota in shaping the host's immune system has been revealed. Microfungi (yeasts and filamentous fungi) residing on the skin and epithelial cells of the body are involved in both physiological and pathological processes.

Inflammatory bowel disease (IBD) is a collection of chronic immune-mediated diseases of the GI tract with a relapsing-remitting disease course. Crohn's disease and ulcerative colitis are the main types of IBD. The global prevalence of these conditions is still rising, while the etiology remains unclear. There is evidence for the impact of environmental triggers impairing gut homeostasis in susceptible individuals. Subsequent dysbiosis and gut barrier damage underlie the development of IBD. Emerging publications suggest that changes in mycobiota composition and diversity occur in parallel to bacterial microbiota alterations.

Considering the need for efficient disease management for the increasing number of diagnosed patients, this thesis focuses on the immune responses to gut mycobiota in IBD. Decoding the role of gut mycobiota in disease pathogenesis could help to improve therapeutic strategies, especially those connected to microbiota (such as fecal microbiota transplantation).

2 Literature review

2.1 Gut (intestinal) barrier

The skin and mucous surfaces of the body form the interface between the internal space of the organism and the external environment. Thus, they must contribute to maintaining the homeostasis and integrity of the organism. The largest interface area inside the body is formed by the GI tract, which is constantly challenged with gut microbes, food or other luminal antigens. The mucosal immune system must recognize antigens that need to be tolerated. A **gut epithelial barrier** is a complex unit comprised of multiple layers – microbial barrier (gut microbiota), humoral barrier (mucus), physical barrier (epithelial cells), and immunological barrier (innate and adaptive immunity). Each of them performs specific tasks in fulfilling mentioned functions. The gut barrier composition is visually presented in **Figure 1**.

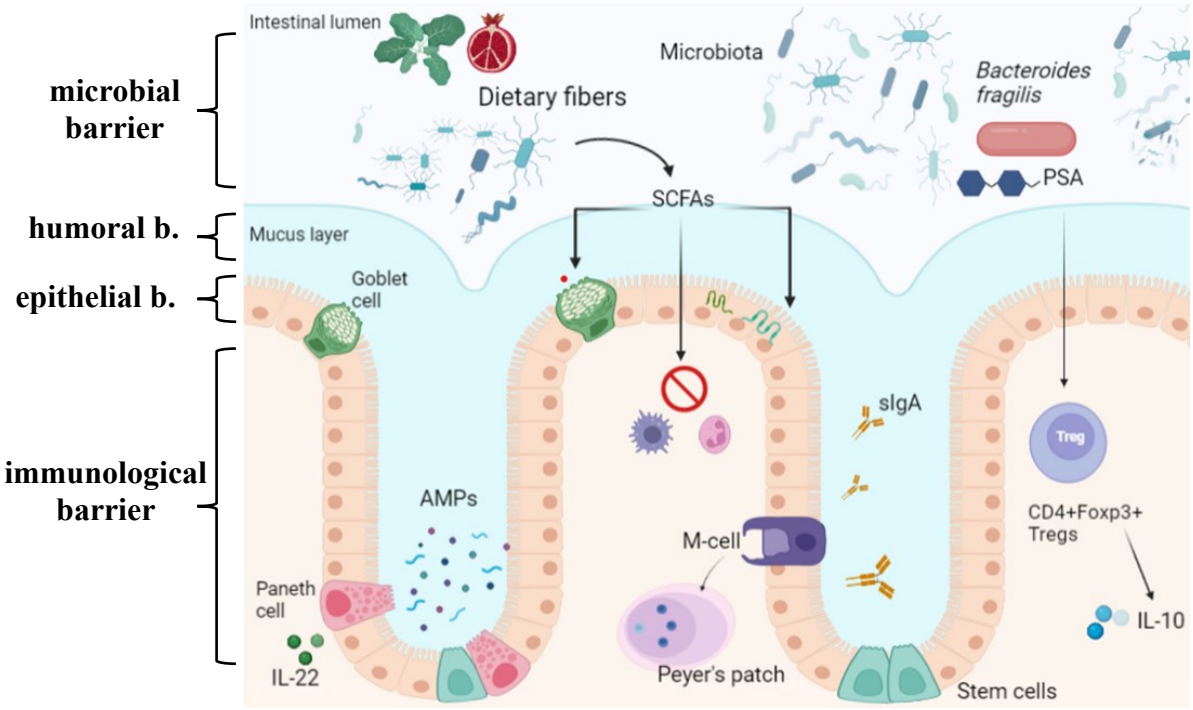


Figure 1. Gut barrier composition

Visualization of gut barrier composition and immune processes occurring there. Adapted from di Tommaso et al. (2021). **Abbreviations:** AMPs – antimicrobial peptides, IL-10 – interleukin 10, IL-22 – interleukin 22, PSA – polysaccharide A, SCFAs – short-chain fatty acids, sIgA – secretory immunoglobulin A, Treg – regulatory T cell.

2.1.1 Gut microbiota

All epithelial surfaces of the human body are covered with a myriad of microorganisms from all domains of life together with viruses, which are commonly called microbiota. The majority of them inhabit the gastrointestinal tract (GI). These complex and diverse communities of microorganisms and their importance in health and disease have been massively studied in the past decades. They are involved in metabolic and protective functions, mainly in the fermentation or anaerobic degradation of undigested dietary fibers, carbohydrates, and proteins (Yadav et al., 2018). Moreover, gut microbes can generate molecules with immunomodulatory properties that provide a wide range of impulses for the intestinal epithelial and immune cells (Delgado et al., 2020). Well-recognized immunomodulatory molecules are produced by bacterial breakdown of undigested complex carbohydrates (for example cellulose, pectin or inulin) in the process of fermentation are short-chain fatty acids (SCFAs). Some SCFAs (butyric, propionic and acetic acids) operate as an energy source for epithelial cells and participate in regulatory functions, such as the increase of mucus production or inducing immunotolerance (Macfarlane & Macfarlane, 2003; Robert & Bernalier-Donadille, 2003; Rooks & Garrett, 2016; Vinolo et al., 2011). Furthermore, commensal bacteria can promote regulatory T cell (Treg) expansion and anti-inflammatory cytokine IL-10 production via another immunomodulatory molecule, polysaccharide A (Round & Mazmanian, 2010).

The crucial immunological role of microbiota is also played in the development of the host's immune system as microbes generate specific metabolites and exhibit conserved patterns, pathogen-associated molecular patterns (PAMPs). PAMPs are recognized by the pattern recognition receptors (PRRs) on the surface of different cell types, predominantly immune and intestinal cells. Hence, intestinal epithelial cells are engaged in dynamic crosstalk between gut microbiota and the host's immune system.

The gut microbiota is, to a significant extent, established in the first years of children's lives. During that time, the diversity and density are dynamically changing. They are under a strong influence of environmental factors – mode of delivery, breastfeeding, antibiotic use in the perinatal period, geography, and others. As an individual gets older, the gut microbiota tends to become less diverse and can be also altered by lifestyle, infections, and medication (Qin et al., 2010a; Scholtens et al., 2012). The disruption of the symbiosis of the host and the microbiota, alterations of the microbial composition or reduction of the

diversity are called dysbiosis. Dysbiosis, especially in early life, contributes to an individual's disease susceptibility (Gomaa, 2020; Rodríguez et al., 2015).

An individual chapter is dedicated to the fungal component of microbiota as it is the main topic of this thesis (see section *2.2 Mycobiota*).

2.1.2 Humoral barrier

The intestinal epithelial layer is covered by mucus, a thick viscous layer that is composed of water and molecules secreted by goblet cells, mainly mucins. Several subfamilies of mucins have been identified, however, highly glycosylated gel-forming MUC2 is the best-characterized mucin of the GI tract (Pelaseyed et al., 2014). Whereas the mucus layer in the small intestine is discontinuous, relatively porous, and penetrable to bacteria, in the large intestine it is organized into the inner and outer layers. The inner layer is firmly attached to the epithelial cells and bacteria are unable to penetrate. The qualities of mucus may also differ depending on the concrete location in the intestines (Paone & Cani, 2020).

The role of the mucus layer is to protect the gut epithelial barrier from external agents by retaining antimicrobial peptides and the secretory immunoglobulin A (sIgA), facilitate nutrient absorption, and prevent direct contact between epithelial cells and microbiota. Gut microorganisms together with the mucus layer are essential to limit pathogen invasion and potential infection (Meyer-Hoffert et al., 2008; Paone & Cani, 2020).

2.1.3 Physical barrier

The intestine is a tube lined by a continuous epithelial cell layer connected to the basal lamina. There is a number of epithelial cell types – the most abundant are enterocytes (intestinal absorptive cells) with characteristic microvilli on the apical membrane increasing the absorption area. The other cell types are unevenly spaced between enterocytes. These include goblet cells necessary for mucus formation, enteroendocrine cells, secretory Paneth cells, chemosensory tuft cells, and microfold cells (M cells) allowing the capture of microorganisms and various luminal molecules and their transport to the underlying lymphoid follicles (Kobayashi et al., 2019; Strunk et al., 2020). The ratio of goblet cells to enterocytes changes along the GI tract – there is a higher proportion of Goblet cells in the large intestine than in the small intestine, which is proportional to the increase of microorganisms (Kim & Ho, 2010). The gut epithelium possesses proliferative crypts with

intestinal stem cells, which enables the turnover of epithelial cells every 4–5 days (van der Flier & Clevers, 2009).

Physiologically, the gut epithelial layer is impermeable to large molecules owing to junctional complexes interconnecting the cells. The transport occurs exclusively via specific transporters. Particularly important junctional complexes are tight junctions that avoid the translocation of commensal and pathogenic organisms. Higher levels of inflammatory molecules in a diseased gut or some enteric pathogens may weaken the gut epithelial barrier by modulating tight junctions (Paradis et al., 2021).

2.1.4 Immunological barrier

In addition to the abovementioned immune mechanisms, the lymphoid follicles or Peyer's patches, both gut-associated lymphoid tissue (GALT), are situated under the intestinal epithelial layer surrounded by follicle-associated epithelium and diffuse cells of innate and adaptive immunity. These sites represent the location of immune response initiation.

The basolateral membrane of microfold cells is invaginated and forms an intraepithelial pocket that facilitates the delivery of transported antigen to immature dendritic cells, macrophages or B cells (Williams & Owen, 2015). Subsequently, dendritic cells undergo a maturation process and migrate to T cell areas of GALT, where the antigen presentation takes place.

Peyer's patches are involved in the generation of B cells and plasma cells producing immunoglobin A (IgA), the most relevant antibody isotype in mucosal immunity (Reboldi & Cyster, 2016).

Paneth cells have antimicrobial and microbiota-modulating activities through the secretion of immunomodulatory proteins, such as defensins, lysozyme, and phospholipase A2 (Ayabe et al., 2000; Elphick & Mahida, 2005).

Many other cell types of innate and adaptive immunity contribute to the immunological barrier function, however, it is beyond the scope of this diploma thesis.

2.2 Mycobiota

Bacteria received the vast majority of research attention as the most abundant microorganisms in the host's microbiota. At the same time, eukaryotic cells appeared as a minor and unimportant part of the entire microbiota. Nonetheless, while being, on average, a hundred times larger cells than bacterial cells, eukaryotes form an appreciable share of intestinal ecosystem biomass. They contribute to metabolisms and might proliferate in response to dietary changes or dysbiosis. Thus, recent studies have indicated the significance of these organisms residing in and on the human body and their role in shaping the immune system (Underhill & Iliev, 2014). Chiefly the role of the fungal component known as **mycobiota** has been examined. The term mycobiota is a combination of the words 'mycology' and 'microbiota' and emerged after the first mention by Ghannoum et al. (2010).

The massive expansion in characterising the fungal communities was made after replacing microscopic and culture-dependent methods with those based on nucleic acids. Stool samples were historically collected and cultured using direct plating as a gut fungi source. This approach had severe drawbacks, noteworthy the inability to culture some microbial organisms. To mention possible causes, the dominant populations could restrict the detection of low-abundance organisms and fungi requiring complex microbe-microbe interactions may be cultivated inadequately (Carraro et al., 2011). Therefore, the development of polymerase chain reaction (PCR) at the end of the 20th century and subsequent novel high-throughput DNA sequencing strategies and bioinformatic analyses revolutionized fungal detection and made it affordable. Species are distinguished by identifying and comparing amplified genome fragments with a database. Commonly used targeted fragments are ribosomal RNA (rRNA) genes (18S small subunit ribosomal DNA, rDNA, and 28S large subunit rDNA) and internal transcribed spacers (ITS1 or ITS2) situated between the subunit genes. Fungal rRNA genes are highly conserved and serve as primer binding sites enabling the amplification of adjacent variable regions. The least conserved are ITS regions considered the primary fungal identification markers (Schoch et al., 2012). Fungal metagenomic sequencing is not well adapted as it needs high sequencing depth and is sensitive to host DNA contamination (Lai et al., 2019). Naturally, some sequencing biases exist; however, it is the most accurate method for determining the mycobiota composition. The culturing is predominantly used for diagnostics since the progress in the development of differential chromogenic media was made. For example, CHROMagar^{TM} Candida can differentiate between clinically relevant species of fungi (*Candida* species).

Every human being is colonized with fungal populations on various body sites, on the skin and the mucosal surfaces – the GI tract, respiratory tract, vagina, urinary tract, and others (Ackerman & Underhill, 2017; Findley et al., 2013; Ghannoum et al., 2010). Human mycobiota is comprised of phylogenetically diverse **microfungi**, morphologically yeasts or filamentous fungi. Microfungi (known as microscopic fungi or *Micromycetes*) are fungi that must be studied under a microscope and develop microscopic reproductive structures (fruiting bodies) containing spores. The fungal nomenclature is governed under the *International code of nomenclature for algae, fungi, and plants*, which has undergone several modifications including the adoption of the 'one fungus – one name' principle in the Melbourne code (McNeill et al., 2012), which aimed to terminate the two multiple name system for some organisms – often for sexual (teleomorph) and asexual (anamorph) states of fungi. These states of the life cycles apply to the pleomorphic fungi (occurring in more forms) in the phyla *Ascomycota* and *Basidiomycota;* the whole fungus is termed holomorph. Many anamorph-teleomorph forms have not been paired yet, and sequencing might reveal they are the same organism in the future. The alternative naming, misidentifications, and misspellings aggravate searching in databases, literature research, and general communication of scientific and medical findings. Therefore, taxonomic instability is an issue of considerable concern that should be improved in the future.

2.2.1 Mycobiota in the human gut

Specific body sites have a unique composition of mycobiota and the local immune system might interact with the fungi in dissimilar ways. The fungal populations vary also over time and with health/disease status.

In at least 70% of healthy adults, fungi are detectable in the GI tract (Schulze & Sonnenborn, 2009). The colonization starts at birth as microfungi are transmitted vertically from the mother and horizontally from the environment (Lupetti et al., 2002). Sequencing has estimated a maximum of 0.1% of eukaryotic genes in human gut mycobiota based on available reference databases (Qin et al., 2010b). In contrast to precise analogous bacterial 16S databases, fungal databases contain frequent errors. Thus, fungal species tend to be highly underrepresented compared to bacteria and might be under-detected in sequencing efforts (Underhill & Iliev, 2014). The diversity of fungal microbiota is lower than bacterial (Lozupone et al., 2013). Nevertheless, the human gut provides a home to at least 60 fungal genera, dominantly yeasts and filamentous fungi (the latter to a lesser extent). Gut fungi

primarily belong to two inversely correlating phyla, *Ascomycota* and *Basidiomycota* with the most prevalent genera being *Saccharomyces*, *Candida*, *Cladosporium* (all *Ascomycota*), and *Malassezia* (*Basidiomycota*). Namely, the most commonly detected species include namely *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. krusei*, *Saccharomyces cerevisiae*, *Cladosporium cladosporioides*, *Penicillium allii*, *Malassezia globosa*, *M. restricta*, *Debaryomyces hansenii*, and *Galactomyces geotrichum* (Suhr & Hallen-Adams, 2015). These species form 'core mycobiota' both across healthy individuals' guts and longitudinally within individuals, strongly suspected as symbionts. *Candida* species are widely accepted as permanent residents of the human gut, however, the symbiotic nature of *Saccharomyces* and *Malassezia* species is still debated owing to the potential attribution to diet (mushroom, bread, beer, and others). The remainder of gut fungi inhabitants is more diverse with a large proportion of well-documented environmental/dietary transients (opportunists) that may originate from food or airborne spores, such as molds (hyphae) genera *Aspergillus*, and *Penicillin*. These might inhabit the gut only transiently (Fiers et al., 2019; Hoffmann et al., 2013a; Nash et al., 2017).

