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Gut barrier in the pathogenesis and diagnostics of necrotizing enterocolitis and inflammatory bowel disease

Role střevní bariéry v patogenezi a diagnostice nekrotizující enterokolitidy a nespecifických střevních zánětů

DOCTORAL THESIS

Supervisor: MUDr. Miloslav Kverka, Ph.D.

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Poděkování

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Abstract

Disruption of gut microbiota, altered mucosal defense, inappropriate immune response and gut barrier damage are all typical features in the pathogenesis of both necrotizing enterocolitis (NEC) and inflammatory bowel disease (IBD). Despite of intensive research, the exact pathogenesis of both diseases remains unclear and the diagnostics and outcome prediction are still problematic. Therefore, we analyzed the role of gut-associated and inflammatory biomarkers, with respect to different aspects of gut barrier dysfunction in the pathogenesis of both disease, with the aim to improve the diagnostics and to predict the disease course and outcome.

Using ELISA, we found that patients who will later develop NEC have significantly higher intestinal fatty acid-binding protein (I-FABP) than infants who will later develop sepsis already in first hours after NEC suspicion. Urinary I-FABP had high sensitivity (81%) and specificity (100%) and its addition to currently used gold standard for NEC diagnosis increased its sensitivity and negative predictive value. We found that serum amyloid A (SAA) was the strongest factor for prediction of the most severe stage of NEC. The combination of intestinal and liver FABP with SAA predicted the length of hospitalization in NEC patients and the low level of SAA predicted short achievement of full enteral feeding.

Using protein array, ELISA and flow cytometry we performed the broad spectrum analysis of serum biomarkers and specific anti-microbial B and T cell response to gut commensal microbiota. We found that proteins of matrix metalloproteinase system were the strongest factors discriminating IBD patients from healthy subjects. The osteoprotegerin was the strongest factor discriminating the patients with UC and PSC-IBD and in the combination with I-FABP, CXCR-1 and TIMP-1 it discriminated the UC from CD. IBD patients responded mostly similarly to selected commensal bacteria as healthy subject, but in CD patients we found lower antibody response, with significant decrease in IgA to *Faecalibacterium* and *Bacteroidetes*. Furthermore, we found increase in T cells response to these bacteria in CD patient.

Thus, we found that I-FABP is capable to distinguish NEC from sepsis and its combination with other biomarkers may be useful in NEC management. Our results stress the importance of gut barrier function and immune response to commensal bacteria and point at the specific differences in the pathogenesis between the different forms of IBD.

Abstrakt

Dysbióza střevní mikrobioty, alterace ochrany střevní sliznice, nepatřičná imunitní odpověď a poškození střevní bariéry jsou typické znaky patogeneze nekrotizující enterokolitidy (NEC) a nespecifických střevních zánětů (IBD). I přes intensivní výzkum, zůstává příčina vzniku těchto chorob nejasná a jejich diagnostika a predikce průběhu jsou stále problematické. Proto jsme analyzovali význam biomarkerů asociovaných se zánětlivou odpovědí ve střevě s ohledem na různé aspekty dysfunkce střevní bariéry v patogenezi obou nemocí s cílem zlepšit diagnostiku, predikovat průběh nemoci.

Pomocí metody ELISA jsme zjistili, že kojenci, u kterých došlo později k rozvoji NEC, mají významně vyšší hladinu proteinu vázajícího mastné kyseliny ve střevě (I-FABP), než kojenci, u kterých došlo později k rozvoji sepse, a to již v prvních hodinách od doby podezření na NEC. Stanovení I-FABP v moči mělo vysokou sensitivitu (81%) a specificitu (100%) a jeho doplnění k současně používanému zlatému standardu pro diagnostiku NEC umožnilo zvýšení sensitivity a negativní prediktivní hodnoty. Zjistili jsme, že sérový amyloid A (SAA) byl nejsilnějším faktorem pro predikci nejzávažnějšího stádia NEC. Kombinace střevní a jaterní formy FABP s SAA predikovala délku hospitalizace u pacientů s NEC a nízká hladina SAA predikovala rychlejší dosažení plného enterálního příjmu.

Pomocí proteinového mikročipu, metody ELISA a průtokové cytometrie jsme dále provedli širokospektrou analýzu biomarkerů v séru a také specifické anti-mikrobiální B a T buněčné odpovědi proti střevním komenzálním bakteriím. Zjistili jsme, že proteiny systému matrix metaloproteináz byly nejsilnějším faktorem umožňující rozlišení pacientů s IBD od zdravých jedinců. Osteoprotegerin umožnil rozlišit pacienty s UC nebo PSC-IBD a v kombinaci s I-FABP, CXCR-1 a TIMP-1 umožnil také rozlišení pacientů s UC od CD. Imunitní odpověď IBD pacientů na vybrané komenzální bakterie byla podobná jako u zdravých jedinců. U pacientů s CD jsme zjistili nižší protilátkovou odpověď s významným snížením protilátek třídy IgA proti *Faecalibacterium* a *Bacteroidetes*. U pacientů s CD jsme dále zjistili zvýšenou T lymfocytární odpověď proti těmto bakteriím.

Zjistili jsme, že pomocí vyšetření hladiny proteinu I-FABP jsme byli schopni rozlišit pacienty s NEC a sepsí, a jeho kombinace s jinými biomarkery může být užitečná při managementu NEC. Naše výsledky zdůrazňují důležitost funkce střevní bariéry a imunitní odpovědi proti komenzálním bakteriím a poukazují na specifické rozdíly v patogenezi mezi různými formami IBD.

Content

List of abbreviations

AMP – Antimicrobial peptide

ccCK18 – Caspase-cleaved CK18

CCL25 – C-C motif chemokine 25

CCR9 – C-C chemokine receptor type 9

CD – Crohn's disease

CK18 – Cytokeratin 18

CRP – C-reactive protein

CTLA-4 – Cytotoxic T-lymphocyte protein 4

CXCR1/ IL8RA - Interleukin-8 receptor, alpha

DC – Dendritic cell

DC-SIGN - Dendritic cell-specific ICAM-3-grabbing non-integrin 1

EEC – Enteroendocrine cell

EG-VEGF - Endocrine-gland-derived vascular endothelial growth factor

GALT – Gut-associated lymphoid tissue

GATA3 – Trans-acting T-cell-specific transcription factor GATA-3

GC – Goblet cell

GIT – Gastrointestinal tract

GWAS – Genome-wide association study

HLA – Human leukocyte antigen

IBD – Inflammatory bowel disease

ICAM-1 – Intracellular adhesion molecule 1

IEC – Intestinal epithelial cell

IECs – Intestinal epithelial cells

IEL – Intraepithelial lymphocyte

I-FABP – Intestinal fatty acid-binding protein

IFN-γ – Interferon gamma

IgA – Immunoglobulin A

IL-1β – Interleukin-1 beta

ILC – Innate lymphoid cell

JAK-2 – Janus kinase 2

LBP – Lipopolysaccharide-binding protein

LFA-1 – Lymphocyte function-associated antigen 1

L-FABP – Liver fatty acid-binding protein

LPS – Lipopolysaccharide

MadCAM-1 – Mucosal addressin cell adhesion molecule 1

MDA5 – Melanoma differentiation-associated protein 5

MHC – Major histocompatibility complex

MIP-1α – Macrophage inflammatory protein-1 alpha

MMP-14 – Matrix metalloproteinase-14

MMP-9 – Matrix metalloproteinase-9

MUC1 – Mucin-1

МΦ - Macrophage

NEC - Necrotizing enterocolitis

NF-κB – Nuclear factor kappa-light-chain-enhancer of activated B cells

NK – Natural killer cell

NKT – Natural killer T cell

NLR – NOD-like receptor

NOD – Nucleotide-binding oligomerization domain

OPG - Osteoprotegerin

PAF – Platelet-activating factor

PAMP – Pathogen-associated molecular pattern

PC – Paneth cell

PD-L1 – Programmed cell death 1 ligand 1

PRR – Pattern recognition receptor

PSC-IBD – Inflammatory bowel disease associated with primary sclerosing cholangitis

PUFA – Polyunsaturated fatty acid

RIG-I – Retinoic acid-inducible gene I protein

RORγ – RAR-related orphan receptor gamma

SAA – Serum amyloid A

SIgA – Secretory immunoglobulin A

STAT-3 – Signal transducer and activator of transcription 3

TCR – T-cell receptor

TFF-3 – Trefoil factor-3

Th1 cells – Type 1 helper T cells

Th17 cells – Type 17 helper T cells

Th2 cells – Type 2 helper T cells

TIMP-1 – Tissue inhibitor of metalloproteinases 1

TJ – Tight junction

TLR – Toll-like receptor

TNF-α – Tumor necrosis factor - alpha

UC – Ulcerative colitis

1. Introduction

The gut mucosa represents the interface between the host and the external environment in the lumen of intestine. It has dual role, it acts as a selective barrier allowing the efficiently absorption of nutrients, electrolytes and water, while still maintain the effective defense against intraluminal toxins, antigens and enteric microbiota (GROSCHWITZ and HOGAN 2009). The gut barrier function is ensured by microbial (commensal microbiota), biochemical (humoral and mechanical (physical) components. The gut barrier defense is mediated by the collaboration of innate and adaptive immune system. Immature infants have underdeveloped gut barrier and thus large quantities of molecules can enter their bodies. Consequently, infants are susceptible to diseases like infectious diarrhea, allergic gastroenteropathy and necrotizing enterocolitis (NEC). It is essential that infant's intestinal barrier matures appropriately because barrier dysfunction in adulthood is a critical factor in predisposition to intestinal diseases and is associated with autoimmune diseases in the other parts of the body (ANDERSON *et al.* 2012).

Both necrotizing enterocolitis (NEC) and inflammatory bowel disease (IBD) are serious inflammatory intestinal diseases with similar features in their pathogenesis, involving disruption of gut microbiota, altered mucosal defense, inappropriate immune response and gut barrier damage (HARPAVAT *et al.* 2012).

Despite of intensive research, the exact pathogenesis of both diseases remains unclear and the diagnostics is still problematic. To date, none ideal single biomarker for the diagnosis of NEC or IBD has been proven. The best biomarker should reflect the major steps in early disease pathogenesis, should be disease specific, able to identify individuals at risk for the disease development, be able to assess the disease activity, complications, relapse or disease recurrence. Moreover, it should be able to monitor the effects of treatment and last but not least it should be easy to measure and cheap (VIENNOIS *et al.* 2015; CALIFF 2018).

Since the gut barrier dysfunction and damage is common mechanism in the pathogenesis of these diseases, we analyzed the role of biomarkers associated with gut barrier and inflammatory response, with respect to different aspects of barrier dysfunction in the pathogenesis of both diseases, to improve the diagnostics of NEC and IBD and to predict the disease course. Moreover, to improve diagnostic values of these tests, we additionally combined several relevant biomarkers to panels.

2. Gut barrier

Gut barrier is a complex functional unit at the interface between the host and the environment in the lumen of intestine. The gut barrier function is ensured by **microbial** (commensal microbiota), **biochemical** (humoral), **mechanical** (physical) and **immunological** components (Figure 1). The gut barrier integrity and defense is maintained by the collaboration of innate and adaptive immune system. For the proper function of the gut barrier is essential that all its components are developed and functional (TLASKALOVÁ-HOGENOVÁ *et al.* 2004; ANDERSON *et al.* 2012).

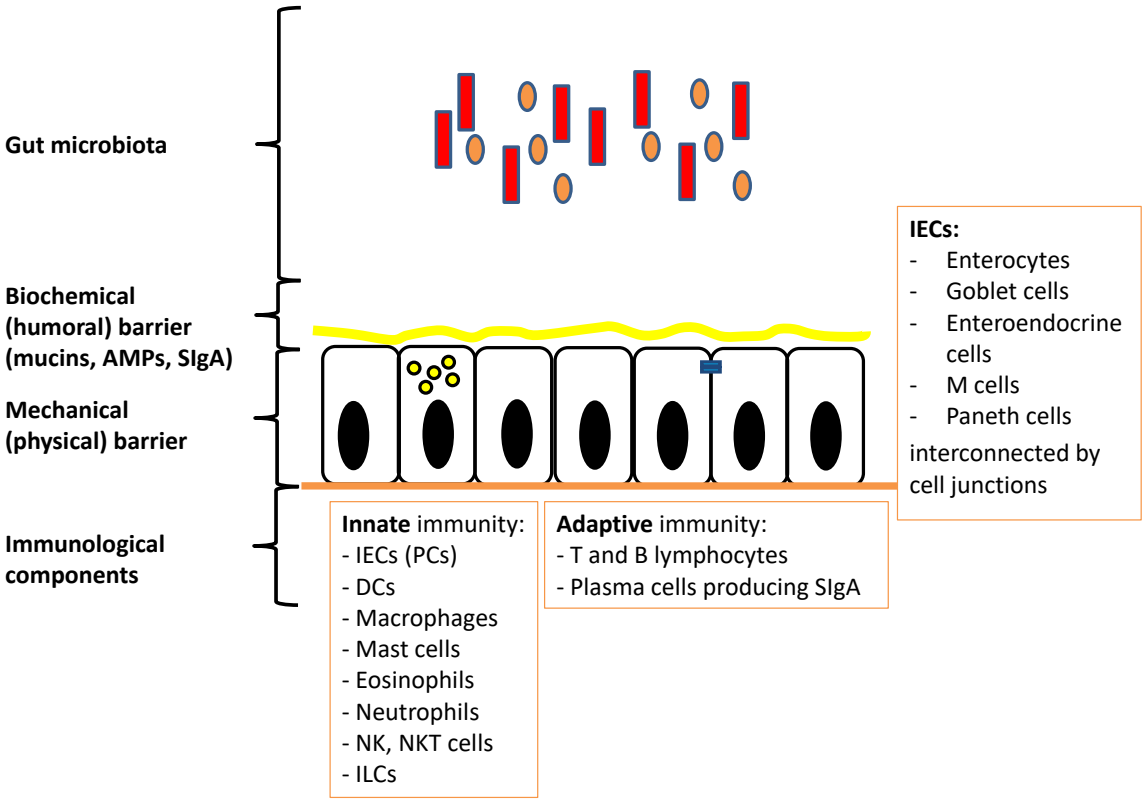


Figure 1: Gut barrier (AMPs – antimicrobial peptides, SIgA – secretory IgA, IECs – intestinal epithelial cells, PCs – Paneth cells, DCs – dendritic cells, NK – natural killer cells, NKT – natural killer T cells, ILCs – innate lymphoid cells) (adapted from Coufal et al. 2016a).

2.1 Microbial barrier

The epithelial surfaces of human body (skin, upper respiratory tract, urogenital and gastrointestinal tract) are colonized by microbes, commonly termed as microbiota. The

most densely colonized part of human body is gut. Gut microbiota is a complex ecosystem that consists of more than 1000 species of bacteria, five genera of Archaea, 66 genera of fungi and non-well defined number of viruses (QIN *et al.* 2010; HOFFMANN *et al.* 2013; COLUMPSI *et al.* 2016; COUFAL *et al.* 2019). Apart from the physiological functions like digestion and absorption, synthesis of vitamins, regulation of lipids metabolism, the gut microbiota participates also in protection against pathogen invasion (colonization resistance) both by competing with pathogens and by driving the development of mucosal immune system. The composition of gut microbiota differs along the gastrointestinal tract (GIT) according to the different environmental conditions. The overall composition of gut microbiota is influenced by the type of birth, nutrition and by environmental factors (TLASKALOVÁ-HOGENOVÁ *et al.* 1983; STEPANKOVA *et al.* 1998; ECKBURG *et al.* 2005; KOZAKOVA *et al.* 2006; WILLIAMS *et al.* 2006; KVERKA *et al.* 2011; KOENIG *et al.* 2011; HANSEN *et al.* 2012; KVERKA and TLASKALOVA-HOGENOVA 2013).

The complex gut microbiota is establishing during the first week after birth and is still maturing and developing until the 3rd year of life. During this period gut microbiota dynamically evolves in diversity, density and activity under the influence of inner (host's genotype, maturity of GIT) and outer factors, including type of delivery, type of feeding, antibiotic usage, the presence of bacteria in the surrounding area (ZOETENDAL *et al.* 2001; PENDERS *et al.* 2006).

Vaginally born newborns are colonized by mother's rectovaginal microbiota. Thus, in their gut dominate the microbes from genus *Lactobcillus* and *Prevotella,* which are less abundant in newborns born by caesarean section. In infants born by caesarean section dominate bacteria, which are typical for the skin – *Staphylococcus*, *Corynebacterium*, *Propionibacterium* spp. This ecological imbalance makes the infants more prone to colonization by pathogenic microbes like *Clostridium difficile* and some strains of *Escherichia coli*. Similarly, the gut microbiota composition of the infant is influenced by the type of feeding. The human milk supports the growth of *Bifidobacteria*, which dominate in breast feeding infants. The formula feeding facilitates the colonization of the gut by *C. difficile*, *Bacteroides fragilis*, *E. coli* and other members of the *Enterobacteriaceae* family (PENDERS *et al.* 2006; FAN *et al.* 2014).

The establishment of the microbial ecosystem in early life is suggested to play an important role in microbiota composition and diseases susceptibility during the life

(OTTMAN *et al.* 2012; COX *et al.* 2014; RODRIGUEZ *et al.* 2015). Each human individual reaches a homeostatic composition, which likely remains relatively stable during most of a healthy adult's life, but can be altered as a result of bacterial infections, antibiotic treatment, lifestyle, surgical and dietary changes. At the late stages of life the microbiota composition becomes less diverse with higher *Bacteroides* to *Firmicutes* ratio, increase in *Proteobacteria* and decrease in *Bifidobacteria* (ZOETENDAL *et al.* 2008; SEKIROV *et al.* 2010; ARUMUGAM *et al.* 2011; OTTMAN *et al.* 2012; SCHOLTENS *et al.* 2012).

2.2 Biochemical (humoral) barrier

The surface of the gut epithelium is covered and protected by thick, viscous and gel-like layer consisting by abundantly O-glycosylated proteins (mucins) called the mucus layer. The mucins are produced by Goblet cells (GCs). There are several subfamilies of mucins with distinct structure and function including the secreted polymeric gel-forming mucins (MUC2, MUC5AC, MUC5B, MUC6, MUC19), secreted non-gel forming mucins (MUC7) and the mucins associated with cell surface (MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17, MUC20, MUC21). For intestine is typical MUC2 mucin (MCGUCKIN *et al.* 2015; CORFIELD 2018). The mucus production is ensured by GCs from the 12th week of gestation and from the 27th week of gestation it resembles the composition of the mucus layer in adults. Mucus layer is composed by inner and outer layer. The thickness of the layers and type of mucins differ along the GIT (CHAMBERS *et al.* 1994; MATSUO *et al.* 1997; BUISINE *et al.* 1998; ATUMA *et al.* 2001). The mucus layer prevents the direct interaction of epithelial cells with gut microbiota. The gut barrier protection is also ensured by the retention of released secretory immunoglobulin A (SIgA) and antimicrobial molecules in mucus layer (MCSWEEGAN *et al.* 1987; GALLO and HOOPER 2012; TLASKALOVÁ-HOGENOVÁ and MĚSTECKÝ 2012; CLEVERS and BEVINS 2013). By retaining digestion enzymes, mucus layer also helps to digest and absorb the nutrients.

2.3 Mechanical (physical) barrier

The GIT is composed of a tube-like structure lined by a continuous epithelial cell layer sitting on a basal lamina that serves as a physical barrier to the external environment. There are several different types of intestinal epithelial cells (IECs). The most abundant epithelial cells in the intestine are enterocytes (~80%) characterized by the presence of villi and microvilli (brush border) and serve to nutrient absorption. Between the enterocytes are

present other IECs: mucus-secreting goblet cells (GCs), antimicrobial molecules secreting Paneth cells (PCs), nutrient-sensing and hormone-producing enteroendocrine cells (EECs), chemo-sensing tuft cells and antigen-sampling microfold cells (M cells). The IECs form the epithelium of both the small and large intestine and contribute in different way to the gut barrier function. Although small and large intestine is interconnected, they can be differentiated in both functional and anatomical ways. While small intestine is responsible for the absorption of nutrients, the large intestine takes a part in water absorption, in further processing of undigested materials and in excretion of solid waste material. Thus, the large intestine is shorter and wider, than small intestine and its internal surface lacks villi, which maximized the nutrients absorption in small intestine. Furthermore, there are also differences in presence and ratio of IECs, e.g. the large intestine lacks PCs, which are typical for small intestine (CLEVERS and BEVINS 2013; CLEVERS 2013; PETERSON and ARTIS 2014; TING and VON MOLTKE 2019).

All IECs originate from the intestinal stem cells residing in the crypts of Lieberkühn. The intestinal stem cells provide a regular pattern of epithelial cells recovery after 4-5 days. New cells differentiate and migrate to the villi peak (crypto-villi axis) to replace the cells that physiologically die by programmed cell death called apoptosis. On the contrary, Paneth cells stay at the basis of the crypts in the proximity of intestinal stem cells, where they participate in maintaining of intestinal stem cell niche (VAN DER FLIER and CLEVERS 2009; SATO *et al.* 2011; ANDERSON *et al.* 2012; CLEVERS 2013).

Epithelial cells are polarized and interconnected by multiprotein complexes including tight junctions (TJs; *zonula occludens*), adherens junctions (*zonula adhearens*), desmosomes (*macula adhearens*) and gap junctions (FARQUHAR and PALADE 1963). These junctions allow the passage of fluids, electrolytes and small macromolecules, but prevent passage of larger molecules. TJs are formed gradually from the 10^{th} week of gestation and are the most apical of all the junctional complexes. Thus, TJs are primarily responsible for permeability control of the paracellular pathway and for prevention of uncontrolled paracelullar transport of microorganism and larger molecules of intestinal content from the gut lumen (POLAK-CHARCON *et al.* 1980; LEBENTHAL and LEBENTHAL 1999; ANDERSON *et al.* 2012).

Under the gut epithelium is a layer of loose connective tissue called lamina propria containing blood and lymphatic vessels and immune cells that mediate innate and adaptive immune response. Gut epithelium together with *lamina propria mucosae* and *lamina muscularis mucosae* form the gut mucosa (*tunica mucosa*). The submucosa (*tunica submucosa*) is dense connective tissue layer containing part of enteric nervous system (*plexus Meissneri*) and connects the *tunica mucosa* with the layer of smooth muscles (*tunica muscularis*), which contains *plexus Auerbachi*, which is responsible for the peristaltic movement of the bowels (Figure 2) (ABBAS *et al.* 2018).

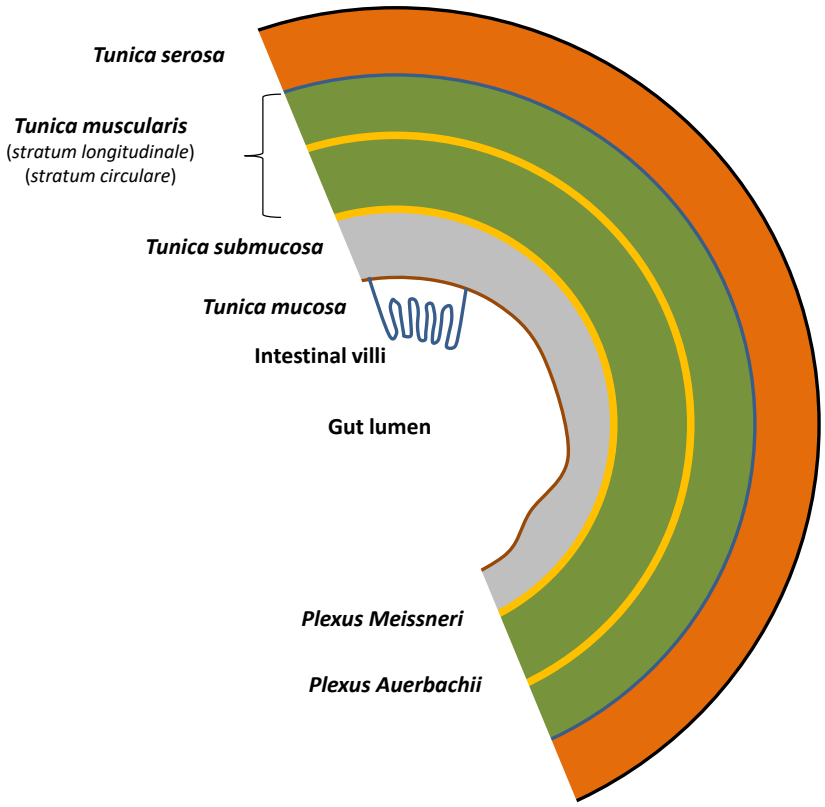


Figure 2: Simplified structure of gut wall (adapted from Coufal et al. 2016a).