2.2.2 Interactions with the host's immune system

Since the GI tract is the habitat of both (symbiotic) microfungi and immune cells, the host's immune system had to adapt to the presence of microorganisms. The recognition of fungal-associated molecular patterns is arranged by a plethora of innate immune receptors, which can mediate cellular signaling cascades resulting in various actions, such as fungal uptake, cytokine and chemokine production, reactive oxygen species production, and pathogen killing. The importance of PRRs in shaping fungal immunity has been established. The same PAMP might interact with cell type-specific and fungus morphotype-specific PRR.

Glycans, glycolipids, and glycoproteins are typical components of fungal cell wells and are recognized by a number of C-type lectin receptors. Although the cell wall components vary between species, it usually comprises an inner chitin layer, followed by a layer of glucans, and an outer layer of mannans or their modifications. Of all C-type lectin receptors, Dectin-1 (CLEC7A, CD369) interacting with β-glucans of some fungi, notably *Candida*, *Aspergillus*, and *Cryptococcus*, in a Ca^{2+} dependent manner appears to be the most crucial (Lionakis & Levitz, 2018). Expression of the Dectin-1 has been observed on myeloid cells (dendritic cells, monocytes, macrophages, and neutrophils), subsets of B and T cells,

mast cells, keratinocytes, and epithelial cells (Cohen-Kedar et al., 2014; Olynych et al., 2006; van den Berg et al., 2014). Signaling via Dectin-1 involves the recruitment of Syk kinase leading to the action of some transcription factors, most importantly NF-κB, and subsequent cytokine production. CARD9 functions as a key transducer in this pathway. Phagocytosis, autophagy, and respiratory burst are other common processes after Dectin-1 ligand binding (Brown, 2005).

The expression of another C-type lectin receptor, DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN, CD209) is restricted to myeloid cells. DC-SIGN forms a tetramer binding mannans and galactomannans of *Candida* and *Aspergillus* species. Its signaling pathways are poorly understood (Lionakis & Levitz, 2018).

Other significant C-type lectin receptor clusters restricted to myeloid cells have been discovered in recent years – Dectin-2 (CLEC6A), Dectin-3 (CLEC4D, MCL), and Mincle (CLEC4E). These are associated with the recognition of α-mannans, O-linked mannoproteins, glyceroglycolipids, and mannitol-linked mannosyl fatty acids mainly from *Candida*, *Aspergillus*, and *Malassezia* species (Lionakis & Levitz, 2018; Nagata et al., 2017). The downstream signaling often complements or antagonizes the signaling via other PRRs (Wevers et al., 2014).

The first receptor proved to react to *Candida* species was the transmembrane mannose receptor (CD206). It is, however, more a binding receptor than a phagocytosis-triggering (Brown, 2011). The soluble oligomeric lectin mannose-binding lectin (MBL) is produced by the liver and circulates in the bloodstream, where it binds microbial carbohydrate structures and mediates the lectin pathway of the complement system activation outcoming in the phagocytosis.

Toll-like receptors and other families of PRRs or scavenger and complement receptors are also engaged in fungal recognition. Moreover, the crosstalk and collaboration between particular receptors were observed. This topic is, nonetheless, omitted in the diploma thesis.

2.2.3 Fungal pathologies and disease susceptibility

Many fungal species can act as pathogens in susceptible and immunocompromised individuals. To give some examples, changes in the diversity of skin mycobiota (traced to *Malassezia* species) may underlie psoriasis, dermatitis or other skin conditions with

compromised barrier functions (Saunders et al., 2012). Next, *C. albicans* causes recurrent vulvovaginal candidiasis in the affected individuals. Lastly, the overgrowth of *C. albicans* in the GI tract and its passage to the bloodstream are believed to initiate susceptibility to systemic candidiasis (Kennedy, 1989). Furthermore, some *Candida*-associated gastric ulcer cases were described (Sasaki, 2012).

In the gut environment, several phagocytic cell subsets are capable to respond to fluctuations in fungal residents' diversity. Especially, CX_3CR1^+ mononuclear phagocytes and $CD103^+$ $CD11b^+$ dendritic cells are essential in the induction of antifungal immunity. Their cooperation can assist in establishing tolerance (Mazzini et al., 2014) and on the other hand, depletion of these cells or a partial loss of function due to SNPs result in antifungal response impairment and susceptibility to disseminated candidiasis (Lionakis et al., 2013). Macrophages are conventionally considered to polarize into M1 or M2 phenotypes. M1 are believed to be pro-inflammatory, classically activated macrophages, fungicidal cells and the M1 polarization may be induced by lipopolysaccharide (LPS), IFN-γ or GM-CSF. M2 appear to be anti-inflammatory, alternatively activated, induced by IL-4 or IL-13 (Lawrence & Natoli, 2011). The phenotype switching between M1 and M2 macrophages has been shown to protect against fungal pathogens (Davis et al., 2013; Salazar & Brown, 2018).

Genetic mutations in the abovementioned fungi recognition receptors or molecules involved in their downstream signaling cascades are connected to increased susceptibility to human fungal infections. In humans, various SNPs in fungal recognition receptors may correlate with pathologies – Dectin-1 polymorphisms with major fungal infections, aspergillosis and mucocutaneous candidiasis (Chai et al., 2011; Ferwerda et al., 2009), Dectin-2 with pulmonary cryptococcosis (Hu et al., 2015), DC-SIGN with pulmonary aspergillosis and fungal keratitis (Qu et al., 2015; Sainz et al., 2012), and many others.

The role of mycobiota in IBD is discussed in the individual chapter (see section *2.4.2 Mycobiota in IBD*).

2.3 Inflammatory bowel disease

IBD is an umbrella term covering a collection of chronic inflammatory disorders of the gastrointestinal (GI) tract. Traditionally, IBD can be divided into **ulcerative colitis (UC)** and **Crohn's disease (CD)**. The natural course of both disorders is marked as relapsing and remitting, which manifests in unpredictable alternating phases of present clinical signs (relapses) and partial or complete recovery (remissions) throughout the lives. Although similar characteristics exist, UC and CD represent different molecular diseases. The divergent mechanisms on the molecular level include Th1 and Th2 immune responses related to CD and UC, respectively, greater damage of the gut barrier in UC, and inconsistencies in signaling (Ruiz Castro et al., 2021). Depending on the form of IBD, symptoms and the features of the inflammatory effects widely differ. CD inflammation may affect any part of the GI tract, whereas localization of UC inflammation is limited to the large intestine. A detailed description of the distribution is presented in **Figure 2**.

As a result of the anatomic heterogeneity, UC patients experience pain in the rectum or on the lower left side of the abdomen, diarrhea, blood in the stool (alternatively rectal bleeding), and weight loss. Histologically, inflammation of the intestinal lining is superficial, restricted to the mucosa/submucosa, causing irritation and ulceration. The severity and frequency of the symptoms positively correlate with the extent of the affected intestinal area. On the contrary, people diagnosed with CD suffer from pain in the lower right abdomen. They might also encounter diarrhea, night sweats, and nutritional deficiencies due to poor absorption. Intestinal wall thickening is a prominent sign, occurring consequent to the swelling and constant blockage. CD tends to extend transmurally and can form fistulas, abnormal connections between two different body parts – the most common is the perianal fistula (fistula-in-ano), a tunnel between the anal canal and the perianal skin (Fakhoury et al., 2014; Yeshi et al., 2020). Moreover, extraintestinal manifestations are seen in up to 40% of IBD patients. These comprise complications associated with nearly any organ system, typically musculoskeletal, renal, dermatologic, and pancreatic problems. Some people may develop the mentioned complications before the onset of colonic symptoms (Rusoniene et al., 2020). Besides the classic extraintestinal manifestations, IBD patients are at significantly higher risk of colorectal cancer development, and also autoimmune and other immune-mediated diseases are more frequent among them (Halling et al., 2017; Stidham & Higgins, 2018).

Considering the burden that patients carry, an issue of major concern is the growing prevalence of IBD around the world. In the second half of the 20th century, the incidence steadily rose in industrialized countries, with the specific lifestyle hypothesized as the driving force. For this reason, IBD has been believed to be a disease of the Western world for a long time. However, the notion is now being dismissed thanks to the changes in global epidemiological patterns that have arisen since the turn of the century. Developed regions (such as North America or Europe) started to report a plateauing incidence of IBD, while the number of new diagnoses in developing and newly industrialized countries, mainly in South America or Asia, is accelerating each year. Possible explanations for the latter trend are the westernization of those societies, awareness, access to healthcare, and advancing infrastructure (Ananthakrishnan et al., 2020; Windsor & Kaplan, 2019). Owing to the gain in life expectancy over time, the prevalent population of IBD patients in the Western world is still growing even if the incidence is stabilized (Kaplan & Windsor, 2021). As stated in the recent systematic review by Ng et al. (2017), the incidence rate in North America ranges between 8.8–23.1 per 100,000 person-years for UC and between 6.3–23.8 per 100,000 person-years for CD; in Western Europe it is 1.9 –17.2 per 100,000 person-years for UC and 1.9–10.5 per 100,000 person-years for CD. The conditions are often diagnosed during adolescence or young adulthood. The pediatric IBD onset rates continue to climb globally (Kuenzig et al., 2022).

Even though IBD etiology alludes to determinants in the Western world, the cooperation of many other environmental and genetic risk factors is needed. Pathogenesis is multifactorial and very complex and requires the presence of many environmental and genetic risk factors. The heritability model of IBD remains unknown, nevertheless, up to 12% of IBD patients have a family history of the diagnosis. Genetics of the individual appears to play a more significant role in CD than UC (Moller et al., 2015). Genome-wide association studies have thus far identified more than 200 single nucleotide polymorphisms (SNPs) with effects on IBD. Many identified genetic risk loci implicate genes necessary for the epithelial barrier function, regulation of immune processes, autophagy, cell survival, and host-microbes interactions in general. Hence, IBD pathogenesis may be linked to gut microbiota to a large extent (Turpin et al., 2018). Any disturbance in the intestinal ecosystem can trigger abnormal immune responses present also in IBD (see section *2.1 Gut barrier*). Regarding the environmental factors, reliable evidence-based recommendations for preventing future IBD initiation are lacking. However, people at risk are advised to modify

some of their lifestyle habits that substantially contribute to the attributable risk of IBD. Still, together they can head towards the prevention of IBD. These include breastfeeding, keeping a consistent sleep schedule, following a nutritious diet, being physically active, maintaining a healthy weight, minimizing antibiotics and nonsteroidal anti-inflammatory drugs, and avoiding smoking and vitamin D deficiency. Air pollution and hypoxia caused by high altitudes are also under investigation as novel potential triggers of IBD (Ananthakrishnan et al., 2018).

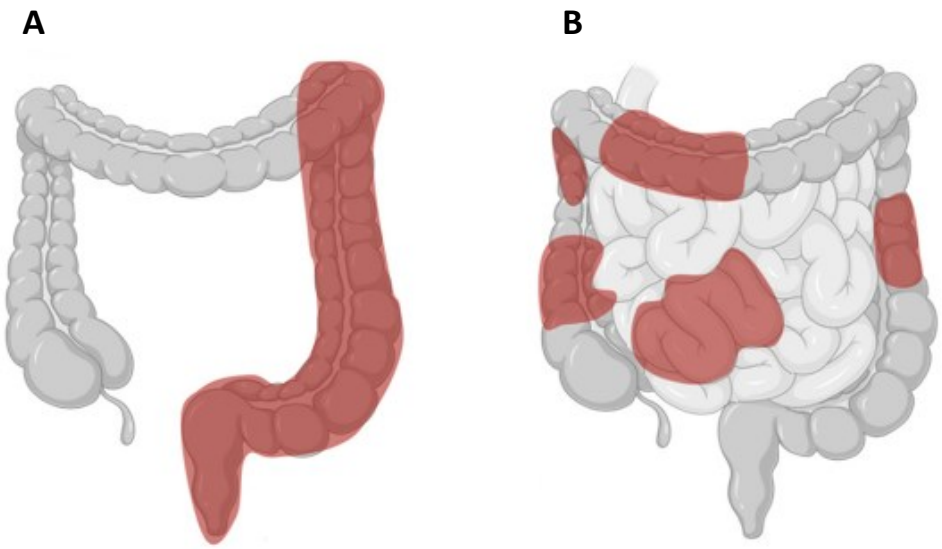


Figure 2. Anatomic distribution of inflammatory bowel disease

(**A**) **Ulcerative colitis**: The condition usually begins in the rectum and progresses proximally in a continuous manner through the large intestine. Classification of the disease is based on the extent of colonic involvement – proctitis, left-sided (distal) colitis, and total colitis; (**B**) **Crohn's disease**: Discontinuous segmental lesions ('skip lesions') are primarily located in the small and large intestine, rarely attacking the rectum and the upper parts of the gastrointestinal tract. As specified by the affected regions, several types of the disease are recognized, such as ileocecal Crohn's disease, Crohn's colitis, or perianal Crohn's disease. Red areas indicate inflammation. Adapted from Ordás et al. (2012) and Yeshi et al. (2020).

Due to the growing prevalent population of IBD, healthcare providers and policymakers are challenged to search for IBD prevention and cost-efficient healthcare management. There is a demand for optimizing the diagnosis of IBD and providing access to emerging treatments (Yeshi et al., 2020). The goal of medications is to alleviate inflammation and prolong the remission afterwards. Currently, the most widely used conventional drugs are biologics and small molecule drugs (SMDs) comprising corticosteroids (for example prednisone), salicylates (for example mesalamine), and

immunomodulators (for example azathioprine). SMDs are easily administered (orally) thanks to their low molecular weight, have a shorter half-life and are also cheaper than biologics (Olivera et al., 2017). Biologics are monoclonal antibodies, recombinant cytokines, specific antagonists of cytokines and other immune modifiers involved during immune-mediated pathologies. They are usually prescribed to patients non-responding to anti-inflammatory drugs or suffering from adverse side effects. Several monoclonal antibodies are approved for IBD treatment, such as integrin antagonist vedolizumab, anti-TNF-α antibodies infliximab, adalimumab, anti-adhesion agent natalizumab, and pro-inflammatory cytokine inhibitor ustekinumab. New therapeutic strategies are in clinical trials each year (Noor et al., 2021). Additional strategies can cover probiotics (for example *Escherichia coli* Nissle 1917), antibiotics for infections, anti-diarrheal medications, pain relievers, and vitamins. The last choice of therapeutic options, especially necessary in severe cases, is surgery. It means the removal of the diseased tissue. Surgical intervention is curative in UC, but only controls the symptoms in CD – owing to the disease site (Kühn & Klar, 2015). The surgical decision-making depends on the extent of the inflamed tissue and potential postoperative complications. A schematic of treatment algorithms in developed countries is displayed in **Figure 3**. The implementation of novel therapies in the developing world still lags behind (Mak & Sung, 2019).

2.3.1 Primary sclerosing cholangitis

IBD is closely associated with several other conditions, noteworthy with the primary sclerosing cholangitis (PSC). PSC is a rare chronic liver disease of unknown cause, where inflammation of the intra- and extrahepatic bile ducts results in obstruction, fibrosis, multifocal scars and strictures within the bile ducts, and liver cirrhosis. Prevalent symptoms also include fatigue, pain, and itch (Karlsen et al., 2017). Cholangitis (inflammation of the bile duct system) manifests with bacteria in patients' bile cultures, which is treatable with antibiotics. On the other hand, *Candida* infection of the bile leads to a poor prognosis and a subsequent need for liver transplantation (Rudolph et al., 2009). PSC patients are also more prone to some co-morbidities, such as tumors (biliary, colorectal or hepatocellular cancer) and gallbladder abnormalities and lesions (Bergquist et al., 2002; Vázquez-Elizondo et al., 2008; Weismüller et al., 2017).

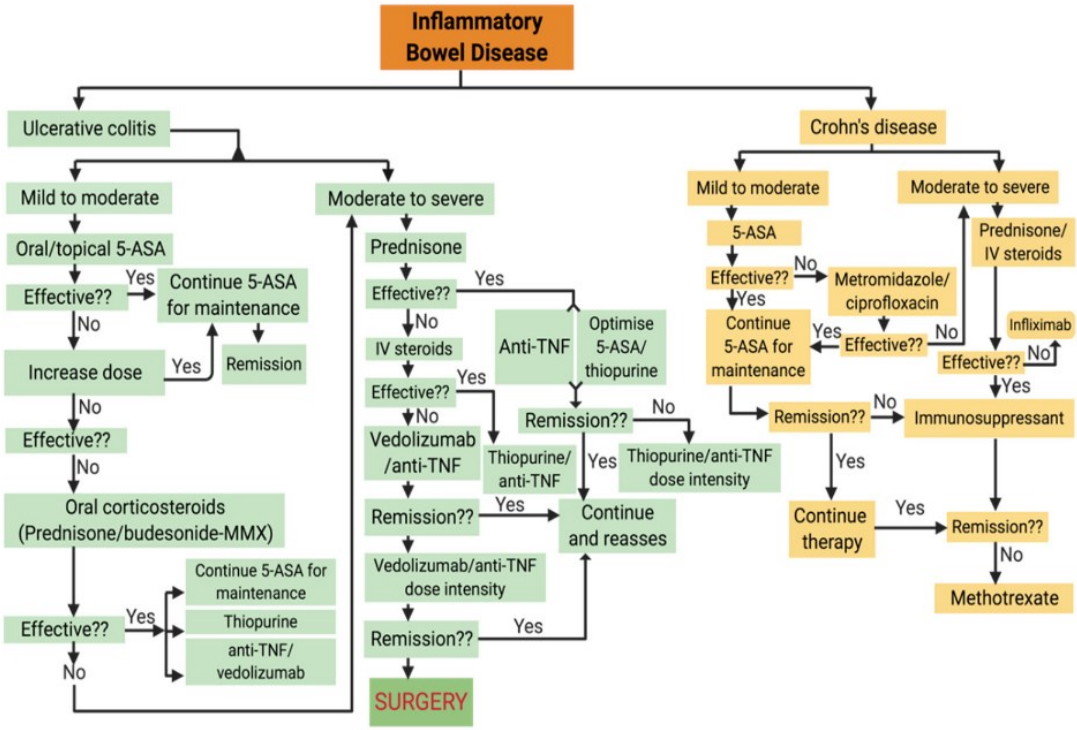


Figure 3. Current treatment options for inflammatory bowel disease

Current therapeutic strategies for ulcerative colitis and Crohn's disease in the developed world. Adapted from Yeshi et al. (2020). **Abbreviations:** 5-ASA – 5-aminosalicylic acid (mesalamine), TNF – tumor necrosis factor alpha.

The incidence rate of PSC in North America and Northern Europe ranges between 0.4–2.0 per 100,000 person-years. PSC probably affects women as often as men, however, in females, it has a clinically more quiescent mode (Lunder et al., 2016; Weismüller et al., 2017). Concerning genetics, siblings of PSC or IBD patients have an increased risk of developing PSC during their lives (Bergquist et al., 2008). More than two-thirds of patients have underlying IBD, most frequently a distinct phenotype with right-sided colonic involvement (de Vries et al., 2015). Possible theories imply that could be caused by the leakage of microbes and microbial components into the circulation and liver (Lichtman et al., 1990; Terjung et al., 2010) or partially by the recruitment of gut-derived T cells to the liver (Grant et al., 2001). Furthermore, the disturbed microbiota can trigger the transformation of bile acids impairing their anti-inflammatory effects on the gut epithelial cells (Duboc et al., 2013). On the whole, the interplay between gut microbiota and bile duct physiology in the context of PSC and related conditions creates considerable research opportunities nowadays.

The disease course of PSC is highly variable. Hence, the choice of treatment strategies is complicated. PSC-IBD patients receive medications typically used as IBD therapy with additional strictures treatment and the possibility of liver transplantation in selected patients (Karlsen et al., 2017). The discovery of biomarkers and advanced imaging technologies could help to implement new diagnostic, prognostic, and therapeutic strategies.

2.4 Immunity in inflammatory bowel disease

2.4.1 Gut barrier in IBD

The microscopic features of CD are transmural inflammation affecting the whole depth of the gut wall, mucosal damage, thickened submucosa, and granulomas (aggregates of macrophages). On the contrary, UC involves superficial inflammation in mucosa and submucosa, distortion of crypts with crypt abscesses formation, and infiltration of immune cells (Fakhoury et al., 2014b; Khor et al., 2011). Although these differences exist, the basis of disease mechanisms is similar in both conditions. The mechanism is described below and visualized in **Figure 4**.

The intestinal leakiness is a characteristic mark of IBD. The reasons for gut barrier failure are dysbiosis, abnormal composition of gut microbiota, mucus defects, alterations in junctional complexes between epithelial cells, and chronic inflammation.

To raise some observations concerning this issue, a loss of mucin sulfation was noticed in the colonic tissue of UC patients (Corfield et al., 1996), which can increase the vulnerability of the mucus layer to degradation by bacterial enzymes. Microorganisms are thus in more intense contact with gut epithelial cells. Decreased expression of tight junction proteins, claudins and occludins (Gassler et al., 2001; Zeissig et al., 2007), was detected suggesting the influence of pro-inflammatory cytokines produced by the underlying inflamed tissue. Additionally, some risk loci associated with IBD in genes for junctional proteins (for example, zonula occludens-1) were identified (Anderson et al., 2011).

Impaired microbial recognition in IBD is implicated by several factors. Firstly, it is a demonstrated IBD susceptibility due to a frameshift mutation in a gene encoding the NOD2 receptor (belonging to PRRs) in CD (Ogura et al., 2001). Furthermore, SNPs in genes encoding NOD2 and some autophagy proteins (ATG16L1 and IRGM) were identified (Cleynen et al., 2013; McGovern et al., 2001). That is important owing to the role of

autophagy in antigen presentation and pathogen clearance. NOD receptors are involved in that process by recruiting autophagy-related proteins. Lastly, CD patients showed reduced antibacterial activity in gut mucosa with a specific decrease in α-defensins secreted by Paneth cells (Wehkamp et al., 2005). Mentioned ATG16L1 prevents loss of Paneth cells by TNF-α-mediated necroptosis (Matsuzawa-Ishimoto et al., 2017).

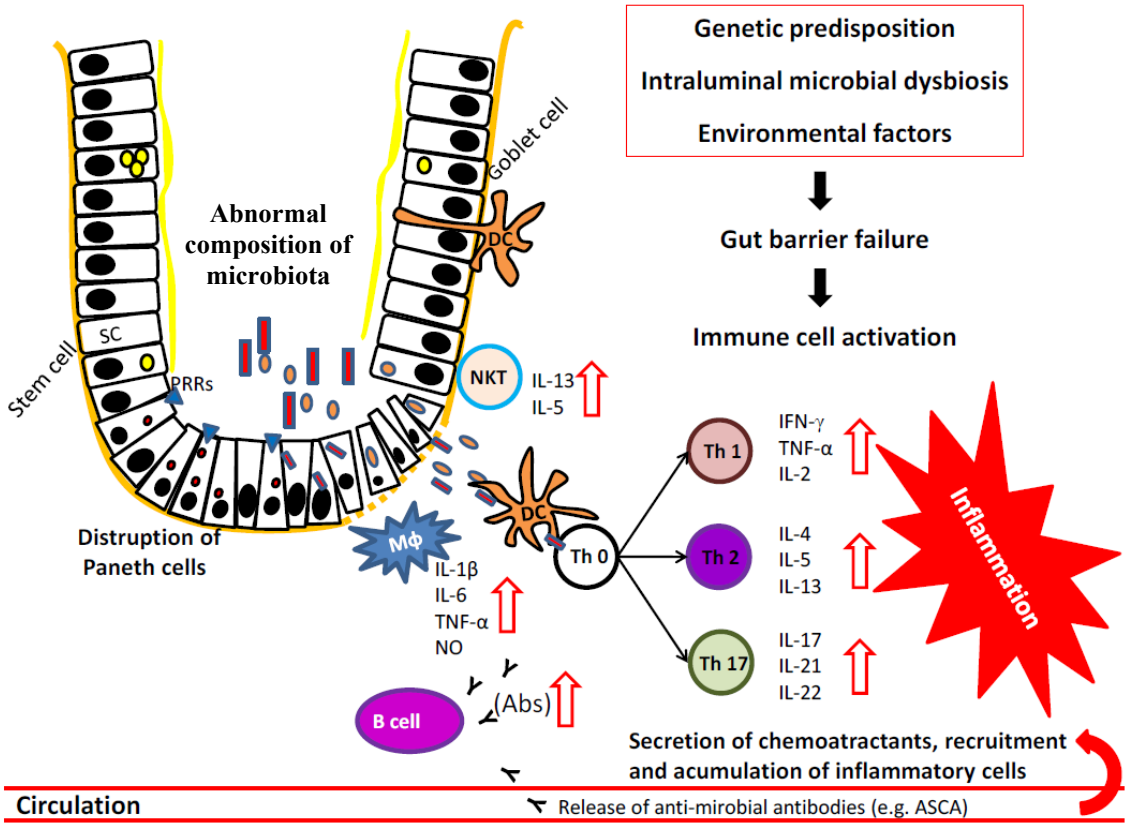


Figure 4. Visualization of immunopathogenic processes in inflammatory bowel disease.

The schematic displays key mediators of inflammatory response in inflammatory bowel disease. Adapted from Coufal (2020). **Abbreviations:** Abs – antibodies, ASCA – anti-*Saccharomyces cerevisiae* antibody, DC – dendritic cell, IL-1β – interleukin 1 beta (and analogically other cytokines), MΦ – macrophage, and PRR – pattern recognition receptor.

2.4.2 Mycobiota in IBD

The advances in sequencing in the past years have made microbiota research in health and disease accessible. Therefore, gut dysbiosis in IBD has been widely studied (focusing mainly on bacteria), with the most consistent observation being the reduction of bacterial diversity. A decrease in bacteria phylum Firmicutes and an increase in phylum

Proteobacteria are observed in patients suffering from IBD. No single pathogenic bacteria species have been detected as a driver of IBD. With the knowledge of identified susceptibility genes, it is not surprising that many clinical therapies correcting dysbiosis (such as probiotics or fecal microbiota transplantation) have been considered beneficial in IBD (Matsuoka & Kanai, 2015). Nonetheless, bacterial composition also indicates the shifts in eukaryotic intestinal populations (Hoffmann et al., 2013b). Commensal bacterial communities can protect the host organisms from fungal overgrowth (Samonis et al., 1993).

Regarding the composition of mycobiota, several papers observed changes in fungal diversity. The investigation of colonic biopsies from IBD patients by Ott et al. (2009) revealed that fungal species are part of the normal gut mycobiota. However, diversity and composition are higher. The increased diversity was detected in stool samples and inflamed surgical specimens of CD patients by Li et al. (2014). The alterations (higher diversity) were observed among *Candida* species, *Aspergillus clavatus*, *Cryptococcus neoformans*, *Alternaria brassicicola*, and *Gibberella moniliformis*. Skewed mycobiota composition was also shown in Sokol et al. (2017), where the ratio of Basidiomycota to Ascomycota was increased. The same study demonstrated no increase in overall fungal diversity in CD and a decrease in UC patients' stool. Another study examined mucosal samples of UC patients and showed an increase in some genera (such as *Candida* and *Aspergillus*) and a decrease in *Alternaria*, *Penicillium* and other genera (Qiu et al., 2017). On the other hand, results from pediatric IBD populations reported different changes in fungal composition – lower diversity was observed in CD patients' fecal samples (Chehoud et al., 2015). The enriched abundance of five fungal species, *S. cerevisiae*, *C. albicans*, *C. ulitis*, *C. lusitaniae*, and *Kluyveromyces marxianus*, was detected in pediatric CD stool samples in another study (Lewis et al., 2015).

Next, *Candida* species are believed to induce strong pro-inflammatory immune responses during IBD. A study on fecal microbiota transplantation in UC thus suggests that this therapy might act at least partially by reducing *Candida* species abundance in the gut and consequently lead to a reduction of disease severity (Leonardi et al., 2020).

Moreover, fungal recognition receptors appear to play a role in IBD. Polymorphism in the gene encoding Dectin-1 strongly relates to the severe form of ulcerative colitis in humans. The experimental colitis in mice is more severe when depleting the receptor (Iliev et al., 2012). Rare variants of CARD9, a downstream component of Dectin-1 signaling, were identified in IBD (Beaudoin et al., 2013).

To sum up, emerging evidence corroborates the theory that fungal dysbiosis occurs in parallel with the changes in bacterial composition in the gut during IBD development. Compositional shifts could be important for understanding the disease mechanism.

2.4.3 Biomarkers in IBD

Numerous biomarkers have been investigated as diagnostic and prediction tools for IBD. Genetic biomarkers could serve as a marker of IBD susceptibility in family members of IBD patients. Biomarkers in serum, stool or urine should mirror the early disease pathogenesis or reflect the effects of treatment. An ideal biomarker should be easily detectable and cheap (Viennois et al., 2015).

This thesis focuses on biomarkers associated with gut barrier failure, microbial (fungal) translocation, and mycobiota. The impairment of gut barrier results in enhanced immune responses to gut microbiota. Hence, a number of serological tests detecting microbial components or immune responses to them were historically examined.

Anti-*Saccharomyces cerevisiae*-antibody (ASCA) is a traditional serum biomarker detected in approximately half of IBD patients (Gao et al., 2001). The generation of ASCA positively correlates with the production of antibodies against *C. albicans*, which was revealed to express ASCA epitopes similar to those of *S. cerevisiae* (Standaert-Vitse et al., 2006a). The abundance of *C. tropicalis* was recently observed to associate with ASCA serum levels in CD patients (Hoarau et al., 2016). There is also a piece of evidence that ASCA levels correlate with disease severity and risk of surgery in adult (Forcione et al., 2004) and pediatric patients (Tang et al., 2017a). A large study analyzing banked sera found that ASCA was present in about a third of CD patients before clinical diagnosis (Israeli et al., 2005).

Concerning the gut barrier damage, typically used biomarkers are fatty acid-binding proteins (FABP), intestinal and liver ones, or matrix metalloproteinases (MMP).

Due to the complex pathogenesis of IBD, there is a demand for developing ‘biomarker signature’ assessing a complex panel of biomarkers.

3 Aims of the thesis

The literature research provided insight into the topic of mycobiota research in disease pathogenesis, especially the pathogenesis of IBD. A connection between mycobiota and IBD is visible. However, further research needs to be performed to understand the underlying immune response. With the knowledge of gut barrier impairment, microbial translocation, increased antimicrobial antibody levels, and abnormal activation of intestinal macrophages in IBD, this diploma thesis set several research goals:

1. to analyze the levels of biomarkers associated with gut barrier damage and microbial translocation in sera of IBD patients and healthy individuals
2. to determine the levels of antifungal antibodies against common fungal mycobiota components in sera of IBD patients and healthy individuals
3. to investigate the effect of fungal lysates on the reactivity of peripheral blood-derived macrophages

4 Materials and methods

4.1 Biological materials

4.1.1 Serum (blood) and stool samples

The study enrolled 52 healthy adult volunteers, referred to as healthy controls (HC), and 198 adult patients diagnosed with IBD (CD or UC), PSC or IBD associated with PSC (PSC-IBD). Blood sampling and clinical data collection were performed by outpatient clinic physicians at the Hepatogastroenterology Department of the Institute for Clinical and Experimental Medicine (Prague, Czech Republic) during regular appointments. Serum samples were then stored at −80 °C. The characteristics of the studied group are depicted in **Table 1**. Some individuals also provided stool samples, which were processed in the laboratory within a few hours.