2.4 Immunological barrier and mechanisms of intestinal immunity

Immunological barrier of the intestine is ensured by both innate and adaptive immunity and their products (see below), which co-operate together to ensure the protection and integrity of the host. Under the intestinal epithelial layer are present lymphoid follicles. These structures are also called organized-gut-associated lymphoid tissue (o-GALT), which represents the site of induction of immune response. On the other hand, diffuse-GALT (d-GALT) consists of widespread leukocytes (e.g. B cells, T cells, macrophages,

natural killer cells, natural killer T cells, neutrophils, eosinophils, basophils and mast cells) scattered throughout the surface epithelium and underlying lamina propria of the mucosa, and constitutes the effector site of immune response (MONTILLA *et al.* 2004).

2.4.1 Inductive site of mucosal immune response

The most prominent o-GALT structures are Peyer's patches, organized lymphoid follicles, found in small intestine. Next to Peyer's patches, there are also isolate lymphoid follicles present throughout the intestine and also in appendix. The formation of Peyer's patches begins in late stages of fetal development via the interaction of lymphoid tissue inducer cells with stromal cells in lymphotoxin-dependent manner, leading to recruitment of all additional cell types. The formation of isolated lymphoid follicles starts after birth in response to antigen stimulation due to colonization of gut commensals. Peyer's patches and isolated lymphoid follicles are connected by lymphatic vessels to the draining lymph nodes. Thus, mesenteric lymph nodes play important role in the immune response to the enteric pathogens (OWEN and JONES 1974; UCHIDA 1988; OWEN *et al.* 1991; RUMBO and SCHIFFRIN 2005).

2.4.1.1. Antigen uptake

The Peyer's patches and isolated lymphoid follicles are covered by follicle-associated epithelium (FAE). The FAE differs from the absorptive epithelium in intestinal tract and is principally composed by columnar epithelial cells and M cells. The transcellular transport by M cells constitutes a major pathway of antigen delivery to the underlying GALT. The M cells have special features that enhance antigen uptake like flattened apical surface with short microvilli, reduced glycocalyx and sparse mucus layer due to reduced frequency of goblet cells in FAE (HEEL *et al.* 1997; RUMBO and SCHIFFRIN 2005). M cells can take up antigens via various mechanisms, including pinocytosis, micropinocytosis and receptor-mediated endocytosis and transport them by transcytosis (OWEN 1977; OWEN *et al.* 1986). The basolateral membrane of M cells is deeply invaginated and adjacent to local cells, including the $CD11c^+$ dendritic cells (DCs) (FARSTAD *et al.* 1994). Thus M cells play crucial role in delivering of antigens to immature DCs (NEUTRA *et al.* 2001; LAMBRECHT *et al.* 2015; WILLIAMS and OWEN 2015). The immature DCs are recruited in chemokine-dependent manner via the constitutively production of CCL20 in FAE. Next to DCs, the antigens can be uptake also by macrophages and B cells located near the M cells (IWASAKI and KELSALL 2000; WILSON *et al.* 2003; RUMBO and SCHIFFRIN 2005). Thus the M cells play important role in the induction of adaptive immune response.

Antigens from gut lumen can be uptake also directly via the protruding trans-epithelial dendrites (TEDs) of $CX3CR1^+$ DCs (RESCIGNO *et al.* 2001). It was shown, that soluble antigens can be also transported from gut lumen by GCs (so called goblet cell associated antigen passage; GAPs) to underlying $CD103^+$ DCs (MCDOLE *et al.* 2012). The $CD103^+$ DCs are responsible for antigen uptake and delivery to the mesenteric lymph nodes, where they can initiate adaptive immune response (SCHULZ *et al.* 2009; SHIOKAWA *et al.* 2017). Transport of luminal antigens across the gut epithelium is facilitated via secretory IgA (SIgA) and IgG (see below). Whereas the SIgA-antigen complexes are taken up from gut lumen by M cells via Dectin-1 receptor (with possible involvement of Siglec-5 as co-receptor), the IgG-antigen complexes are transported to lamina propria via neonatal Fc receptor (FcR)-dependent epithelial transcytosis (RATH *et al.* 2013; ROCHEREAU *et al.* 2013). The soluble antigens with low molecular weight can also leak between IECs by paracellular diffusion (KNOOP *et al.* 2013).

Thus, different pathways deliver antigens with specific characteristics as well as different pathways may be associated with specific immune outcome.

2.4.1.2. Antigen driven priming of naïve T and B lymphocytes

After activation, immature DCs undergo a maturation process, leading to the decrease of antigen uptake capacity and increase in the capacity of antigen processing and presentation of MHC-I and II-peptide complexes as well as enhancement of the costimulatory molecule expression. During this maturation process the DCs migrate to T cell areas of o-GALT or draining MLNs, where they present the processed antigens to naïve T cells. The high levels of MHC-peptide complexes, adhesion molecules (e.g. ICAM-1, ICAM-2, LFA-1, LFA-3, DC-SIGN), and costimulatory molecules (e.g. CD80, CD86) on the surface of the mature DCs allow effective stimulation of naïve T cells leading to their activation and differentiation in variety of phenotypes (e.g. Th1, Th2, Th17, Tregs). The differentiation is based on cytokine signals derived from DCs or other cells (e.g. basophils, mast cells, epithelial cells, ILCs) in response to the antigen. Furthermore, the DCs also provide signals for differentiation of IgA-producing B cells (MACPHERSON and UHR 2004; LAMBRECHT *et al.* 2015; WILLIAMS and OWEN 2015).

Effector lymphocytes generated in GALT and mesenteric lymph nodes are imprinted with a specific gut homing phenotype, which enable them to migrate back from the circulation to the lamina propria via CCR9 – CCL25 and α4:β7 – MadCAM interaction. This gut

homing phenotype is imprinted by DCs via production of retinoic acid from vitamin A by retinalaldehyde dehydrogenase (DE CALISTO *et al.* 2012).

In addition to organized lymphoid tissue, the intestine contains also diffusely distributed immune cells, scattered throughout the surface epithelium and underlying lamina propria of the mucosa, which constitute the effectors of immune response.

3. Components of immunological barrier

The protection of gut barrier is ensured by components of both innate and adaptive immunity.

3.1 Innate immunity

Intestinal macrophages are positioned to intercept the microorganisms and foreign debris that breach the gut barrier. They have important role in the maintaining of mucosal homeostasis by efficient clearance of apoptotic or damaged cells from lamina propria (SAVILL *et al.* 2002; HENSON and HUME 2006; SMITH *et al.* 2011). Next to this, the intestinal macrophages have also important role in the immunoregulation. It was shown, that intestinal macrophages can express retinaldehyde dehydrogenase as intestinal DCs, which has important role in production of retinoic acid and thus intestinal macrophages can also participate on induction of Tregs (MANICASSAMY and PULENDRAN 2009). Resident intestinal macrophages have downregulated innate response receptors and do not produce pro-inflammatory cytokines in response to inflammatory stimuli, but they retain their phagocytic and bactericidal activity ensuring the gut barrier defense in a non-inflammatory manner in the close proximity of gut microbiota (SMYTHIES *et al.* 2005; SMITH *et al.* 2011).

Mast cells and basophils are abundant in human GIT and have important role in the immune response to viral, bacterial, fungal and parasitic infection. They are involved in first inflammatory responses generated during infection. Mast cells and basophils contain large number of preformed mediators stored in the cytoplasmic granules. Both cells express histamine, leukotriene C4, platelet-activating factor. Furthermore, the spectrum of mediators that is produced differs according the type of stimuli involved in their activation (MARSHALL 2004; SHELBURNE and ABRAHAM 2011).

Eosinophils are common resident cells of the lamina propria and are important effector leukocytes implicated in both mucosal defense and allergic reactions. They express broad range of PRRs and also Fc receptors (FcRs), allowing them to be stimulated by various

pathogens even coated by antibodies. Eosinophils produce several specific cytotoxic molecules such as eosinophil peroxidase, major basic protein, eosinophil cationic protein and eosinophil-derived neurotoxin, which are released from granules upon activation. Eosinophils can also produce a variety of cytokines (e.g. IL-2, IL-4, IL-5, IL-12, TGF-β), chemokines (e.g. eotaxin, MIP-1α) and neurotransmitters (e.g. substance P, vasoactive intestinal peptide) which can regulate both innate and adaptive immune response (HOGAN *et al.* 2008; JUNG and ROTHENBERG 2014). Moreover, they can also present antigens to T cells via MHC II and regulate SIgA production by plasma cells (LUCEY *et al.* 1989; TAMURA *et al.* 1996; CHU *et al.* 2014). It was shown, that eosinophils can release DNA to trap intestinal bacteria and thus contribute to their clearance (JUNG and ROTHENBERG 2014).

Neutrophils are most abundant leukocyte population in circulation. They are recruited from bloodstream via inflammatory mediators, which are released in response to invading microbes to elaborate acute inflammatory response. Thus, neutrophils represent important first-line response against microbial invasion. However, excessive recruitment and accumulation of activated neutrophils in the intestine under pathological conditions, such as inflammatory bowel disease, is associated with mucosal injury (FOURNIER and PARKOS 2012). Neutrophils have specialized mechanisms for killing the pathogens. These include phagocytosis for intracellular killing, production of reactive oxygen species and cytokines, release of granules with antimicrobial and toxic substances (e.g. lysozyme, lactoferin, bactericidal/permeability increasing protein, neutrophil gelatinase-associated lipocalin, cathelicidin hCAP18) and formation of extracellular traps (netosis) (BORREGAARD *et al.* 2007; AMULIC *et al.* 2012).

Innate lymphoid cells (ILCs) participate in the maintaining of the integrity of the intestinal barrier and protection against viral, bacterial, fungal and parasitic infection. ILCs play also important role in the intestinal inflammation (CHOY *et al.* 2017). There are 3 main groups of ILCs. ILC1 are dependent of T-bet transcription factor and produce the Th1-related cytokines, including IFN-γ; ILC2 are dependent on GATA3 and produce the Th2-associated cytokines (IL-5, IL-13); ILC3 are dependent on RORγt and produce the Th17-related cytokines (IL-17A, IL-22 and IL-23) (SPITS *et al.* 2013; CHOY *et al.* 2017).

Natural killer (NK) cells are involved in immune response against intracellular pathogens. The effector functions of these cells are to kill infected but also stressed cells of the

organism and to produce IFN-γ, which is important for activation of the macrophages for killing of the phagocytosed microbes. Thus, NK cells have important role in response to intestinal infections. Interestingly, via production of IL-22 they may also have role in the regulation of gut homeostasis. IL-22 may be implicated in the restoration of gut barrier function upon epithelial damage during inflammation and it also enhances the production of AMPs and chemokines by IECs (MIDDENDORP and NIEUWENHUIS 2009; STRIZ *et al.* 2014; POGGI *et al.* 2019). **Natural killer T (NKT) cells** represent a minor subset of T cells that share cell-surface molecules with conventional T cells and NK cells recognizing lipids antigens presented by the MHC class I-like antigen presenting molecule CD1d. In gut, various cell type including IECs, DCs and B cells can express the antigen-presenting molecule CD1d. Upon activation NKT cells can rapidly produce large amount of Th1, Th2, but also regulatory cytokines. Thus, NKT cells may promote or suppress innate and adaptive immune response according specific conditions. On the other hand, uncontrolled activation of NKT cells may have an important role in the pathogenesis of IBD (MIDDENDORP and NIEUWENHUIS 2009; LIAO *et al.* 2013).