Peripheral blood used for isolation of peripheral blood mononuclear cells (PBMCs) followed by a derivation of macrophages was donated by 4 healthy voluntary blood donors. The health professionals at the Department of Blood Transfusion Medicine of the Thomayer University Hospital collected the blood samples and extracted the buffy coats that were then processed in the laboratory.

4.1.2 Ethics statement

All subjects provided written informed consent before any study-related procedures were performed. The study was conducted in line with the principles stated in the Declaration of Helsinki (World Medical Association, 2013) and the project was approved by the Ethics Committee of the Institute for Clinical and Experimental Medicine and the Thomayer University Hospital.

Table 1. Characteristics of the studied healthy controls and inflammatory bowel disease patients

	HC	CD	UC	PSC-IBD	PSC
Count [number]	52	112	49	32	5
Sex [% of males]	48.1	46.4	63.2	75.0	100.0
Age [mean ± SD; years]	36.5 ± 10.8	41.1 ± 10.8	41.7 ± 13.8	36.4 ± 11.1	34.0 ± 3.7
Activity [% of active]	0.0	22.3	28.6	15.6	N/A
BMI [mean ± SD; kg/m^2]	24.5 ± 4.3	25.9 ± 5.1	26.0 ± 4.5	23.4 ± 4.5	23.1 ± 1.7
Therapy					
5-ASA [%]	0.0	75.0	89.8	81.2	40.0
Glucocorticoids [%]	0.0	30.4	24.5	50.0	40.0
AZA [%]	0.0	40.1	34.7	25.0	0.0
Vedolizumab [%]	0.0	3.6	6.1	3.1	0.0
Ustekinumab [%]	0.0	1.8	0.0	N/A	N/A
Anti-TNF-α [%]	0.0	42.0	40.8	0.0	0.0
***E. coli* Nissle 1917 [%]**	0.0	17.0	26.5	15.6	0.0

Differences between the control group (HC) and each diagnosis were statistically tested for age and BMI using ANOVA or Kruskal-Wallis tests with multiple comparisons. Results indicated non-significant differences.
Abbreviations: 5-ASA – 5-aminosalicylic acid (mesalamine), AZA – azathioprine, BMI – body mass index, CD – Crohn's disease, *E.* – *Escherichia*, HC – healthy controls, N/A – not applicable, PSC – primary sclerosing cholangitis, PSC-IBD – inflammatory bowel disease associated with primary sclerosing cholangitis, SD – standard deviation, TNF-α – tumor necrosis factor alpha, and UC – ulcerative colitis.

4.2 Laboratory equipment and supplies

4.2.1 Reagents and chemicals

3,3',5,5'-Tetramethylbenzidine (TMB), Merck KGaA, Germany

Agar, Dr. Kulich Pharma, Czech Republic
Bovine serum albumin (BSA), Merck KGaA, Germany
Chloramphenicol, Merck KGaA, Germany
CHROMagar™ Candida, Kanto Chemical, Japan
Dulbecco's Modified Eagle Medium (DMEM), Merck KGaA, Germany
Ethylenediaminetetraacetic acid (EDTA), Merck KGaA, Germany
Ficoll-Paque™ PLUS, GE Healthcare, Sweden

Gibco fetal bovine serum (FBS), Thermo Fisher Scientific, USA

Human macrophage colony-stimulating factor (M-CSF), Merck KGaA, Germany

Hydrogen peroxide, Lach-Ner, Czech Republic

LPS from *Escherichia coli* O111:B4, Merck KGaA, Germany

Malt extract, Sladovna, Czech Republic

Mannan from *Saccharomyces cerevisiae*, Merck KGaA, Germany

PCR water, Thermo Fisher Scientific, USA

Penicillin-Streptomycin, Merck KGaA, Germany

Peroxidase AffinniPure F(ab')$_2$ Fragment Goat Anti-Human IgA/IgG/IgM, Fcγ fragment specific, Jackson ImmunoResearch Laboratories, USA

Primers, Generi Biotech, Czech Republic

Sterile physiological saline solution, Ardeapharma, Czech Republic

Sterile water, Ardeapharma, Czech Republic

Sulfuric acid, Merck KGaA, Germany

UltraComp eBeads™ Plus Compensation Beads, Thermo Fisher Scientific, USA

Zymosan A from *Saccharomyces cerevisiae*, Merck KGaA, Germany

4.2.2 Solutions and buffers

Cell culture medium:

DMEM containing 10% FBS and 1% antibiotics (Penicillin-Streptomycin).

ELISA blocking buffer:

PBS containing 1% BSA.

ELISA wash buffer:

PBS containing 0,05 % Tween® 20.

Liquid malt extract medium:

Distilled water containing 4% malt extract and 0,01% chloramphenicol. Sterilized by autoclaving (antibiotic added after sterilization).

MACS buffer:

Sterile PBS containing 0.5% BSA and 2 mM EDTA. Filtered through Millex Syringe Filter, 0.22 μm.

Phosphate-buffered saline 1X (PBS), pH 7.2:

Distilled water containing 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4.

PBS-EDTA:

Distilled water containing 1 mM EDTA.

Solid malt extract agar medium:

Distilled water containing 4% malt extract, 15% agar, and 0,01% chloramphenicol. Sterilized by autoclaving (antibiotic added after sterilization).

4.2.3 Kits and sets

CD14 MicroBeads UltraPure, human, Miltenyi Biotec, USA

DNA Clean & Concentrator® kit, Zymo Research, USA

DNeasy® UltraClean® Microbial Kit, Qiagen, Germany

ELISA kits – **Table 2**

KAPA2G Robust HotStart® PCR Kit, Merck KGaA, Germany

Qubit™ dsDNA Broad Range and High Sensitivity Assay Kits, Thermo Fisher Scientific, USA

Table 2. Kits used for ELISA

Marker	Kit	Manufacturer
CD14	**Human CD14 DuoSet ELISA**	R&D Systems
IL-1ß	**Human IL-1 beta/IL-1F2 DuoSet ELISA**	R&D Systems
IL-6	**Human IL-6 DuoSet ELISA**	R&D Systems
IL-10	**Human IL-10 DuoSet ELISA**	R&D Systems
IL-18	**Human Total IL-18 DuoSet ELISA**	R&D Systems
I-FABP	**I-FABP, Human, ELISA kit**	Hycult Biotech
LBP	**Human LBP DuoSet ELISA**	R&D Systems
MBL	**Human MBL DuoSet ELISA**	R&D Systems
MMP-9	**Human MMP-9 DuoSet ELISA**	R&D Systems
TNF-α	**Human TNF-alpha DuoSet ELISA**	R&D Systems

Abbreviations: IL-1ß – interleukin 1 beta, IL-6 – interleukin 6, IL-10 – interleukin 10, IL-18 – interleukin 18, I-FABP – intestinal fatty acid-binding protein, LBP – lipopolysaccharide-binding protein, MBL – mannose-binding lectin, MMP-9 – matrix metalloproteinase-9, and TNF-α – tumor necrosis factor alpha.

4.2.4 Antibodies

Antibodies used for flow cytometry analysis are listed in **Table 3**.

Table 3. Antibodies used for flow cytometry

Antibody	Conjugate	Clone	Cat. number	Manufacturer
CD11b	Alexa Fluor 700	ICRF44	301356	BioLegend
CD16/CD32	-	93	14-0161-86	Thermo Fisher Scientific
CD13	BV 605	WM15	301728	BioLegend
CD14	PE	M5E2	301850	BioLegend
CD38	BV 711	HIT2	303528	BioLegend
CD40	PE	HB14	313006	BioLegend
CD80	Pe-Cyanine 7	2D10.4	2106778	Thermo Fisher Scientific
CD209	BV 421	9E9A8	330118	BioLegend
CD369	PerCP-eFluor 710	15E2	2011136	Thermo Fisher Scientific
Fixable Viability Dye	eFluor 780	-	65-0865-14	Thermo Fisher Scientific

4.2.5 Plastic

autoMACS® Columns, Miltenyi Biotec, USA

Lysing Matrix Y 2ml tubes, MP Biomedicals, USA

Millex Syringe Filter, 0.22 µm, Merck KGaA, Germany

Nunc™ MicroWell™ 96-well, flat-bottom microplate, Thermo Fisher Scientific, USA

Nunc™ MicroWell™ 96-well, Nunclon Delta-treated, flat-bottom microplate, Thermo Fisher Scientific, USA

Pre-Separation Filters, 30 µm, Miltenyi Biotec, USA

TTP® tissue culture test plates 96-well, TPP, Switzerland

4.2.5 Instruments

Centrifuges: **Microcentrifuge Minispin™**, Eppendorf, Germany
Universal 30RF, Hettich, Germany
Universal 320R, Hettich, Germany

Cycler: **Biometra TAdvanced thermal cycler**, Analytik Jena, Germany

Flow cytometer: **LSR II Flow Cytometer with a high-throughput sampler**, BD, USA

Fluorometer: **Qubit**, Thermo Fisher Scientific, USA

Homogenizer:	**FastPrep®-24 system**, MP Biomedicals, USA
Incubator:	**MCO-15AC**, Sanyo, Japan
Lyophilizer:	**Lyovac GT 2 lyophilizer**, Leybold Heraeus, Germany
Microplate reader:	**Thermo Multiskan Ascent 96/38**, Thermo Fisher Scientific, USA
Separator:	**autoMACS® Pro Separator**, Miltenyi Biotec, USA
Vortex:	**Vortex-Genie 2**, Scientific Industries, Czech Republic
Washer:	**HydroFlex™ Plus microplate washer**, Tecan, Switzerland

4.2.6 Software

Microsoft Excel, Microsoft, USA

GraphPad Prism version 9.3.1, Dotmatics, USA

Ascent version 2.4.1, Thermo Fisher Scientific, USA

BD FACS DIVA version 6.1.2, BD, USA

FlowJo version 7.2.5/9.9.4, BD, USA

4.3 Methods

All reagents, solutions, and buffers used in the protocols are defined in the section *4.2 Laboratory equipment and supplies*.

4.3.1 Quantitative enzyme-linked immunosorbent assay (ELISA) for serum biomarkers and cytokines in supernatants from macrophages stimulation

The serum concentrations of selected markers were examined by quantitative sandwich ELISA with a streptavidin-biotin detection system using ELISA kits listed in **Table 2**. All of the dilutions were used according to the manufacturer's recommendations. The biomarkers analyzed in the IBD patients' sera and control sera were CD14, I-FABP, IL-18, LBP, MBL, and MMP-9. Supernatants from macrophages stimulated for 24 h with fungal lysates and control stimuli were measured for concentrations of selected cytokines (IL-1ß, IL-6, IL-10, and TNF-α). Capture antibodies from the kits were diluted in PBS and coated on the 96-well flat-bottom plates overnight. After the overnight incubation, the plates were washed with the ELISA wash buffer using a HydroFlex™ Plus microplate washer and blocked with the ELISA blocking buffer for 1 h. The dilution of the samples (supernatants) was pre-tested, and the optimal dilution was applied to the wells in duplicates together with the standards in duplicates (in 2-fold serial dilutions), all in volume 50 µl/well, and incubated for 2 h. After the washing, the corresponding detection antibodies from the kit diluted in 1%

BSA were applied to each well (50 µl/well) and incubated for 2 h. After the washing step, the working solution of Streptavidin-HRP (horseradish peroxidase) from the kits was added (50 µl/well) and the plates were incubated for 20 min in the dark. TMB substrate solution was added after the washing and incubated for 5–30 min (depending on the particular kit) in the dark. The reaction was stopped afterwards with sulfuric acid. Optical density (OD) values were measured at 450 nm using Thermo Multiskan Ascent 96/384 microplate reader. The concentration of analyzed marker was determined by creating the standard curve using computer software. A solution of 1% BSA was used as a blank and subtracted. All procedures were performed at RT.

4.3.2 Fungal lysates preparation

Stool samples from IBD patients and HC were collected, and a piece of each sample (about 1g) was homogenized and diluted in a sterile physiological saline solution three times. Petri dishes (90mm diameter) with solid malt extract agar and chloramphenicol were inoculated with the prepared stool sample solutions (100µl per plate; 15 plates per sample) and incubated in aerobic conditions at two different temperatures (RT and 37 °C). After a few days, various single colonies of yeasts and molds were visible. New Petri dishes were inoculated with isolated colonies and incubated again to obtain purified cultures. CHROMagar^{TM} Candida, a selective chromogenic culture medium, was used for the preliminary direct identification of major clinical-significant *Candida* species on a single isolation plate. Yeast differentiation was interpreted after 48 hours of aerobic incubation at 37 °C (as stated in the manufacturer's documents). Purified cultures were transferred to the liquid malt extract medium (transferred on a sterile pipette tip into 50ml of the medium in a large Erlenmeyer flask) and incubated aerobically on the shaker (temperature 37 °C, speed of the shaker 150 RPM) until the OD at 600 nm reached around 1.0.

After the incubation, a small volume (1ml) of the fresh culture was used for DNA isolation, followed by sequencing (see *4.3.3 Identification of fungal species*). The fungal cells were harvested from the rest by centrifugation (400 × g, 20 min, RT). Washing of the pellets was performed by repeated re-suspension in sterile water (centrifugation; 400 × g, 3 × 10 min, RT) and inactivated by lysis in Lysing Matrix Y 2ml tubes using the FastPrep®-24 system. The homogenization procedure was performed three times (3 × 60 s, 6.5 m/s), and subsequently, the suspensions were collected. Lastly, the samples were freeze-dried in the Lyovac GT 2 lyophilizer and stored in aliquots at −20 °C.

4.3.3 Identification of fungal species

DNA of fungal species was isolated from the pure fungal cultures (the process of growing is described in *Fungal lysates preparation*) using DNeasy® UltraClean® Microbial Kit according to the manufacturer's instructions and stored at −20 °C until further analyses. The concentration of yielded DNA was measured on the Qubit Fluorometer applying Qubit™ dsDNA Broad Range and Qubit™ dsDNA High Sensitivity Assay Kits. All DNA samples were adjusted to a concentration of approximately 10 ng/µl. Next, the yielded DNA served as a template for amplification of the ITS regions of fungal rDNA that served as representative sequences for taxonomic identification. Both ITS1 and ITS2 were amplified by PCR utilizing ITS1-F forward primer (Gardes & Bruns, 1993) and ITS4 reverse primer (White et al., 1990) –position of the primers and their sequences are shown in **Figure 5** and **Table 4**, respectively. The PCR reaction was performed with the KAPA2G Robust HotStart® PCR Kit in a volume of 25 µl (exact measurements are listed in **Table 5**) using the Biometra TAdvanced thermal cycler. The cycling parameters were set up as follows: the initial denaturation at 94 °C for 4 min, cycling of denaturation (94 °C, 1 min), annealing (53 °C, 1 min), and extension (72 °C, 1 min) steps (35 times), and the final elongation at 72 °C for 10 min. PCR products were purified according to the manufacturer's protocol for DNA Clean & Concentrator® kit and their concentration was measured on the Qubit Fluorometer, again applying the Qubit™ dsDNA Broad Range and Qubit™ dsDNA High Sensitivity Assay Kits. The success of the PCR reaction was confirmed by agarose gel electrophoresis, which also allowed the estimation of the amplified DNA fragments' length. Samples were then sequenced and compared with reference databases. For further experiments, fungal species relevant for human gut microbiota studies were selected according to the literature.