Not only leukocytes protect the gut mucosa. The **IECs** are of central importance in gut barrier defense by providing both a mechanical/physical and immunological barrier. They can also actively modulate immune response at mucosal surfaces to maintain tolerance and homeostasis as well as control the direction of the immune response. IECs express several pattern recognition receptors (PRRs) to recognize pathogen associated molecular patterns (PAMPs). The PRRs can be present on the cellular (e.g. TLR 1, 2, 4, 5, 6) or endosomal membrane (e.g. TLR 3, 7, 8, 9) or in the cytoplasm (e.g. NOD1, NOD2, RIG-I, MDA5) of the IECs (PETNICKI-OCWIEJA *et al.* 2009; FUKATA and ARDITI 2013). Upon recognition of respective PAMPs are triggered appropriate downstream signaling events leading to the production and secretion of inflammatory cytokines, type I interferons, antimicrobial molecules and chemokines allowing the recruitment of leukocytes (KAWAI and AKIRA 2010, 2011; STRIZ *et al.* 2014; SHI and WALKER 2015). The unresponsiveness of IECs to the TLR stimuli under homeostatic condition is ensured by several mechanism, for example by lower expression of TLRs, together with the presence of inhibitors of TLR signaling pathway (e.g. Toll-interacting protein, peroxisome proliferators-activated receptor-γ, Ig LI-1 R-related protein) (OTTE *et al.* 2004; BISWAS *et al.* 2011; DE KIVIT *et al.* 2014). Furthermore, the IECs contribute to the transepithelial transport of

immunoglobulins and antigens, and they can also contribute to the antigen presentation of MHC-restricted peptides as well as CD1d-restricted lipids (ZEISSIG *et al.* 2015).

Paneth cells (PCs) are unique epithelial cell type, residing at the base of Lieberkühn's crypts in small intestine. By production of antimicrobial molecules: α-defensins (HD-5, HD-6) (PORTER *et al.* 1997; SALZMAN *et al.* 1998), lysosyme (ERLANDSEN *et al.* 1974), and phospholipase A2 (NEVALAINEN and HAAPANEN 1993) PCs participate not only in gut barrier defense, but also in the regulation of composition and distribution of intestinal microbiota (AYABE *et al.* 2000). Moreover, the proper function of PCs is essential for the maintaining of homeostasis in the Lieberkühn's crypt, where reside the intestinal stem cells (KRAUSOVA and KORINEK 2014). PCs control the stem cells via production of bactericidal products and important niche signals like epidermal growth factor, transforming growth factor-α, Wnt-3 and Notch ligand DII4 (SATO *et al.* 2011; TAKAHASHI and SHIRAISHI 2020). Another important class of antimicrobial molecules ensuring gut barrier protection constitutes from cathelicidins. Cathelicidin LL-37 can be produced by granulocytes and epithelial cells and it could be released on mucosal surfaces. Next to its antimicrobial effect it has also immunomodulatory properties (chemotaxis of granulocytes and $CD4^+$ T-lymphocytes). Moreover, together with other antimicrobial molecules in amniotic fluid, LL-37 helps to the protection of fetus during the gestation (KAI-LARSEN *et al.* 2014). The level of LL-37 increases in neonate's plasma during birth. There was described the correlation between the level of LL-37 in the mother's plasma and the plasma isolated from cord blood. This is explained by the transfer of LL-37 in the late phase of pregnancy and during the birth. In vaginally born neonates was found higher LL-37 than in neonates born by cesarean section. This increase is caused probably by the stress during the spontaneous birth, which stimulates the production of LL-37 in fetus/newborn. The production of LL-37 as well as other antimicrobial molecules (e.g. HD-5, HD-6, lysozyme) increases further after the birth (HERSON *et al.* 1992; MANDIC HAVELKA *et al.* 2010). Thus, gut epithelium produces a diverse collection of antimicrobial molecules, including α- and β-defensins, lysosyme, cathelicinds and C-type lectins (REGIIIα), which are released in the mucus layer to prevent microbial invasion (MCSWEEGAN *et al.* 1987; GALLO and HOOPER 2012; TLASKALOVÁ-HOGENOVÁ and MĚSTECKÝ 2012; CLEVERS and BEVINS 2013).

3.2 Adaptive immunity

Effector T and B lymphocytes generated in the o-GALT and mesenteric lymph nodes are imprinted with selective integrin and chemokine receptor-dependent gut homing properties and they migrate from the bloodstream back into lamina propria of the gut, where they participate on gut barrier defense and intestinal homeostasis.

Primary role of effector T cells is to amplify and coordinate immune response initiated by innate immunity. Effector T cells provide second line of defense aimed to pathogen eradication. Furthermore, memory T cells provide long-term memory ensuring robust immune response, which counteract subsequent encounters with the same pathogen.

In the GIT, **T cells** are found within the epithelial layer, scattered throughout the lamina propria and submucosa. **Intraepithelial lymphocytes (IELs)** are subset of T lymphocytes residing between IECs in the intestinal epithelium with limited antigen receptor diversity. In human, most of IELs are $CD8^+$ T cells. The IELs can eliminate infected or damaged (stressed) epithelial cells and they can also produce pro-inflammatory cytokines. Thus, IELs play important function in intestinal epithelium defense and homeostasis (CHEROUTRE 2004).

There are different subsets of effector **$CD4^+$ T cells** in lamina propria (e.g. Th1, Th2, Th17). They are induced by and protect against different type of microbes. Th1 cells are involved in the activation of intracellular killing by macrophages via IFN-γ and TNF-α and thus are essential to control virus and intracellular bacterial infections. They are relatively sparse in healthy lamina propria in comparison with Th2 or Th17. Th2 cells are important in eradication of parasitic helminthes. Via production of IL-4, IL-5 and IL-13 they mediate enhancement of fluid and mucus production, smooth muscle contraction and bowel motility, recruitment of eosinophils to lamina propria, induction of B cell class switching to IgE and thereby priming basophils and mast cells for release their granules. The Th17 are essential in orchestrating of clearance of extracellular bacteria and fungi. In intestine, Th17 cell have important role in maintaining of mucosal epithelial barrier integrity. Via production of IL-17 and IL-22 they stimulate production of mucins and β-defensins (SHALE *et al.* 2013; MAYNARD and WEAVER 2015; STŘÍŽ and HOLÁŇ 2015).

Thus, different effector $CD4^+$ T cells ensure defense against different type of microbial invasion. The immune responses mediated by these cells need to be controlled in order to

reestablish and to maintain homeostasis upon eradication of pathogens. In gut are abundant **regulatory T cells**, they are responsible for ensuring of the intestinal homeostasis via prevention of inflammatory response against intestinal commensal microbiota and orally ingested food antigens (oral tolerance). The most relevant types of regulatory T cells in the intestine are inducible and thymus-derived $Foxp3^+$ regulatory T cells, type 1 regulatory (Tr1) cells, iTreg35 and regulatory Th17 cells (GAGLIANI *et al.* 2015). Gut mucosal DCs have been shown to have important role in the induction of Treg cells via production of TGF-β and retinoic acid (COMMINS 2015). Treg cells control immune response and ensure oral tolerance by several mechanisms: production of inhibitory cytokines IL-10, TGF-β, high level of IL-2 consumption from microenvironment, and by binding of B7 molecules on antigen presenting cells via CTLA-4 on Tregs. The latter mechanisms then lead to competitive inhibition B7-CD28 mediated costimulation and ultimately to T cell anergy (BOLLRATH and POWRIE 2013; GAGLIANI *et al.* 2015). The failure of regulation of the mucosal immune system may result in the unwanted mucosal inflammation or allergic response (MONTILLA *et al.* 2004; SHI and WALKER 2015; STAGG 2018).

Thus, the $CD4^+$ T cells in lamina propria represent heterogeneous population of effector, effector memory and regulatory T cells, which also act to provide help to B cell in IgA production to maintain tolerance and homeostasis and to protect the mucosa from antigen invasion (WERSHIL and FURUTA 2008).

The humoral immunity in gastrointestinal tract is dominantly ensured by **secretory IgA (SIgA)** produced by plasma cells. The abundance of intestinal IgA-producing plasma cells is due to selective induction of IgA class switch in GALT and mesenteric lymph nodes. SIgA is secreted as polymeric IgA (dimer connected via J chain) by plasma cells in lamina propria, transported by poly-Ig receptor (poly-IgR) through gut epithelium into the mucus layer (biochemical barrier). The released SIgA is enriched by secretory component, which arises by proteolytic cleavage of poly-IgR and protects the SIgA against enzymatic digestion in gut lumen. SIgA can bind microbes in antigen-specific and non-specific manner by Fab or by glycans, respectively (MESTECKY 2001; MATHIAS and CORTHÉSY 2011). The mechanism of SIgA action is mainly the neutralization of microbes and toxins, in processed called immune exclusion. It also facilitates the antigen uptake from gut lumen. In infants, the intake of SIgA in human milk represents a great benefit for the gut barrier defense. SIgA is secreted into the human milk by plasma cells adjacent to the mammary gland epithelium. The origin of these cells is in the GALT. This explains the

specificity of these antibodies to the intestinal microbiota antigens and points out the importance of common mucosal immune system (ROUX *et al.* 1977; WEAVER *et al.* 1998; ELLA *et al.* 2011; BRANDTZAEG 2013).

However, most of the IgA is present at mucosal surfaces (SIgA), the IgA is also the second most abundant immunoglobulin isotype in circulation (WINES and HOGARTH 2006). As opposed to the role of SIgA in immune exclusion, systematic IgA may provide powerful opsonisation, phagocytosis, respiratory burst, degranulation and cytokine and chemokine production via FcαRI. These findings suggest the presence of common IgA compartment through body, where by their functions both, IgA and SIgA protect against microbes (REINHOLDT and HUSBY 2004; HANSEN *et al.* 2019).

All mentioned gut barrier components create in its mature form a functional protective complex.

4. Necrotizing enterocolitis

Necrotizing enterocolitis is one of the most severe acute gastrointestinal diseases affecting mainly preterm newborns. NEC occurs in 1-3 per 1000 live births, and the surgical treatment is necessary in 20-40% cases. The mortality rate is up to 50% (HOLMAN *et al.* 1989; YEE *et al.* 2012). For NEC is typical rapid onset and progression with devastating consequences. The early signs are non-specific and may delay the NEC treatment by misdiagnosing it as neonatal sepsis. The specific signs for NEC, such as *pneumatosis intestinalis* or gas in the portal vein, appear rather later in the course of the disease and their absence must be interpreted with extremely caution (SHANBHOGUE *et al.* 1991; COUFAL *et al.* 2020). Therefore, NEC is one of common cause of surgery intervention and it is also one of the leading causes of morbidity and mortality in neonatal intensive care units (GEPHART *et al.* 2012).