Figure 5. Structure of the nuclear ribosomal RNA genes and their internal transcribed spacers

Positions of used forward (left) and reverse (right) primers are depicted on the map of the ribosomal RNA genes with internal transcribed spacers. Adapted from Toju et al. (2012). **Abbreviations:** ITS1 – internal transcribed spacer 1, ITS2 – internal transcribed spacer 2, LSU – large subunit, and SSU – small subunit.

Table 4. Sequences of used ITS primers

Primers	
Name	**Sequence (5' to 3')**
ITS1-F	CTTGGTCATTTAGAGGAAGTAA
ITS4	TCCTCCGCTTATTGATATGC

Table 5. Individual components of one PCR reaction

PCR		
Component	**Concentration of stock solution**	**Volume [µl]**
Buffer – KAPA2G Buffer B[a]	5X	5
Enhancer – KAPA ENHANCER 1[a]	5X	5
Mix of dNTPs – KAPA dNTP Mix[a]	10 mM each	0.5
Polymerase – KAPA2G Robust HotStart DNA Polymerase[a]	5 U/µl	0.1
PCR water	N/A	10.4
Forward primer (ITS1-F)	10 µM	1
Reverse primer (ITS4)	10 µM	1
Template DNA	10 ng/µl	2

Letter 'a' indicates a component obtained from the KAPA2G Robust HotStart® PCR Kit.

4.3.4 Indirect semiquantitative enzyme-linked immunosorbent assay (ELISA)

The serum concentrations of anti-fungal antibodies (IgA, IgG, and IgM isotypes) were examined by the in-house indirect semiquantitative ELISA protocol (Kverka et al., 2011). Fungal lysates (*Saccharomyces cerevisiae*, *Candida albicans*, *C. tropicalis*, *C. parapsilosis*, *C. colliculosa*, *C. dubliniensis*, *C. kefyr* and *Galactomyces geotrichum*) were dissolved in PBS and coated on the 96-well flat-bottom plates overnight. The concentration of the coated fungal lysates was pre-tested with the sera of HC and IBD patients before the experiment. The appropriate final concentration was determined as 10 µg/ml (50 µl/well). After the overnight incubation, the plates were washed with the ELISA wash buffer using a Tecan HydroFlex™ Plus microplate washer and blocked with the ELISA blocking buffer for 1 h. The dilution of the human sera in the blocking buffer was also pre-tested, and the optimal dilution was applied in duplicates to the wells (50 µl/well) and incubated for 2 h. After the washing, the corresponding HRP-labelled secondary antibody was added (50 µl/well) and plates were incubated for 1 h in the dark. The dilutions of secondary antibodies were tested and determined as follows: 1:20000 in 1% BSA for IgA and IgM and 1:40000 for IgG. After the washing step, TMB substrate solution was applied (50 µl/well) and plates were incubated for 10 min in the dark. The reaction was stopped afterwards with sulfuric acid (50 µl/well). The OD values were measured at 450 nm using Thermo Multiskan Ascent 96/384 microplate reader. The relative antibody concentration was presented as OD in arbitrary units (AU) and was determined by the serial dilution of a reference serum sample (marked as standard serum) applied on each ELISA plate. A solution of 1% BSA was used as a blank and subtracted. All procedures were performed at RT.

4.3.5 Derivation of macrophages and in vitro co-cultivation with stimuli

Buffy coat of healthy donors (around 40 ml) was diluted 1:1 with PBS-EDTA prewarmed to RT. Prewarmed Ficoll-Paque™ PLUS (15 ml) polysaccharide media solution was placed into a 50 ml centrifugation tube and 25 ml of diluted blood was carefully layered onto the polysaccharide layer in each tube. Tubes were centrifugated (400 × g, 30 min, RT) with slow acceleration and the brake was turned off. The ring of mononuclear cells formed between the plasma layer and the Ficoll-Paque™ PLUS media layer was precisely transferred using a serological pipette into a clean 50 ml centrifugation tube and filled up to the final volume of 40 ml with PBS-EDTA. Mixed solutions were centrifuged (400 × g, 10 min, RT), from this step with higher acceleration and the brake turned on, then resuspended in 20 ml of PBS-EDTA and centrifuged one more time (300 × g, 10 min, RT). After the last

centrifugation, cell pellets were resuspended again in 5 ml of PBS-EDTA and preparation for magnetic separation (MACS) of $CD14^+$ cells followed according to the slightly modified manufacturer's instructions for CD14 MicroBeads UltraPure, human. Single-cell suspensions (for optimal performance of the magnetic labelling) were obtained by passing cells through 30 µm nylon mesh, the filter was moistened with MACS buffer before use. Single-cell suspensions were centrifuged (300×g, 10 min, RT) and pellets were resuspended 1:1 in PBS-EDTA. To this small volume, 40 µl of CD14 Microbeads UltraPure was added, the cell suspensions were mixed and incubated for 30 min in a refrigerator (2–8 ℃). Labelled cell suspensions were washed by adding 7 ml of MACS buffer and centrifuged (300×g, 10 min, RT). Supernatants were aspirated and cell pellets were resuspended with 1 ml of MACS buffer and proceeded to the separation step. $CD14^+$ cells were positively separated with the autoMACS® Pro Separator. Samples were placed in the chilled rack and instructions on the separator were followed.

Separated cells were counted and seeded on the Nunc™ MicroWell™ 96-well flat-bottom microplate (150,000 cells/well; 100 µl/well) and cultured in the cell culture medium at 37 °C in the incubator. To differentiate the $CD14^+$ cells into macrophages human M-CSF was added once in 24 h in the final concentration of 20 ng/ml. Cells without M-CSF and negatively separated cells were also cultured. After 5 days, cells were stimulated *in vitro* with prepared fungal lysates and control stimuli (zymosan, mannan, and LPS) in the final concentration of 500 ng/ml and co-cultivated for 24 h. Various concentrations and different cultivation periods were tested and optimized. The separation efficiency and stimulation's effect on differentiated macrophages were measured with flow cytometry and ELISA from cell supernatants.

4.3.6 Flow cytometry

After 24 h cultivation, stimulated macrophages were transferred to 96-well plates (duplicates from the same donor were pooled into one well) and supernatants were transferred into tubes and frozen at −20 °C until ELISA analysis. Macrophages were washed with PBS-EDTA and centrifuged (310×g, 3 min, 10 °C). Fc receptors were blocked by adding a human CD16/CD32 monoclonal antibody into each well (diluted 1:200 in PBS; 20 µl/well) and incubating for 30 min at 4 °C (the plate was gently tapped to loosen the cell pellets after adding the antibody). Antibody mixtures (mix with all antibodies, mix for 'Fluorescence Minus One' controls, and mix for single stained controls) were prepared in

the meantime. The optimal dilution of the antibodies in PBS-EDTA was tested by antibodies titration. The list of chosen antibodies is displayed in **Table 3**. UltraComp eBeads™ Plus were added to the wells selected for the single stained bead controls (10 µl/well). After incubation, wells were washed with PBS-EDTA and centrifuged (310 × g, 3 min, 10 °C). Each well was stained with the appropriate antibody mix (10 µl/well), gently tapped, and incubated for 30 min at 4 °C in the dark. One well remained completely unstained and served for unstained control. After incubation, wells were washed with PBS-EDTA twice and each time centrifuged (310 × g, 3 min, 10 °C). Subsequently, wells were resuspended in PBS-EDTA (100 µl/well) and samples were acquired on a BD LSR II flow cytometer with a high-throughput sampler employing BD FACS DIVA software. The measurement was performed with the assistance of Dr. Jan Svoboda and Žaneta Slavíčková.

4.3.7 Statistical analysis

Flow cytometry results were analyzed using FlowJo software – the analyses included appropriate compensation controls (unstained control, single stained cells and single stained compensation beads) and FMO controls helpful in setting the upper boundary for background signals. The gating strategy is displayed in **Supplementary figure 1**.

The analysis of obtained data was performed utilizing Microsoft Excel and GraphPad Prism software. The normal distribution of data was tested by the Shapiro-Wilk test and depending on the results appropriate parametric or non-parametric tests were used for further analyses. Particular tests and other additional information are mentioned in the legend of each graph. Results were considered statistically significant according to **Table 6**.

Table 6. P-values, their signs and interpretations

Sign	P-value	Interpretation
ns	≥ 0.05	Nonsignificant
*	< 0.05	Significant
**	< 0.01	Very significant
***	< 0.001	Extremely significant

5 Results

5.1 Analysis of the levels of serum biomarkers associated with gut barrier and microbial translocation

In order to determine the degree of the organism's integrity disruption in IBD patients, the levels of selected biomarkers in the sera were analyzed using a quantitative ELISA approach. Various biomarkers associated with gut barrier damage (I-FABP, MMP-9) and microbial translocation (CD14, MBL, LBP, and IL-18) were measured in the sera of patients diagnosed with CD, UC and PSC-IBD and in the sera of healthy volunteers, labelled as HC in the graphs. The characteristics of the studied population are described in **Table 1** in section *4.1.1 Serum (blood) and stool samples*. The analysis revealed a pattern of increased levels of the serum biomarkers in the samples of IBD patients in comparison to healthy individuals (**Figure 6**). In most of the examined biomarkers (MMP-9, CD14, MBL, and LBP), the difference was statistically significant. Next, a detailed examination of serum biomarker levels in different types of IBD indicated differences between the groups (**Figure 7**). The biomarkers associated with inflammation and microbial translocation, CD14 and MBL, were higher in PSC-IBD patients than in CD and UC patients. Significant differences in biomarkers connected to the epithelial damage and remodeling were also demonstrated while focusing on the PSC-IBD diagnosis group – this group possessed higher serum levels of I-FABP and lower serum levels of MMP-9 as compared to CD and UC groups. Although an overall trend toward higher biomarkers in CD patients compared to UC patients was seen, this observation did not reach statistical significance.

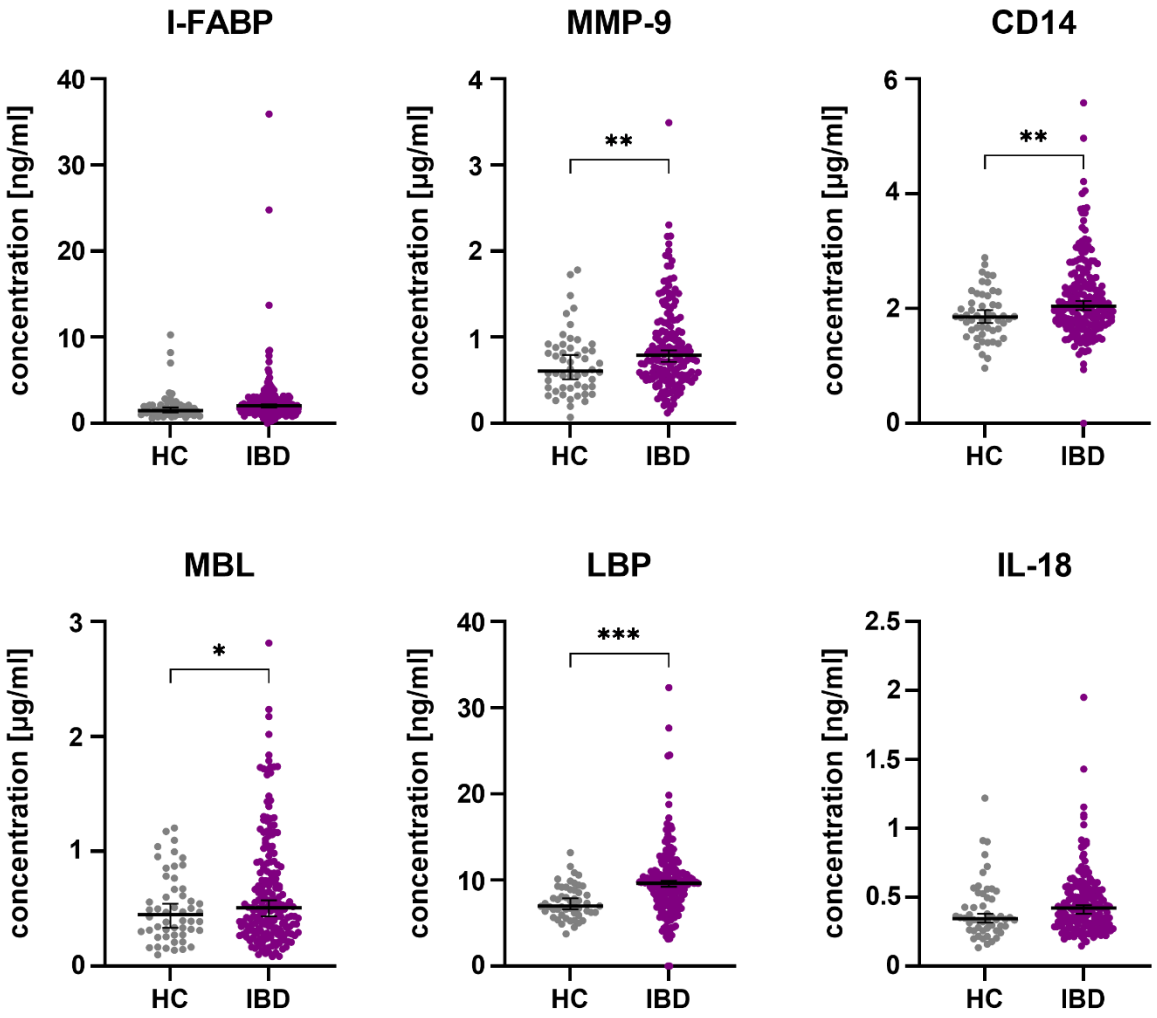


Figure 6. Quantitative comparison of serum biomarker levels in healthy individuals and IBD patients

Quantitative plots of the selected serum biomarkers measured by ELISA in healthy individuals ($n_{HC} = 52$) and patients with IBD ($n_{IBD} = 195$). Individual values and median together with its 95% confidence interval are displayed for each group. Differences were analyzed using the Unpaired t-test. Significance levels are represented as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). **Abbreviations:** ELISA – enzyme-linked immunosorbent assay, HC – healthy control, IBD – inflammatory bowel disease, I-FABP – intestinal-type fatty acid-binding protein, IL-18 – interleukin 18, LBP – lipopolysaccharide-binding protein, MBL – mannose-binding lectin, and MMP-9 – matrix metalloproteinase-9.