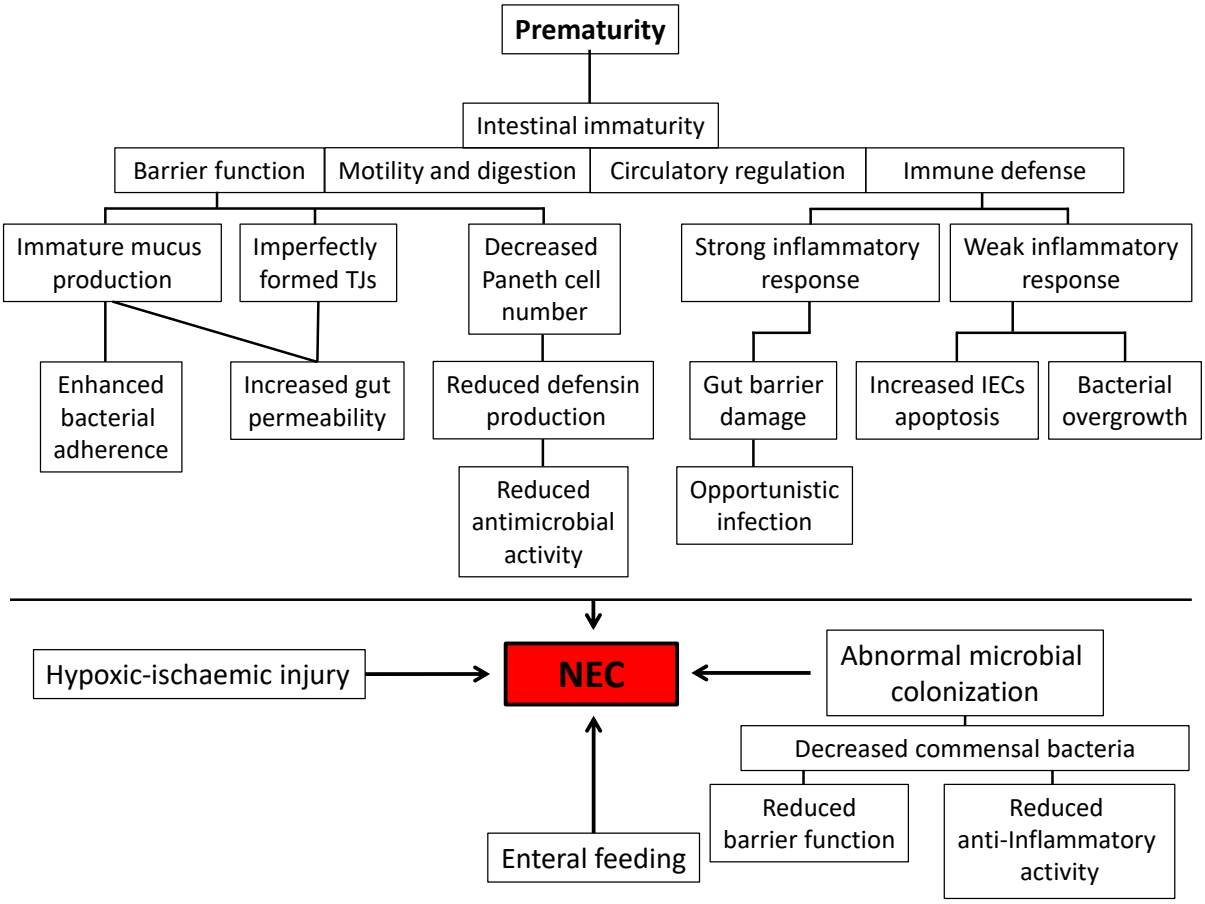
During NEC occurs coagulative necrosis in *tunica mucosa* and *tunica submucosa* of the gut wall with the risk of gut perforation. The most often affected areas are the distal part of small intestine (terminal ileum) and proximal part of the large intestine (*caecum*, *colon ascendens*). In the most severe form, called NEC totalis, is affected the entire gut and the mortality could reach 100% (BALLANCE *et al.* 1990; SHO *et al.* 2014). Macroscopically, the gut appears to be irregularly enlarged with a thinned wall and with a dark-red to black coloration in areas affected by necrosis. In *tunica subserosa* can be gas-filled deposits

called *pneumatosis intestinalis*, which is one of the main features of NEC diagnosis in X-ray or ultrasound (MUCHANTEF *et al.* 2013). These gas deposits are formed by fermentation of undigested sugars by bacteria that penetrated the intestinal wall through the damaged gut barrier (KLIEGMAN and FANAROFF 1984). The most serious complication is perforation of the necrotic gut and subsequent inflammation of the peritoneum caused by the gut content, so called stercoral peritonitis (PEREL *et al.* 1988; BALLANCE *et al.* 1990).

The main histopathological signs are: leukocytes infiltration in the *tunica mucosa* and *submucosa*, mucosal edema and coagulation necrosis, which in later stages affects the entire intestinal wall. Initial mucosal lesions lead to enlargement of villi and in the advanced stage to separation of the epithelial layer and destruction of the villi (KOSLOSKE *et al.* 1980; KLIEGMAN and FANAROFF 1984; BALLANCE *et al.* 1990).

4.1 Pathogenesis and risk factors

The pathogenesis of NEC remains elusive and is likely multifactorial. The main risk factors are immaturity of gut barrier and immune system together with enteral feeding and abnormal bacterial colonization (Schema 1). Premature neonates (born before 37th week of gravidity; most frequently between 24th – 37th weeks) represent main risk group (90%). These neonates develop NEC mostly between 14th to 21st day after birth, in the majority of cases after the initiation of enteral feeding. According to the birth weight are at the most risk of NEC development neonates with extremely low birth weight (under 1000 g) or neonates with very low birth weight (under 1500 g) (STOLL *et al.* 2004; LIN and STOLL 2006; YEE *et al.* 2012). The term neonates represent 10% of all NEC cases. Term neonates develop NEC earlier, within the first week after birth (OSTLIE *et al.* 2003; LIN and STOLL 2006; YEE *et al.* 2012). At high risk are also neonates with congenital developmental disorder of heart and GIT (MCELHINNEY *et al.* 2000; OSTLIE *et al.* 2003; ERDOĞAN *et al.* 2012), intrauterine growth retardation (KARAGIANNI *et al.* 2010), respiratory stress or perinatal asphyxia (WILSON *et al.* 1983).



Schema 1: Overview of NEC risk factors (adapted from Lin and Stoll 2006; Lin et al. 2008).

4.2 Gut barrier in NEC

The development of gut microbiota in preterm neonates is complicated by their critical condition and by related complications requiring long term hospitalization in neonatal intensive care units. The microbiota development is thus influenced by hospital environment, antibiotic therapy, necessity of parenteral nutrition and formula feeding instead of human milk usage. These circumstances lead to abnormal composition of gut microbiota (intraluminal dysbiosis between commensal and potentially pathogenic bacteria) in the early stage of life, when the gut microbiota is developing (Figure 3) (ELGIN *et al.* 2016; WARNER *et al.* 2016).

The study of bacterial isolates from blood and stool revealed the possible connection of NEC and the presence bacteria like *Escherichia coli*, *Klebsiella* species, *Clostridia* species, *Staphylococcus* species and *Enterobacter* species (DE LA COCHETIERE *et al.* 2004; STEWART *et al.* 2012, 2013; BIZZARRO *et al.* 2014). The current studies using next-generation sequencing indicate that the NEC is not caused by one single bacterium, but

rather by whole complex of changes in the gut microbiota composition (ELGIN *et al.* 2016). To abnormal gut microbiota composition may also contribute other signs of the prematurity: low production or activity of proteolytic enzymes (e.g. pepsin, trypsin), low gastric acidity and imperfect gut peristalsis.

However, the microbes have important role in the NEC pathogenesis, they can act also protective. There are studies showing the beneficial role of probiotics in the prevention of NEC in preterm infants (DESHPANDE *et al.* 2007; LIN *et al.* 2008a; ALFALEH and ANABREES 2014; NEVORAL 2015; OLSEN *et al.* 2016). In spite of these successes, there is still certain risk in the administration of probiotics to preterm newborns, because probiotics are live bacteria and the premature individuals have reduced defense mechanisms. Therefore, there is possibility of risk of sepsis development via probiotics administration in premature neonates (OHISHI *et al.* 2010; BERTELLI *et al.* 2015; DANI *et al.* 2016).

Although the anatomical differentiation of the human fetus intestine is almost complete at 20th week of gestation and all main components of gut mucosal immune system are established by the 29th week of gestation, the final steps in gut barrier development are performed during perinatal period. This process is strongly influenced by organism maturation during late pregnancy and then by interactions with microbial components and breast feeding (ROUWET *et al.* 2002; FOXX-ORENSTEIN and CHEY 2012).

Thus, the preterm neonates do not have fully developed all components of gut barrier complex, predisposing it for increase permeability and more susceptible to the damage by strong burden of microbial and food antigens. Moreover, the long term hospitalization in neonate intensive care units, administration of antibiotics, formula feeding or delayed enteral feeding interfere with proper development and maturing of gut barrier and with proper adjustment of function and regulation of neonate's immune system. These events also support the abnormal development of gut microbiota composition in the critical period of development of the individual as was described above.

The immature mucus layer (insufficient production or composition) can lead to the inadequate protection of gut epithelium, bacterial adhesion and possible damage of gut barrier by pathogenic or non-pathogenic stimuli. Although there is a little known about the maturation of TJs in human fetus, there was reported that premature individuals have increased intestinal permeability compared to full term neonates (ROUWET *et al.* 2002). Disruption or alteration of TJ complexes leading to higher gut barrier permeability can be

also caused by pathogenic bacteria, which were described in association with the NEC pathogenesis (e.g. *Clostridium difficile, Clostridium perfringens, Escherichia coli)* (HECHT *et al.* 1988, 1992; SONODA *et al.* 1999; SIMONOVIC *et al.* 2000; NUSRAT *et al.* 2001; MIRSEPASI-LAURIDSEN *et al.* 2016). There was also reported increased intestinal epithelial tight junction permeability caused by tumor necrosis factor-α (TNF-α) *in vitro* (MA *et al.* 2004). Together with platelet activating factor (PAF) was TNF-α described as important mediator in the inflammatory cascade leading to intestinal epithelium damage in the pathogenesis of NEC (CAPLAN *et al.* 1990; PENDER *et al.* 2003; TRAVADI *et al.* 2006).

Paneth cells appear around 13th week of gestation and mature further from 22th - 24th week of gestation, at the same time the number of PCs starts increasing. The increase in PCs further continues to the adulthood (together with intestinal growth). There was reported decrease production of α-defensins in premature neonates in comparison with term neonates. In intestinal samples taken during the surgery for NEC was found increase number of Paneth cells as well as α-defensins expression but no increase was detected at the protein level (SALZMAN *et al.* 1998). Other studies reported decreased number of Paneth cells in intestinal samples from NEC neonates in comparison with neonates suffering from intestinal atresia or spontaneous intestinal perforation (COUTINHO *et al.* 1998; ZHANG *et al.* 2012). The deficiency or developmental defect associated with the function of Paneth cells thus can make immature intestine more vulnerable. The insufficient production of antimicrobial molecules leads to microbial overgrowth, higher bacterial adherence and greater burden of immature gut barrier.

Another limitation of innate immunity in preterm neonates is deficiency in complement system (low amount of complement factors in serum) and lower bactericidal activity of leukocytes (McCracken and Eichenwald 1971; Wright *et al.* 1975).

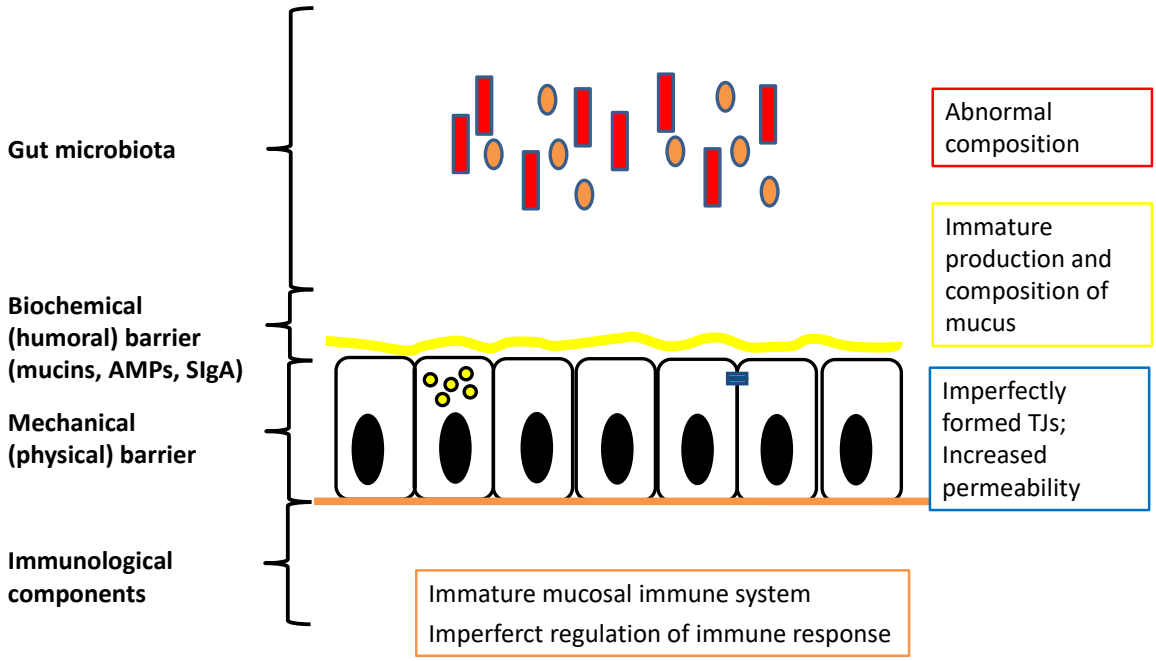


Figure 3: Gut barrier in NEC (AMPs – antimicrobial peptides, SIgA – secretory IgA, TJs – tight junctions) (adapted from Coufal et al. 2016a).