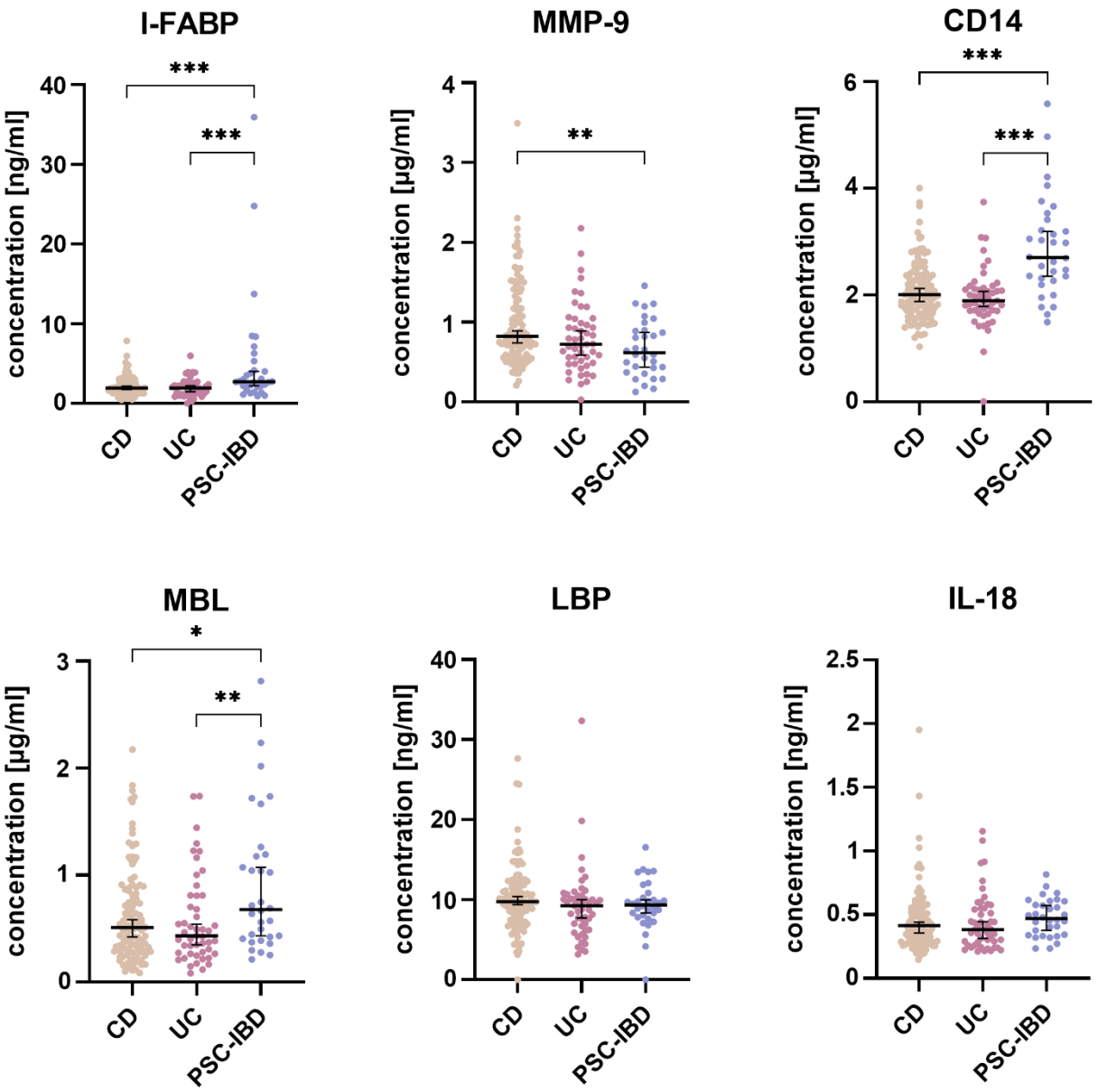


Figure 7. Dissimilarities in serum biomarker levels between different types of IBD

Quantitative plots of the selected serum biomarkers measured by ELISA in patients with CD, UC, and PSC-IBD. Individual values and median together with its 95% confidence interval are displayed for each group. Group differences were analyzed using One-Way ANOVA with Tukey's post hoc test. For all graphs, $n_{CD} = 114$ (CD patients), $n_{UC} = 49$ (UC patients), and $n_{PSC\text{-}IBD} = 32$ (PSC-IBD patients). Significance levels are represented as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). **Abbreviations:** ANOVA – analysis of variance, CD – Crohn's disease, ELISA – enzyme-linked immunosorbent assay, I-FABP – intestinal-type fatty acid-binding protein, IL-18 – interleukin 18, LBP – lipopolysaccharide-binding protein, MBL – mannose-binding lectin, MMP-9 – matrix metalloproteinase-9, PSC-IBD – inflammatory bowel disease associated with primary sclerosing cholangitis, and UC – ulcerative colitis.

5.2 Fungal cultivation

In an effort to obtain lysates of fungal strains commonly found in the human gut, stool samples of 3 IBD patients and 2 healthy individuals were cultured under conditions appropriate for fungal growth. Various colonies of yeasts and molds appeared on agar plates after a few days of incubation. Pure cultures of cultivated fungi were identified by sequencing (**Table 7**). Some of the species are considered to be foodborne, thus, lysates were prepared only from the relevant gut-associated fungal species.

Table 7. List of fungal species isolated from the stool samples of IBD patients and healthy individuals

Name of the fungal species (alternate name if exists)	Subject of origin
Aspergillus niger, welwitschiae	HC
Candida albicans	CD, HC
Candida colliculosa (Torulaspora delbrueckii)	HC
Candida dubliniensis	HC, PSC-IBD
Candida kefyr (Kluyveromyces marxianus)	UC
Candida parapsilosis	CD, HC
Candida sojae	UC
Candida tropicalis	HC, UC
Galactomyces geotrichum	UC
Penicillium citrinum	HC
Saccharomyces cerevisiae	CD, HC
Trichoderma citrinoviride	HC

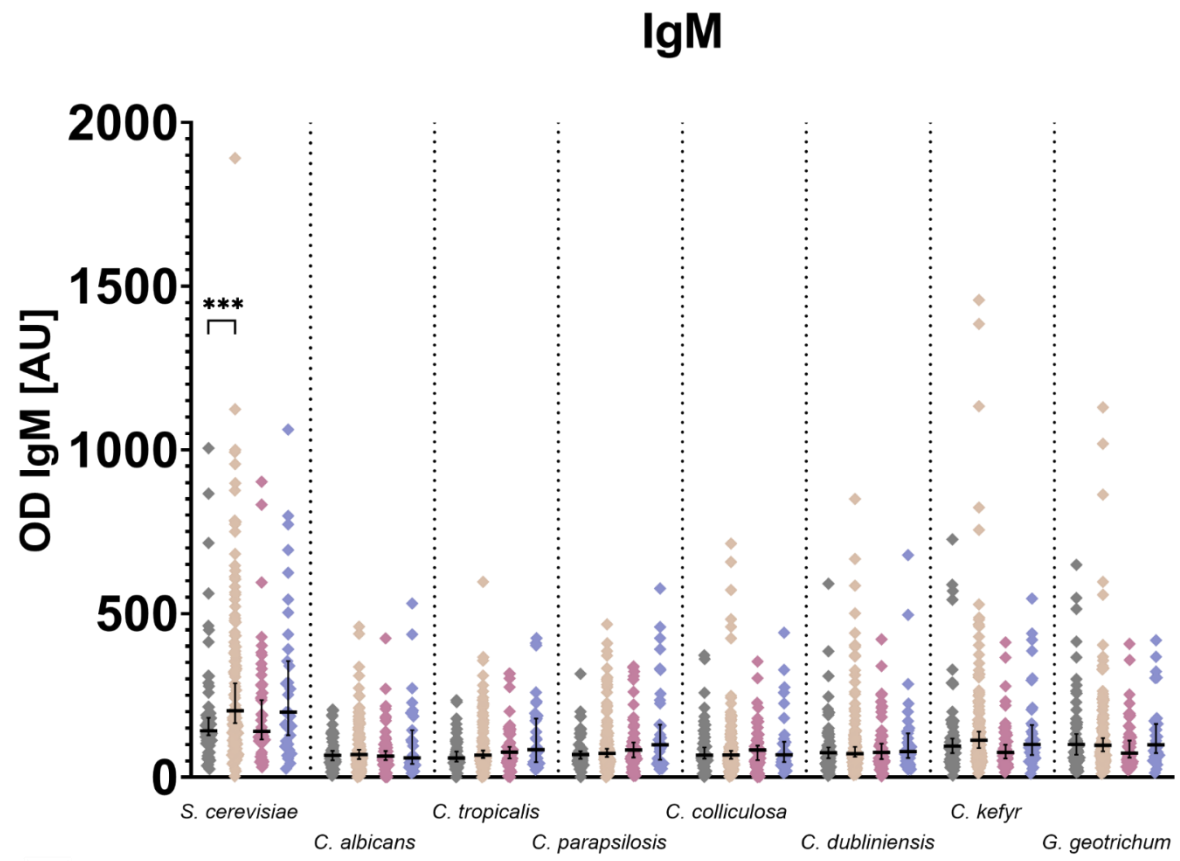
Abbreviations: CD – Crohn's disease, HC – healthy control, IBD – inflammatory bowel disease, PSC-IBD – inflammatory bowel disease associated with primary sclerosing cholangitis, and UC – ulcerative colitis.

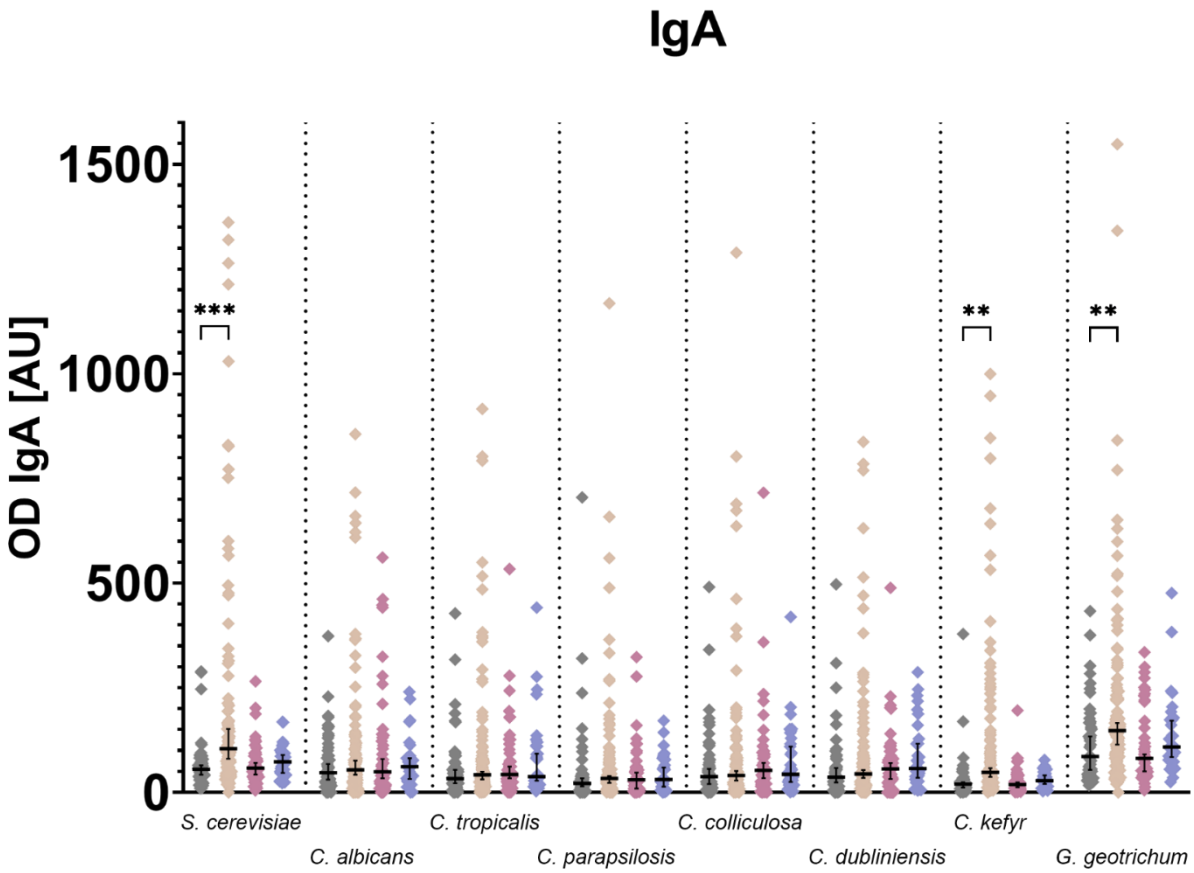
5.3 Determination of the serum antimicrobial antibodies levels

In addition to the measurement of serum biomarkers associated with gut barrier damage and microbial translocation, antibody response to gut fungi (*Saccharomyces cerevisiae*, *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. colliculosa*, *C. dubliniensis*, *C. kefyr*, and *Galactomyces geotrichum*) was determined using the semiquantitative ELISA method in the sera of healthy individuals and IBD patients. The prepared fungal lysates were used for antigen coating. The levels of anti-fungal antibodies against most of the examined

fungal species were higher in IBD patients' sera compared to healthy subjects' sera across all antibody isotypes (**Figure 8**). The most noticeable increase was observed in Crohn's disease, where the difference compared to healthy individuals was significant in some cases.

Further, to find the connection between antibody responses against particular fungal species across the three antibody isotypes (IgM, IgA, IgG), a correlation analysis was performed. All samples (sera of healthy individuals and all diagnoses) were analyzed together creating a correlation matrix (**Figure 9**). The antibody responses against fungi correlate well within one isotype, especially among the least specific isotype, IgM. Weaker correlations were detected across different isotypes.


IgM
OD IgM [AU]
2000
1500
1000
500
0
***
S. cerevisiae
C. albicans
C. tropicalis
C. parapsilosis
C. colliculosa
C. dubliniensis
C. kefyr
G. geotrichum


IgA
OD IgA [AU]
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**
S. cerevisiae
C. albicans
C. tropicalis
C. parapsilosis
C. colliculosa
C. dubliniensis
C. kefyr
G. geotrichum

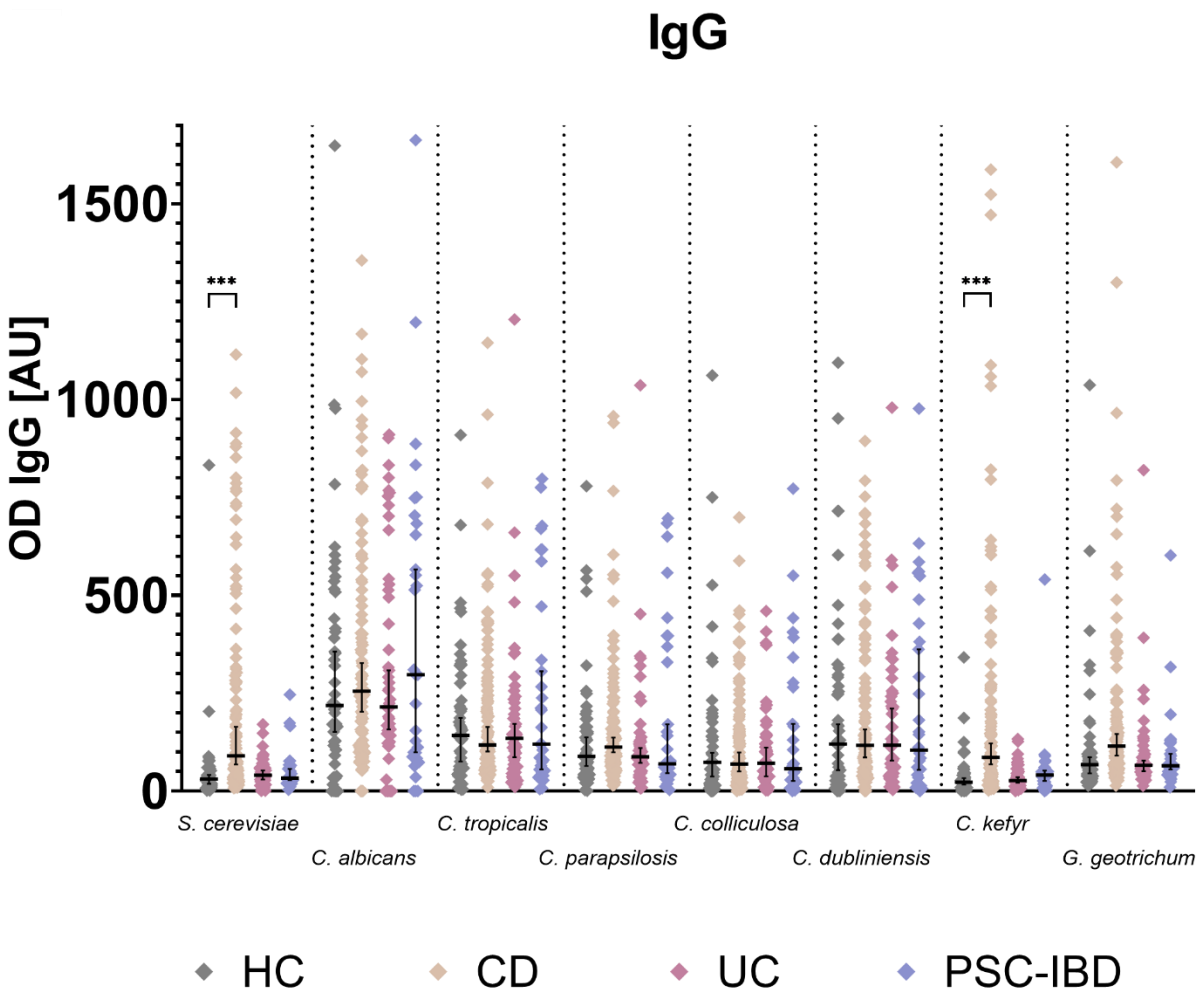

Figure 8. Dissimilarities in antibody response against various fungal species between healthy individuals and patients with different types of IBD

Comparison of specific anti-fungal antibody responses (IgM, IgA, IgG) among healthy individuals (HC) and patients with CD, UC, and PSC-IBD. Antibody concentrations were measured by semiquantitative ELISA and are displayed as OD in AU. Individual values and median together with its 95% confidence interval are displayed for each group. Group differences were analyzed using One-Way ANOVA with Šidák's post hoc test. For all graphs, $n_{HC} = 52$ (HC) $n_{CD} = 114$ (CD patients), $n_{UC} = 49$ (UC patients), and $n_{PSC\text{-}IBD} = 32$ (PSC-IBD patients). Significance levels are represented as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). **Abbreviations:** ANOVA – analysis of variance, AU – arbitrary units, CD – Crohn's disease, ELISA – enzyme-linked immunosorbent assay, HC – healthy control, IBD – inflammatory bowel disease, OD – optical density, PSC-IBD – inflammatory bowel disease associated with primary sclerosing cholangitis, and UC – ulcerative colitis.

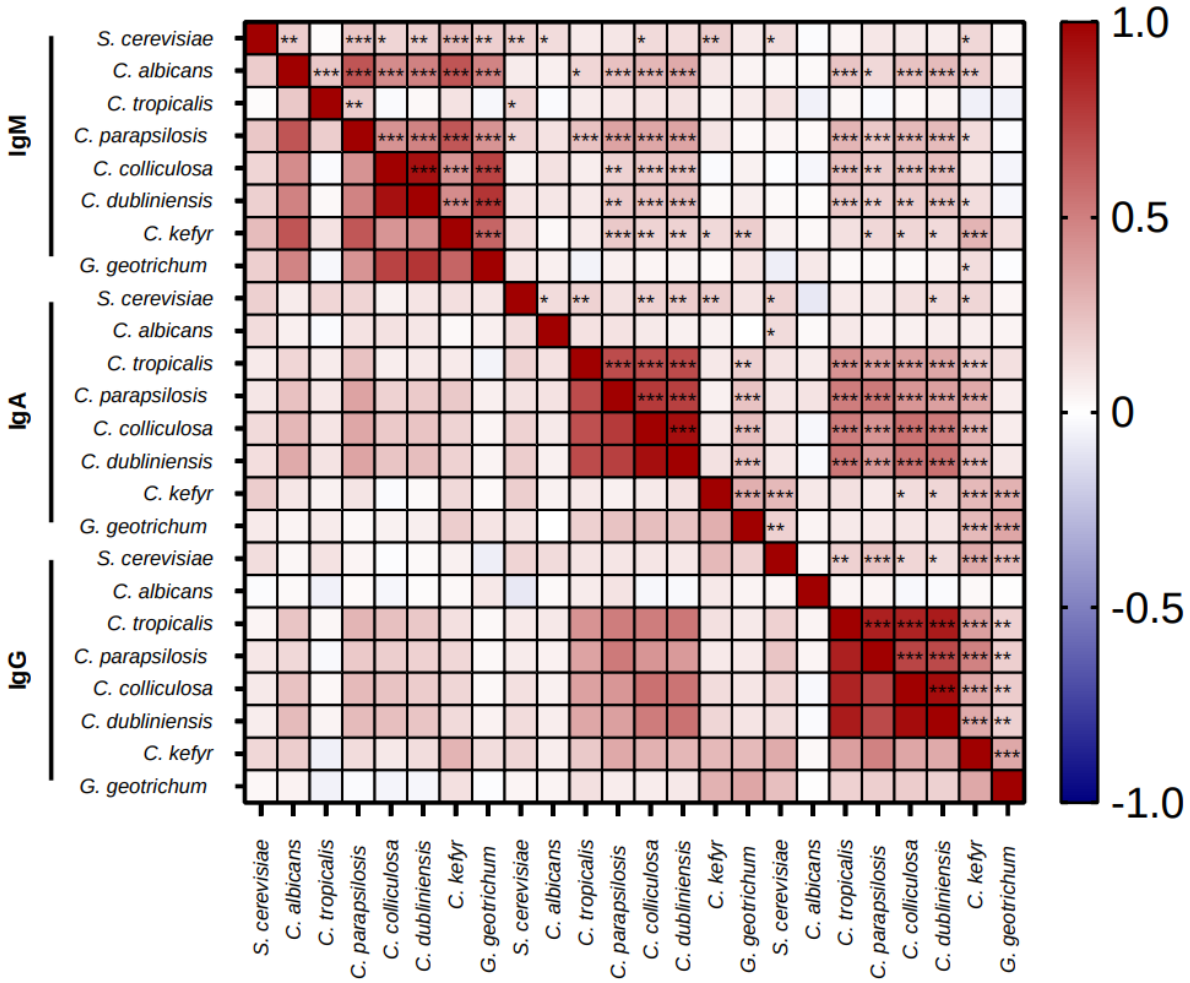

Figure 9. Correlations of all anti-fungal serum antibodies

The correlation matrix based on Spearman's rank correlation coefficients for all samples (healthy individuals and different IBD types) analyzed together. Significance levels are represented as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). **Abbreviations:** *C.* – *Candida*, *G.* – *Galactomyces*, IBD – inflammatory bowel disease, and *S.* – *Saccharomyces*.

5.4 Analysis of macrophage reactivity after stimulation with fungal lysates

To investigate the effect of fungal lysates on macrophage reactivity, peripheral blood-derived macrophages were co-cultivated in vitro with stimuli, prepared fungal lysates and control stimuli (zymosan, mannan, and LPS). After co-cultivation, the phenotype of stimulated macrophages was assessed by flow cytometry. The expression of a number of macrophage activation markers together with the expression of fungal recognition receptors Dectin-1 and DC-SIGN was measured.

No distinct macrophage polarization to the M1 or M2 phenotype was observed. Almost no statistical differences in expression of the measured markers were found after the co-cultivation with fungal lysates compared to unstimulated cells (**Figure 10**).

Moreover, macrophage cytokine production was analyzed using a quantitative ELISA approach. Selected cytokine levels (IL-1β, IL-6, TNF-α, and IL-10) were measured in the supernatants of macrophages co-cultivated with fungal stimuli. As plotted in **Figure 11**, increased levels of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) were observed after stimulation with some of the prepared fungal lysates compared to unstimulated macrophages. A significant increase in TNF-α production by macrophages co-cultivated with *C. dubliniensis* lysate was detected. Macrophages stimulated with *C. dubliniensis* lysate produced also significantly higher levels of anti-inflammatory cytokine IL-10.

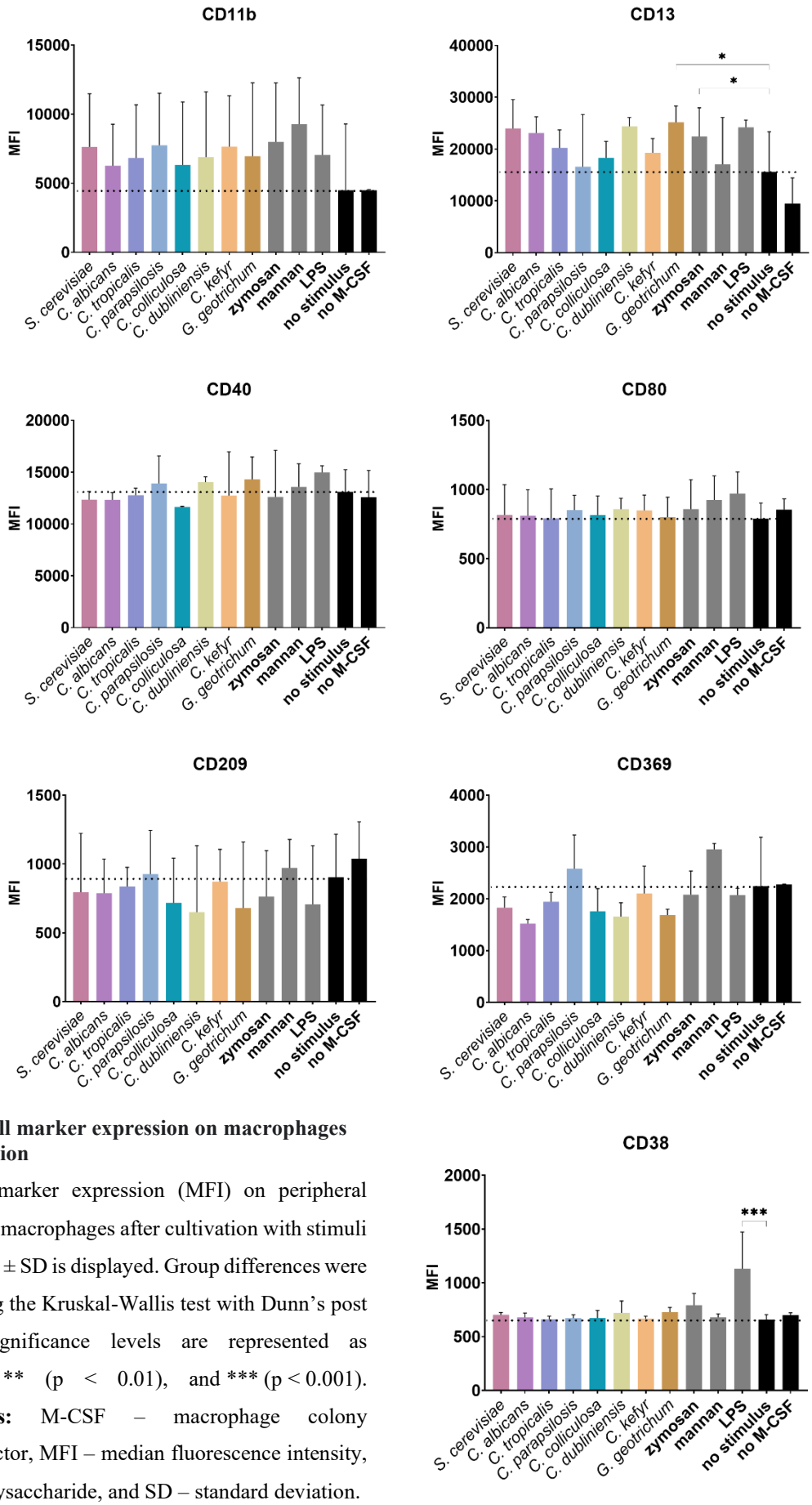

Figure 10. Cell marker expression on macrophages after stimulation

Surface cell marker expression (MFI) on peripheral blood-derived macrophages after cultivation with stimuli for 24 h. Mean ± SD is displayed. Group differences were analyzed using the Kruskal-Wallis test with Dunn's post hoc test. Significance levels are represented as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). **Abbreviations:** M-CSF – macrophage colony stimulating factor, MFI – median fluorescence intensity, LPS – lipopolysaccharide, and SD – standard deviation.

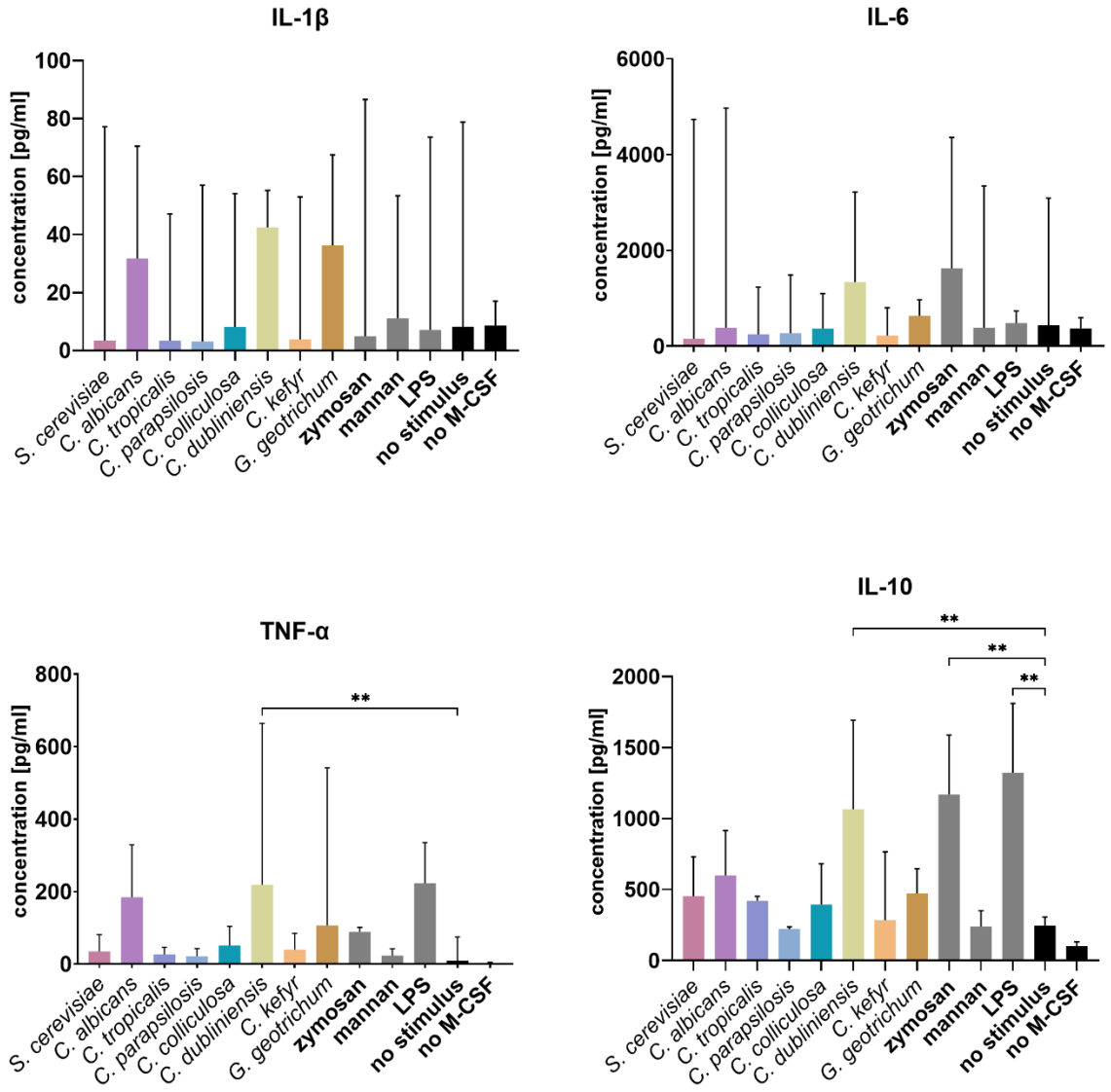

Figure 11. Cytokine production by macrophages after stimulation

Qualitative plots of the selected cytokines produced by peripheral blood-derived macrophages after cultivation with stimuli for 24 h, measured by ELISA. Mean ± SD is displayed. Group differences were analyzed using the Kruskal-Wallis test with Dunn's post hoc test. Significance levels are represented as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). **Abbreviations:** *C.* – *Candida*, ELISA – enzyme-linked immunosorbent assay, *G.* – *Galactomyces*, IL-10 – interleukin 10, IL-1β – interleukin 1 beta, IL-6 – interleukin 6, LPS – lipopolysaccharide, M-CSF – macrophage colony-stimulating factor, *S.* – *Saccharomyces*, SD – standard deviation, and TNF-α – tumor necrosis factor alpha.