Human milk, thanks to its unique composition, including SIgA, growth factors, hormones, enzymes, transporters and cytokines, contributes to the maturation of neural tissue, gastrointestinal tract and immune system. In comparison with formula, human milk does not burden the mucosal surface of the gut and via growth factors helps to repair eventual disruption of the gut barrier. For these reasons is the human milk in conservative feeding practice described as one of the few options in the prevention of NEC (Moran *et al.* 1983; Saito *et al.* 1993; Moya *et al.* 1994; Schanler *et al.* 1999; Kverka *et al.* 2007; Groer *et al.* 2014).

4.3 Inflammation

There are currently two models describing the development of NEC. In both models is the immature gut barrier burdened by intraluminal bacterial dysbiosis. The "Top down" model describes the beginning of the NEC in the area of the tips of intestinal villi. The second "Bottom up" model takes into account described insufficiency of PCs in premature neonates and neonates suffering from NEC and describes the gut barrier disruption in the crypts of Lieberkühn (Figure 4).

In both models, excessive burden of immature gut barrier by intraluminal bacterial dysbiosis leads to stimulation of PRRs and to activation of NF-κB signaling pathway and production of pro-inflammatory cytokines (e.g. IL-1β, IL-6, IL-12, TNF-α) and chemokines (e.g. IL-8, CXCL-2), which then stimulate the inflammatory response and recruitment of leukocytes (STEFANUTTI *et al.* 2005; LE MANDAT SCHULTZ *et al.* 2007; HUNTER and DE PLAEN 2014).

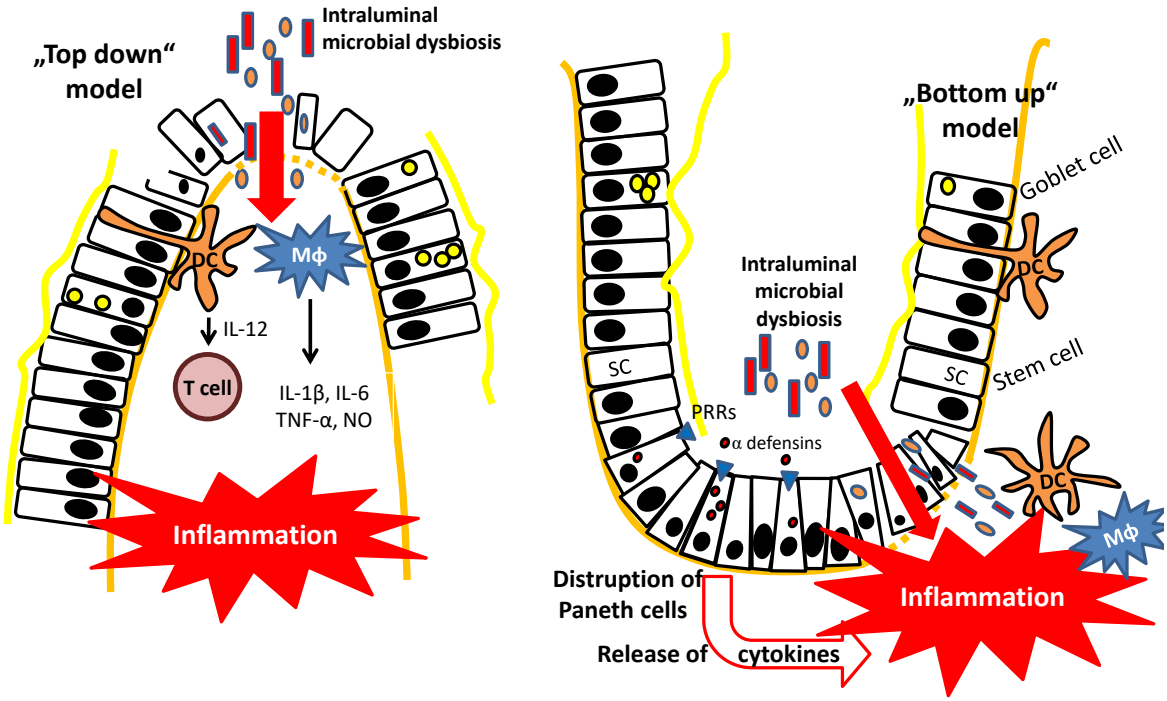


Figure 4: Models of NEC pathogenesis (DC - dendritic cell, MΦ – macrophage, PRRs – pattern recognition receptors, NO – nitric oxide (adapted from McElroy et al. 2014; Coufal et al. 2016a).

Further, burdening of immature gut barrier by intraluminal bacterial dysbiosis may lead to excessive apoptosis or necrosis of gut epithelial cells, and to gut barrier failure allowing the bacterial translocation, which in turn amplifies the inflammatory response and finally leads to the intestinal wall destruction and NEC. The activation of immature or inappropriately regulated immune system lead to strong inflammatory response, which further damage the gut barrier (MCELROY *et al.* 2014; AFRAZI *et al.* 2014).

4.4 Diagnostics of NEC

The first diagnostic and clinical staging criteria were established in 1978 by Bell et al. and were further modified in 1986 by Walsh and Kliegman (BELL *et al.* 1978; WALSH and KLIEGMAN 1986).

The diagnosis of NEC is based on the presence combination of clinical symptoms, (i.e. abdominal distension and blood in stool), radiologic or sonographic findings of *pneumatosis intestinalis* or gas in portal vein (WALSH and KLIEGMAN 1986). For NEC is characteristic unexpected onset and rapid progression with the risk of gut perforation and the infant's death. The clinical signs are in the early stage non-specific and thus easily interchangeable with neonatal sepsis or other medical emergency, which may cause the delay in the NEC treatment. The specific signs, such as *pneumatosis intestinalis* or gas in the portal vein, appear rather later in the disease course and their absence must be interpreted with extremely caution (SHANBHOGUE *et al.* 1991; TAM *et al.* 2002; COUFAL *et al.* 2020).

Laboratory examination

The complete blood cell count with differential shows that white blood count may be normal but is frequently either elevated with an increased left shift or low (leukopenia). The thrombocytopenia is often present. The coagulopathy screening may show prolonged prothrombin time, partial thromboplastin time, decreased fibrinogen and increased fibrin spilt products most likely indicating that disseminated intravascular coagulation take place. It was shown that infants with proven or severe NEC have high levels of CRP. Lasting high levels of CRP during the therapy indicate the development of complication. However, there is not possible to distinguish the CRP elevation caused due to NEC or due to sepsis. Moreover, CRP may not be elevated initially or in cases of severe NEC, because an infant

may be unable to produce an effective inflammatory response. The electrolytes imbalances such as hyponatremia and hypernatremia as well as hyperkalemia are also common (GOMELLA *et al.* 2009).

Early diagnosis of NEC allows more efficient intervention, consisting of cessation of enteral feeding, administration of broad-spectrum antibiotics, and supportive care, which has major impact on the disease prognosis (BELL *et al.* 1978; RICKETTS 1984; LIN and STOLL 2006; ELTAYEB *et al.* 2010). Therefore, there is strong need for identification of new biomarkers, suitable for early diagnosis of NEC, which would give the opportunity for early and proper intervention without necessity of surgery, as in the case of late diagnosed NEC.

5. Inflammatory bowel disease (IBD)

Inflammatory bowel disease is a collection of chronic, immune-mediated inflammatory disorders of the gastrointestinal tract that are usually classified in two major, relapsing conditions – ulcerative colitis (UC) and Crohn's disease (CD).

The incidence of UC in North America is 8.8 - 23.1 per 100 000 persons-years and 6.3 – 23.8 cases of CD per 100 000 person-years. Incidence in Northern Europe is 1.7 - 57.9 cases of UC per 100 000 person-years and 0 – 11.4 cases of CD per 100 000 person-years. The highest counts of patients are in North America and Europe. The incidence of both diseases is increasing also in previously low-incidence countries in Southern Europe, Asia and also in newly industrialized countries whose society has become more westernized (NG *et al.* 2017). The pediatric IBD patients represent 7-20% of all IBD cases. Among children is more prevalent CD than UC, although the UC is more prevalent than CD in the whole population (KELSEN and BALDASSANO 2008).

The UC and CD differ in anatomic localization, intensity and range of gut mucosa damage. Whereas CD can affect any part of the small and large bowel (most commonly affects the terminal ileum or perianal region), the UC is limited to the large bowel, beginning in the rectum, spreading proximally and frequently involves the periappendicular region. Histologically, is UC associated with superficial inflammatory changes restricted to the mucosa and submucosa, with inflammatory infiltrates composed by lymphocytes, plasma

cells and polymorphonuclear leukocytes, there is depletion of goblet cells, architectural crypt distortion and ulceration. The histological examination in CD reveals the thickened submucosa and transmural inflammation (LODDENKEMPER 2009; KHOR *et al.* 2011). The signs and symptoms of UC and CD depend on the location and severity of the disease including abdominal pain, diarrhea, rectal bleeding and weight loss. The onset of symptoms is gradual, typically followed by periods of remission and subsequent relapse (VERMEIRE *et al.* 2012; FUSS and STROBER 2015). Up to 50% of IBD patients have also extraintestinal manifestation (e.g. musculoskeletal, mucocutaneous, eye, hepatobiliary, pancreatic, cardiac and neurological manifestation). The presence of extraintestinal manifestation is associated with severe course of the disease and worse prognosis. The extraintestinal manifestation can be present also several years before the diagnosis of IBD (FARMER *et al.* 1993; LEVINE and BURAKOFF 2011). The patients suffering from IBD can also develop severe complication during the disease course including toxic colitis, fistulas, abdominal abscesses, malignancy or pouchitis (MARRERO *et al.* 2008).

The disease course can be positively modulated thanks to well-established headlines in IBD therapy. However the discontinuation of pharmacological intervention due to the inefficiency or adverse events is still common. The prediction of the disease relapse and complications or suggestion of the ideal therapy for a particular patient would be of great value in IBD diagnostics (STEIN and HANAUER 2000; ROTHFUSS *et al.* 2006; COUFAL *et al.* 2019).

5.1 Pathogenesis and risk factors

The pathogenesis of IBD is complex and multifactorial. Three major mechanisms are involved in IBD pathogenesis, gut microbiota dysbiosis, aberrant immune response and gut barrier failure. In recent years genetic, environmental and immunological factors were identified that have effect on the development and maintenance of chronic inflammation in IBD.

5.1.1 Genetic susceptibility

Among complex diseases, genome-wide association studies (GWAS) identified 163 IBD disease loci. Of the 163 risk loci, 110 genetic risk loci were associated with both UC and CD, whereas 23 are specific for UC and 30 are specific for CD. The overlaps in genetic risk loci represent similarities in the basis of both diseases, whereas the presence of specific risk loci indicates, that despite common genetic traits are the UC and CD

genetically distinguishable and unique units (FUSS and STROBER 2015). In the complexity of disease mechanisms, a susceptibility allele often need also other genetic and non-genetic incentive to manifest the disease (KHOR *et al.* 2011; JOSTINS *et al.* 2012; FUSS and STROBER 2015).

The GWAS revealed the risk loci associated with activation and regulation of innate and adaptive immune system, mucosal immune response, epithelial barrier function, HLA abnormalities and cell survival transcription factors (Table 1) (KHOR *et al.* 2011; ABEGUNDE *et al.* 2016).

UC loci	CD loci	IBD shared loci
Immune cell activation		
TNFRSF9 *TNFRSF14* *IRF5* *FCGR2A* *IL7R* *IL8RA/IL8RB* *IFN/IL-26/IL-22* *LSP1*	*CCR6* *CCL2/CCL7* *PTPN22* *IL18RAP* *IL27TNFSF11* *BACH2* *TGAP* *VAMP3*	*SMAD3* *IL1R2* *ICOSLG* *PTPN2* *PRDM1* *TNFSF15* *IL10* *CARD9* *IL12B* *TYK2* *STAT3* *IL23R* *PTGER4*
Epithelial barrier function		
HNF4A *LAMB1* *CDH1* *GNA12* *ECM1*	*MUC1/SCAMP3*	*NKX2-3*
HLA abnormality		
DR2	*DR7*	*DRB03*
Macrophage/Dendritic cell activation		
	LRRK2 *IRGM* *NOD2* *ATG16L1*	
Cell survival transcription factors		
OTUD3/PLA2GE *DAP* *PIM3* *CAPN10*	*THADA* *ZPF36L1* *GCKR* *ZMIZ1* *PRDX5* *SP140* *CPEB4* *FADS1* *DENNDIB*	*CREM* *ZNF365* *RTEL1* *ORMDL3* *XBP1*

	IBD5 *DNNT3A*	

Table 1: The risk loci associated with IBD revealed by genome-wide association studies (adapted from Lees et al. 2011; Fuss and Strober 2015).

5.1.2 Environmental factors

Genetic susceptibility does not completely explain the variance in disease incidence, indicating the importance of non-genetic factors in the development of IBD. Environmental factors including smoking, drug usage (e.g. antibiotics, non-steroidal anti-inflammatory drugs), stress and diet have been investigated in IBD pathogenesis (ABEGUNDE *et al.* 2016).

It was shown that cigarette smoking increases the risk for the CD development, whereas the cessation of smoking was associated with reduction of the risk (HIGUCHI *et al.* 2012). Smoking is associated with higher relapse rate in CD patients and also increases the risk of penetrating intestinal complications, fistulae and need for surgical interventions. On contrary to CD, there was reported beneficial and protective effect of smoking in UC (JOHNSON *et al.* 2005; MAHID *et al.* 2006; HIGUCHI *et al.* 2012). Another risk factor with similar effect on the IBD development is surgical intervention called appendectomy (ANDERSSON *et al.* 2003). There was reported a relation between previous usage of antibiotic therapy or long term non-steroidal anti-inflammatory drug therapy and the development of CD and UC (CHAN *et al.* 2011; ANANTHAKRISHNAN *et al.* 2012; ANIWAN *et al.* 2018).

The increased risk for chronic illnesses is thought to be associated with the shift to a high-fat and high-sugar diet in Western countries. It was shown, that high intake of saturated fats, total polyunsaturated fatty acids (PUFA), omega-6 fatty acids and meat (high amount of animal protein) can increase the risk for the development of IBD. The diet significantly influences the composition and function of gut microbiota, which can lead to microbial dysbiosis and to perturbation in the immune system homeostasis (DEVKOTA *et al.* 2012; ANNAHÁZI and MOLNAR 2014).

5.2 Gut barrier in IBD

Both, animal studies and clinical observation confirmed the role of gut microbiota and its dysbiosis in the IBD pathogenesis. The studies revealed the reduction in diversity, decrease in abundance of bacterial taxa within *Firmicutes* and *Bacteroides* and increase in *Gammaproteobacteria*. Further studies found increase of *Enterobacteriaceae*, *Veillonellaceae* and *Fusobacteriaceae* together with decrease of *Bacteroidales*, *Clostridiales* and *Erysipelotrichales* in IBD patients (FRANK *et al.* 2007, 2011; SEPEHRI *et al.* 2007; MORGAN *et al.* 2012; GEVERS *et al.* 2014). In CD patients was found adherent-invasive *E.coli* (BOUDEAU *et al.* 1999; DARFEUILLE-MICHAUD 2002), while diffusely adherent *E.coli* was found in patients with UC (BURKE and AXON 1987; SOKOL *et al.* 2006; MIRSEPASI-LAURIDSEN *et al.* 2019). It was shown that UC associated *E. coli* P19A produces α-hemolysin that cause rapid loss of tight junction integrity (MIRSEPASI-LAURIDSEN *et al.* 2016). Furthermore, high abundance of adherent-invasive *Fusobacterium* species was found in colonic mucosa of UC patients (OHKUSA *et al.* 2002, 2009). It was shown, that the invasive potential of *Fusobacterium nucleatum* positively correlates with IBD status of the host (STRAUSS *et al.* 2011). In IBD patients was also found high abundance of sulfate-reducing bacteria showing its possible role in the IBD pathogenesis (GIBSON *et al.* 1991; ZUO and NG 2018).

While some bacteria are associated with the IBD development, other may have a role in protection against it. It was shown that *Faecalibacterium prausnitzii* can protect against mucosal inflammation via several mechanisms including the stimulation of IL-10 production (SOKOL *et al.* 2008). *F. prausnitzii* was found significantly decreased in CD patients and its presence decreased the risk of IBD relapse after surgery (SOKOL *et al.* 2008, 2009; WILLING *et al.* 2009). The restoration of *F. prausnitzii* abundance in UC patients was associated with maintenance of clinical remission (VARELA *et al.* 2013). Next to *F. prausnitzii*, there was reported also decrease in other bacteria producing the short-chain fatty acids (SCFAs) (e.g. *Bifidobacterium* species, *Phascolarctobacterium*, and *Roseburia*) in IBD patients. SCFAs including acetate, propionate and butyrate are important source of energy for colonic epithelial cells and they can also induce expansion of colonic Treg cells (AHMAD *et al.* 2000; GUEIMONDE *et al.* 2007; MORGAN *et al.* 2012; ATARASHI *et al.* 2013; SMITH *et al.* 2013).

The gut barrier failure is characteristic feature in IBD. The perturbation can affect different levels of the gut barrier complex, including defective mucus layer, alteration in TJs organization, abnormality in PRRs, decreased antimicrobial peptides (AMPs) production and abnormality in autophagy (Figure 5). These defects increase gut barrier permeability allowing the excessive contact of the luminal antigens with the immune cells, resulting in chronic intestinal inflammation. It is also responsible for many IBD symptoms even during later phase of mucosal healing (ZEISSIG *et al.* 2007; TLASKALOVÁ-HOGENOVÁ *et al.* 2011; JOHANSSON *et al.* 2014; CHANG *et al.* 2017; COUFAL *et al.* 2019).

The failure of gut barrier can result from a variety of mechanisms.

UC patients have thinner and less continuous mucus layer with altered mucin glycosylation and sulfation, where the glycans are shorter with less complex structure. Since the sulfation enhancing the resistance of the glycans against the enzymatic degradation, reduction in sulfation can cause vulnerability of mucus layer to enzymatic degradation by bacteria (CLAMP *et al.* 1981; RAOUF *et al.* 1992; PULLAN *et al.* 1994; CORFIELD *et al.* 1996; LARSSON *et al.* 2011). These abnormalities can result from an underlying genetic abnormality involving the mucus production and goblet cell function. The reduced number of mature goblets cells in active UC patients, can be caused by the defects in goblet cell differentiation (GERSEMANN *et al.* 2009), or alternatively as a result of inflammation (STROBER *et al.* 2002; ANTONI *et al.* 2014; JOHANSSON *et al.* 2014).

Increased intestinal permeability in IBD patients can also occur as a result of various abnormalities in cell junctions of gut epithelial cells. The TJs in IBD patients exhibit reduced complexity characterized by decreased expression of sealing claudins (claudin-5 and -8) and occludins and increased expression of pore-forming claudin-2 (SCHULZKE *et al.* 2009; ODENWALD and TURNER 2013). These abnormalities can be caused by inflammatory cytokines produced by underlying inflammatory cells, which drive the inflammation in UC (IL-9, IL-13) and CD (TNF-α, IFN-γ), at the level of expression or by the reorganization of TJs components (ZEISSIG *et al.* 2004; BRUEWER *et al.* 2005, 2006; WEBER *et al.* 2010; PARKER *et al.* 2013). Moreover, the pro-inflammatory cytokines can also increase intestinal barrier permeability via induction of gut epithelial cells apoptosis (DI SABATINO *et al.* 2003; HELLER *et al.* 2005; SIPOS *et al.* 2005).

Next, there are abnormalities in PRRs and antimicrobial peptides in IBD patients leading to decreased defense of gut barrier. In CD patients were found genetic abnormalities in the nucleotide-binding oligomerization domain 2/caspase recruitment domain-containing protein 15 gene (*NOD2/CARD15*), which are risk factors for the development of CD (HUGOT *et al.* 2001; OGURA *et al.* 2001). NOD2 belongs to the PRRs family called NOD-like receptors (NLRs). NOD2 is an intracellular sensor for muramyl dipeptide and has an important role in innate immunity and in regulation of gut microbiota composition via inducing of AMPs and pro-inflammatory cytokines production (REHMAN *et al.* 2011). It was found, that *Nod2*-deficient mice have reduced levels of α-defensins in Paneth cells (KOBAYASHI *et al.* 2005). The reduced levels of Paneth cells α-defensins was also found in CD patients (WEHKAMP *et al.* 2005). Moreover, in CD patients was found reduced expression of β-defensins and cathelicidin LL-37 (WEHKAMP *et al.* 2003; SCHAUBER *et al.* 2006).

Autophagy is an intracellular degradation process that is important not only during cellular stress caused by starvation, it also participates in the clearance of ingested microbes and in the processing of antigens for presentation to the immune cells. There was found polymorphism in genes *ATG16L1* and *IRGM* leading to defects in autophagy in CD patients (HAMPE *et al.* 2007; PARKES *et al.* 2007; CLEYNEN *et al.* 2013). It was shown, that polymorphism in *ATG16L1* affects also the function and survival of Paneth cells (MATSUZAWA-ISHIMOTO *et al.* 2017). Travassos et al. demonstrated that NOD1 and NOD2 are critical for the autophagy response to invading bacteria by recruiting the autophagy protein ATG16L1 to the plasma membrane at the bacterial entry site (TRAVASSOS *et al.* 2010; ANTONI *et al.* 2014).

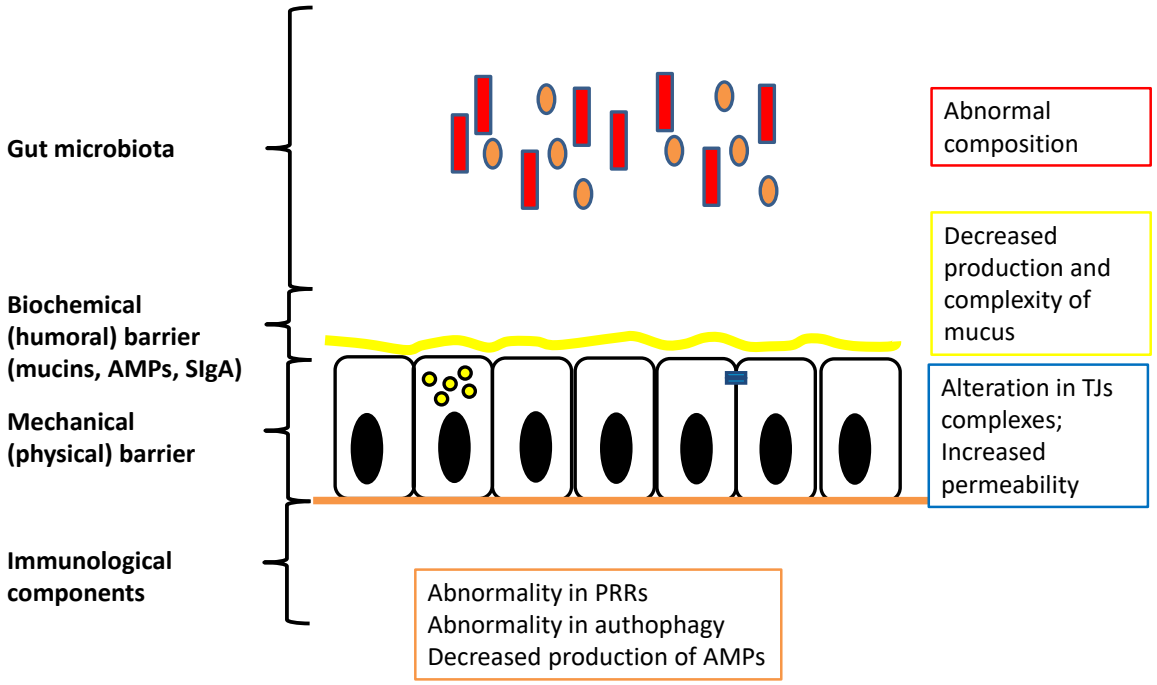


Figure 5: Gut barrier in IBD (AMPs – antimicrobial peptides, SIgA – secretory IgA, PRRs – pattern recognition receptors, TJs – tight junctions) (adapted from Coufal et al. 2016a).