6 Discussion

The epithelial surfaces of the body (such as skin, upper respiratory tract, or GI tract) represent the perfect habitat for a considerable number of diverse microorganisms. Complex interactions between these countless communities of bacteria, archaea, fungi, and viruses enable physiological functions like digestion, metabolism, and the development of the mucosal immune system. The gut epithelium forms a barrier between the host and the ecosystem in the intestinal lumen. Under healthy conditions, an interplay between microbial, physical, and biochemical components guarantees the efficiency of this barrier. Effective sealing of the junctional complexes between gut epithelial cells and balancing proliferation and cell death are necessary (Peterson & Artis, 2014). Impairment of this homeostasis is associated with several diseases, including IBD (Podolsky, 2002).

Another crucial and well-documented link between gut microbiota and IBD is dysbiosis, characterized mainly by variations in Proteobacteria and Firmicutes bacterial phyla abundance (Santana et al., 2022). Although most attention has been placed on the bacterial composition of microbial dysbiosis, current evidence also reveals the changes in IBD fungal diversity in parallel (Richard et al., 2015; Sokol et al., 2017b). That raises the question of how yeasts and filamentous fungi and the trans-kingdom interactions shape the immune response in IBD.

With knowledge of the presented importance of gut mycobiota in health and disease, this diploma thesis focused on studying the host's immune response to gut mycobiota and its relevance in IBD. The study was conducted on 52 healthy individuals, 112 CD patients, 49 UC patients, 32 PSC-IBD patients, and 5 PSC patients; all of them were adults. PSC patient group was excluded from the analyses owing to its small sample size. No statistically significant difference in age or BMI between the HC and each diagnosis group was found. This fact prevents misinterpretations caused by age- and BMI-related alternations in gut microbiota observed in the past (Haro et al., 2016; Odamaki et al., 2016). Patients were receiving different medical therapies.

The persistent mucosal immune system stimulation due to the intestinal epithelium's increased permeability is a hallmark of IBD. It was discovered a long ago that UC and CD represent distinct conditions on the molecular level (Lawrance et al., 2001). The recent publication by Ruiz Castro et al. (2021) found a possible explanation (at least a partial one)

for this phenomenon, suggesting the divergent mechanisms may lie in inconsistencies of the discussed gut barrier dysfunction. According to the IBD network models designed by the authors, the barrier dysfunction is more pronounced in UC patients. Thus, using an ELISA approach, the selected biomarkers connected to this issue were quantified in a large group of UC, CD, PSC-IBD, and healthy individuals' sera. The final selection of biomarkers corresponds to the published literature on IBD biomarkers.

For the epithelial barrier damage, serological I-FABP and MMP-9 were quantified. FABPs were previously used as early biomarkers for some diseases, and several papers concerning the levels of these circulating proteins also in IBD patients had been published. The results indicated higher serum biomarkers in CD, UC or PSC-IBD adult patients compared to HC (Al-Saffar et al., 2017; Coufal et al., 2019; Wiercinska-Drapalo et al., 2008) and also in pediatric CD patients (Logan et al., 2022). However, inconsistencies occurred while taking into account disease activity or localization. Some experiments proved no statistical differences between active disease and remission (alternatively remission and healthy controls) or between different disease locations in CD and UC patients, while some authors observed higher concentrations of I-FABP in UC remission than in active disease (Bodelier et al., 2016; Sarikaya et al., 2015).

Regarding the MMP system, these proteins are heavily regulated and involved in fibrogenesis of the intestinal wall. At the same time, the imbalance between MMPs and their inhibitors, TIMPs, might contribute to the inflammatory and remodeling processes typical for IBD, such as the formation of ulcers, development of fibrosis or subsequent organ destruction (Kapsoritakis et al., 2008; G. Lakatos et al., 2012). Analogous to I-FABP, some discrepancies in the existing publications associated with MMP-9 were found. Papers report increased serum MMP-9 concentrations in adult and pediatric IBD patients, which are higher in active UC than in active CD and positively correlated with disease activity (Kofla-Dlubacz et al., 2012; G. Lakatos et al., 2012; Matusiewicz et al., 2014). On the contrary, Coufal et al. (2019) pointed out decreased MMP-9 levels and increased MMP-14 levels as important distinguishing markers of IBD.

The evaluation in this thesis demonstrated higher levels of I-FABP and MMP-9 in IBD patients compared to controls (the latter difference was statistically significant), suggesting a higher rate of gut barrier damage, with the PSC-IBD patient group

controversially evincing the highest concentration of I-FABP and the lowest concentration of MMP-9 among different IBD types.

Increased intestinal barrier permeability and dysbiosis explain the common microbial translocation from the gut lumen into the bloodstream, generating an uncontrolled inflammatory response in IBD patients (Swank & Deitch, 1996). Some serum biomarkers playing a role in this process have been assessed over the years. According to (Lakatos et al., 2011), LBP and soluble CD14 are markers of disease activity and predictors of clinical relapse. Risen levels of MBL and CD14 had been detected in the sera of patients with PSC-IBD compared to healthy individuals' sera (Coufal et al., 2019). In line with previous research, this thesis reports a significantly higher concentration of CD14, MBL, and LBP, all biomarkers related to microbial translocation, in IBD patients' sera compared to control sera. The highest concentrations of CD14, MBL, and IL-18 were detected in PSC-IBD patients suggesting the most enormous inflammatory response. That could be caused by the most compromised gut barrier function among studied patient groups combined with bile duct epithelium leakiness, and abnormalities in liver function.

Moreover, the abovementioned gut barrier failure gives rise to a substantial anti-microbial immune response after the abnormal exposure of immune cells to microbial epitopes, including fungal epitopes. To investigate the role of gut mycobiota, colonies of yeasts and molds were cultured from stool samples of IBD patients and healthy individuals and identified. Remembering the impossibility of culturing all fungal strains, this culture-dependent technique only aimed to obtain fungal lysates of some fungal strains frequently found in the gut and use them for further analyses. As an outcome, 12 different fungal strains were cultured, with the majority of them being yeasts from the phylum *Ascomycota*. Among identified species, some strains are constantly detected as inhabitants of the gut (*C. albicans*, *C. dubliniensis, C. parapsilosis*, and *C. tropicalis*), some strains are probably foodborne or plant-associated but have still been found in several studies of human gut microbiota (*C. colliculosa*, *C. kefyr*, *Galactomyces geotrichum*, and *Saccharomyces cerevisiae*), and some strains that have been reported maximally once (*Aspergillus niger*, *welwitschiae*, *Candida sojae*, *Penicillium citrinum*, and *Trichoderma citrinoviride*) and, thus, were excluded from further experiments (the references to gut fungi are summarized in Suhr & Hallen-Adams, 2015). The literature research on the presence of particular species in the healthy or diseased gut mycobiota was hindered by the improper nomenclature system.

Past research indicated serum ASCA as a serological biomarker for IBD and higher serum and fecal levels of ASCA in adult and pediatric CD patients (Kansal et al., 2019; Tang et al., 2017b). There was still a need to address the situation with other fungal strains. For the purpose of determining the anti-fungal immune response in IBD patients, the selected lysates of cultured fungi were used for ELISA plate coating and subsequent measurement of anti-fungal antibody levels. The antibodies against the tested fungi appear to be higher in IBD patients than in controls across all strains and antibody isotypes (IgM, IgA, and IgG). The largest difference was observed in CD, which is consistent with the literature (Pang et al., 2020). The rationale behind that could be the relation of antimicrobial antibodies to the small intestine, which is inflamed majorly in CD. However, together with the trend of higher serum biomarker levels in CD compared to UC, it contradicts the abovementioned model (Ruiz Castro et al., 2021) of the more significant gut barrier dysfunction in UC patients.

The correlation analysis of antibody responses across different isotypes showed a more noticeable positive correlation within isotypes, with the strongest correlation among the least specific antibody isotype, IgM, as expected. These results proposed that the individual isotypes act independently. The statistically significant positive correlations in antibody responses may also imply that studied fungal species share some antigenic determinants, which has to be considered while interpreting measured antibody responses. Concretely, some species could mimic each other – the overexpression of ASCA epitopes by *C. albicans* was revealed previously (Standaert-Vitse et al., 2006b).

As partially mentioned above, some authors of papers related to both serum biomarkers and anti-microbial antibodies stratified IBD patients into subgroups based on the disease activity, severity, and location. One limitation of this thesis is the lack of this stratification, as some features may mirror the disease activity. Also, no pediatric patients were included in the study. Lastly, studied IBD patients undergo several therapeutic approaches and those might influence serum biomarker levels or immune responses since some effects were already confirmed. Mesalamine, *E. coli* Nissle 1917, and anti-TNF-α therapy affected the levels of serum biomarkers and antibodies against some bacterial genera when assessing IBD patients' sera (Coufal et al., 2019).

In an uninflamed tissue, the resident intestinal macrophages ensure the microbicidal activity and clearance of damaged or apoptotic cells in a non-inflammatory manner. PRRs of those macrophages may be downregulated along with their downstream signaling

cascades in this state, termed inflammation anergy. Nonetheless, inflammation is induced in the event of epithelial barrier damage or invasion of microorganisms (Smith et al., 2010). In IBD, chronic inflammation of intestinal tissue occurs, providing the need for action. Hence, macrophages in IBD overexpress Toll-like receptors and various activation molecules and produce pro-inflammatory cytokines, such as IL-1β, IL-6 or TNF-α (Ahluwalia et al., 2018). Concerning the mycobiota, CX_3CR1^+ mononuclear phagocytes have been recently identified as key players in the initiation of immune responses to gut fungi – acting both at a steady state and inflammation. SNPs in gene encoding CX_3CR1 are associated with a detrimental antifungal response in IBD (Leonardi et al., 2018).

To explore the effect of common fungal inhabitants of the GI tract on the reactivity of macrophages, the macrophages derived from peripheral blood were stimulated with prepared fungal lysates. Zymosan, mannan (fungal ligands), and LPS (bacterial ligand) were used as control stimuli. The phenotype of macrophages was determined afterwards by flow cytometry analysis and the production of cytokines was measured using ELISA from cell supernatants. The expression of macrophages markers CD13, CD11b, and CD38, activation markers CD80 and CD40, and fungal recognition receptors dectin-1 (CD369) and DC-SIGN (CD209) was measured. Almost no differences in expression of the selected markers were proved after stimulation with different fungal lysates. That could be caused by the pre-stimulation of tested macrophages by M-CSF during their differentiation (Jaguin et al., 2013).

Despite the fact that M2 macrophage polarization was shown as protection against fungal pathogens (Davis et al., 2013; Salazar & Brown, 2018), no clear polarization was observed in this thesis. That is in good agreement with the novel observations that macrophage activation after stimulation is a continuum of activation states (Martinez & Gordon, 2014).

Although differences are visible, no statistical significance was detected while comparing the levels of IL-1β and IL-6 cytokine production in supernatants from unstimulated cells and from cells stimulated with different fungal lysates. TNF-α levels were elevated after stimulation by some fungal lysates, with a significant increase after stimulation with *C. dubliniensis* compared to unstimulated cells. This supports the hypothesis that fungal residents of the gut may contribute to inflammation in IBD. Cells stimulated with

C. dubliniensis produced also significantly higher levels of anti-inflammatory cytokine IL-10.

The issue of donor-to-donor heterogeneity must be highlighted. The analyses revealed major variability in immune responses or macrophage phenotype among donors. There is a number of possible directions to address this drawback in the next stage – (i) more blood donors could be involved in the study or (ii) the assay could be optimized to provide a comparable homogenous differentiation state of cells or (iii) human cell lines could be used for the assay to ensure the absolute consistency.

Altogether, experiments utilizing prepared fungal lysates broadened the knowledge of immune reactions to gut mycobiota in IBD. Increased antifungal antibodies in the sera of IBD patients compared to controls emphasize the involvement of mycobiota in the disease pathogenesis. Enhanced pro-inflammatory cytokine production in response to macrophage stimulation by common gut fungal species supports this hypothesis.

Further research is required to uncover the underlying mechanisms of mycobiota's influence on the immune system in health and disease. Obtained data could be useful in designing novel, microbiota-related, treatment strategies.

7 Conclusions

Following the recent research, this study aimed to investigate the immune responses to gut mycobiota with respect to IBD. The performed experiments showed increased antifungal antibodies and microbial translocation-associated biomarkers in the sera of IBD patients compared to healthy controls. Lysates of common fungal residents enhanced the production of pro-inflammatory cytokines by peripheral blood-derived macrophages.

The results obtained in this thesis correspond to the emerging evidence that gut mycobiota play a role in IBD etiology and chronic inflammation. Future observations might focus on developing new therapeutic strategies considering the mycobiota alterations.

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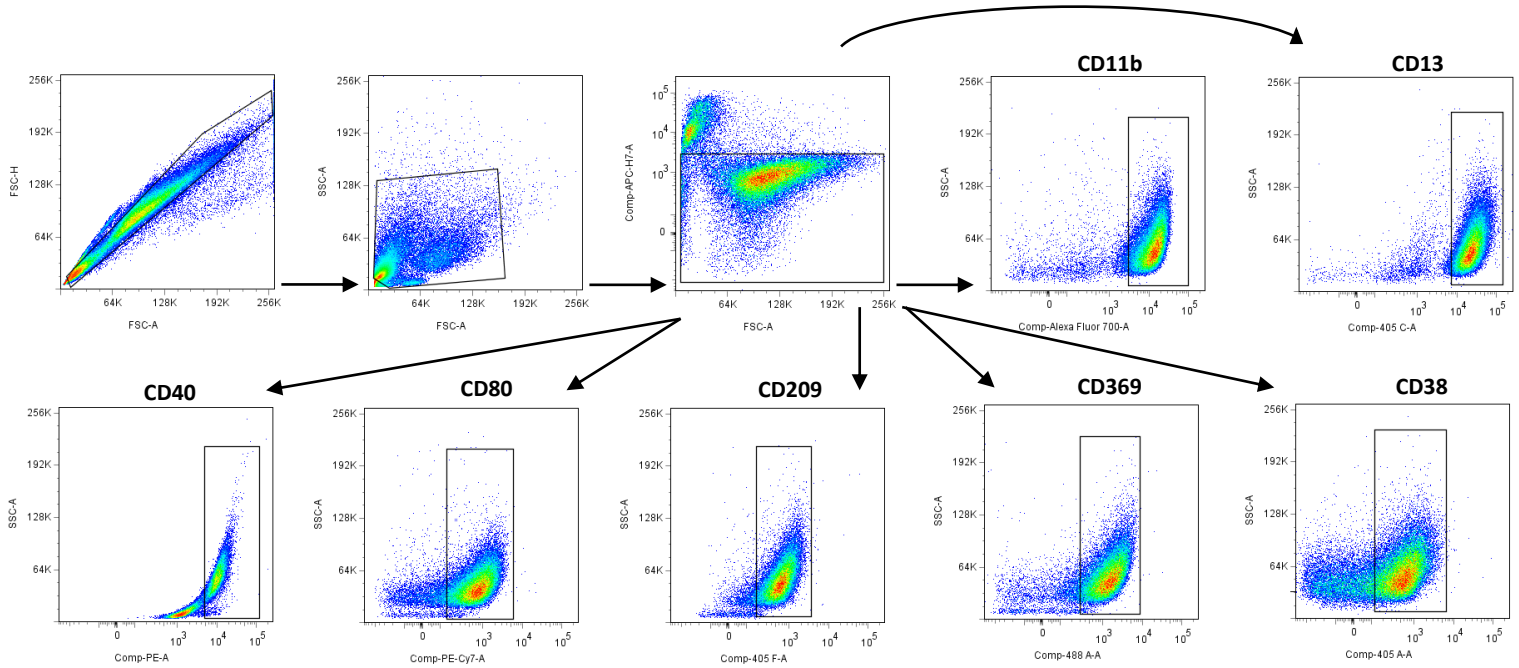
Supplements



Supplementary figure 1. Gating strategy

Flow cytometry data were analyzed as depicted.