The Th1 inflammation was reported in immunopathogenesis of CD. There was found higher expression of *T-bet and STAT-4* transcriptional factors in lamina propria $CD4^+$ T cells as well as high production of IFN-γ by T cells in CD patients as compared with UC patients or controls (Breese *et al.* 1993; Fuss *et al.* 1996; Parrello *et al.* 2000). In UC has important role Th2 response and NKT cells producing high amounts of IL-13 (Fuss *et al.* 2004; Heller *et al.* 2005; Nemeth *et al.* 2017).

The Th17 cells and its cytokines were also implicated in the pathogenesis of IBD. The GWAS studies found several polymorphisms in genes coding proteins involved in IL-23/Th17 pathway (*IL-23R, IL-12B, STAT-3, JAK-2*) (Strober, Warren; Fuss 2011; Choy *et al.* 2017). Next studies found high levels of Th17 cells together with high levels of IL-17, IL-21 and IL-23 in both UC and CD compared to controls, indicating the role of Th17 cells in the pathogenesis of both diseases (Kobayashi *et al.* 2008; Monteleone *et al.* 2011; Olsen *et al.* 2011; Verdier *et al.* 2012; Neurath 2015).

Except the role of T cells in pathogenesis of IBD, the presence of antimicrobial antibodies in sera of IBD patients suggests a broader spectrum of immune reactions in IBD pathophysiology (ADAMS *et al.* 2008; COUFAL *et al.* 2019).

5.3 Inflammation

However there are differences in pathogenesis between UC and CD, the general principles are similar (Figure 6). Genetic abnormalities resulting in the reduced production of mucus in UC leading to defective, thinned mucus layer allows increased contact of microbes with gut epithelial cells. The abnormalities in defensin production and secretion by Paneth cells adversely affect the innate defense of gut barrier in CD patients. In UC glycolipids presented by intestinal epithelial cells lead to the stimulation of NKT cells. NKT cells can cause damage of gut barrier via direct cytotoxicity or via production of high amounts of IL-13 leading to epithelial cell apoptosis. The released pro-inflammatory cytokines can cause the alteration of the TJs. Defects in bacterial recognition and autophagy impair antimicrobial defense and can also lead to the alteration of the microbiota composition. Thus, the failure of gut barrier allows excessive contact of the luminal microbes with the host's immune cells resulting in inflammation. This results in vicious cycle, where the gut barrier damage allows further entry of luminal antigens, which in turn promotes the inflammatory response leading to further damage of gut barrier (FASANO and SHEA-DONOHUE 2005; MATRICON *et al.* 2010; ANDERSON *et al.* 2012; FUSS and STROBER 2015; WEHKAMP *et al.* 2016; AHLUWALIA *et al.* 2018).

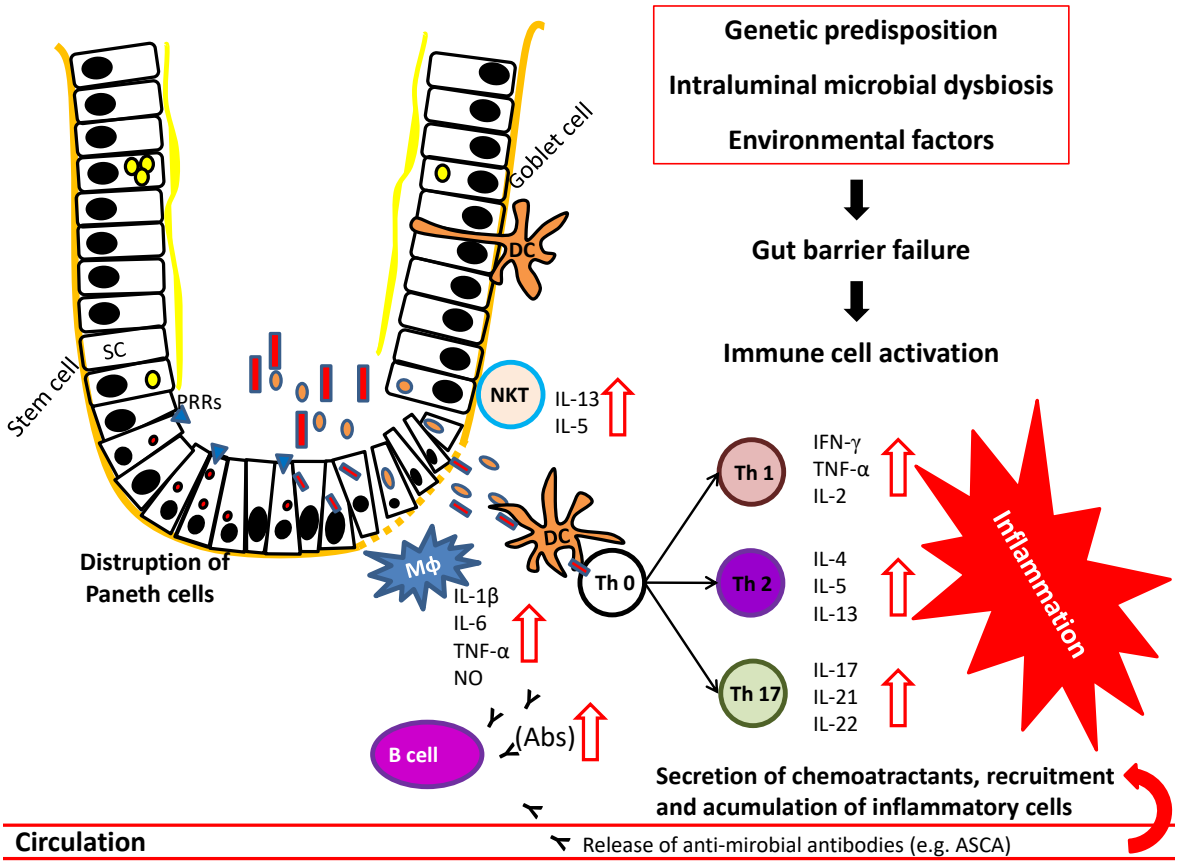


Figure 6: Model of IBD pathogenesis (MΦ – macrophage, DC – dendritic cell, NKT – Natural killer T cell, PRRs – pattern recognition receptors, Abs – antibodies, ASCA – anti-Saccharomyces cerevisiae antibody) (adapted from AHLUWALIA *et al. 2018).*

5.4 Diagnostics

The diagnosis of IBD and its clinical staging is made on the basis of patient's history and medical examination, where the endoscopy plays an important part. The addition of the ultrasonography and magnetic resonance enterography of the small intestine complete the initial diagnostic evaluation for the extent of the disease. Histological examination offers additional information, where the architectonic abnormalities help to confirm the diagnosis. The laboratory tests include examination of a complete blood count, inflammatory markers (e.g. CRP, fecal calprotectin, S100A12), tests of renal and hepatic functions (WEHKAMP *et al.* 2016).

Increasing efforts are being made to discover new biomarkers that can help to discriminate between the types of IBD, help in differential diagnosis, planning of treatment and predict

response to therapy and outcome. To date, no single biomarker has been proven to possess all of the desired qualities (VIENNOIS *et al.* 2015).

6. New biomarkers

Although numerous biomarkers have been investigated and some currently used in clinical practice, none of them is an ideal tool. New biomarkers are being investigated for diagnostics and prediction of the course of NEC and IBD, such as inflammatory mediators and gut-associated biomarkers in blood, urine and stool. The best biomarker should reflect the major steps in early disease pathogenesis, should be disease specific and able to identify individuals at risk for the disease development. Moreover, it should be able to monitor the effects of treatment and last but not least it should be easy to measure and cheap (VIENNOIS *et al.* 2015; CALIFF 2018).

NEC is characterized by gut barrier damage, destruction of mucosal layer and transmural necrosis of the intestinal wall (BALLANCE *et al.* 1990). During the enterocyte damage, the cytokeratin-18 (CK18), an intracellular protein of epithelial cells, is released into the circulation. If the epithelial cells died by apoptosis, higher proportion of the caspase cleaved form of CK18 (ccCK18) could be found in the circulation (LEERS *et al.* 1999). In animal model of NEC, the excessive apoptosis of gut epithelial cells was suggested as a major form of cells death before necrosis (JILLING *et al.* 2004). Therefore ccCK18 is an interesting marker for early NEC diagnosis.

Fatty acid-bindings proteins are small (14-15 kDa), tissue specific cytoplasmic proteins involved in the fatty acid metabolism. The intestinal fatty acid-binding protein (I-FABP) is specifically expressed in enterocytes where it constitutes up to 2% of cytoplasmic protein content. The liver fatty acid-binding protein (L-FABP) is produced in similar pattern of tissue distribution along the duodenal-colonal axis, with the higher tissue content than I-FABP (PELSERS *et al.* 2003). During the damage of enterocyte are both, I-FABP and L-FABP, released into the circulation and thus they can be used as markers of gut epithelium damage (GOLLIN and MARKS 1993; GUTHMANN *et al.* 2002; PELSERS *et al.* 2003). Both I-FABP and L-FABP are elevated in patients with NEC, but could be elevated also in patients with sepsis and otherwise healthy people after abdominal surgery or trauma

(THUIJLS *et al.* 2011; MACHADO *et al.* 2012; BINGOLD *et al.* 2015; CHENG *et al.* 2015; KOKESOVA *et al.* 2019).

The Trefoil factor-3 (TFF-3) is produced in intestinal tract, where it is associated with maintaining of mucosal barrier integrity, promotion of mucosal barrier restitution, cell migration and also with gastrointestinal inflammation (KINOSHITA *et al.* 2000; VEREY *et al.* 2011; GE *et al.* 2015; NAKOV *et al.* 2019). It was shown that patients suffering from severe sepsis or IBD have elevated TFF-3. Further, its level correlates with the activity of ulcerative colitis localized to the colonic mucosa, suggesting the site-specific up-regulation of TFF-3 in inflamed tissue (GRØNBÆK *et al.* 2006; SUN *et al.* 2016). Moreover, it was shown the protective effect of TFF-3 in intestinal tract injury in animal model of NEC via protection against excessive apoptosis (YI *et al.* 2016).

Serum amyloid A (SAA) is an acute-phase protein, which increases in response to infection, inflammation and trauma. It was suggested as a more sensitive inflammatory biomarker than CRP in various inflammatory conditions (CHAMBERS *et al.* 1983; MAYER *et al.* 2002; BOZINOVSKI *et al.* 2008). Thus, the SAA was shown to be helpful in diagnostics of acute diseases, including sepsis and NEC (ARNON *et al.* 2007; ÇETINKAYA *et al.* 2009, 2011).

Thanks to the small size, I-FABP, L-FABP, TFF-3 and SAA can pass through the kidney to urine, giving the unique opportunity for non-invasive measurement and constant monitoring without necessity to stress the infants from the repeated blood sampling (COUFAL *et al.* 2020).

Gut barrier failure in IBD leads to strong immune response to gut commensal microbes. Thus, several serological tests aiming at microbial antigens were suggested for the IBD diagnostics, including anti-Saccharomyces cerevisiae antibodies (ASCAs) (ZHOLUDEV *et al.* 2004; ADAMS *et al.* 2008; DUARTE-SILVA *et al.* 2019). Serum antibodies to *E. coli* outer membrane porinC (anti-OmpC), anti-flagellin (anti-Cbir1) and the anti-I2 component of *Pseudomonas fluorescens* (anti-I2) were also suggested as potential biomarkers in IBD diagnostics (SITARAMAN *et al.* 2005; ILTANEN *et al.* 2006). Next to their possible role in diagnostics, this also suggests the importance of antimicrobial immune response in IBD pathogenesis (COUFAL *et al.* 2019). But the utility of these markers in

daily clinical practice is still rather low (PAPP *et al.* 2007; PEYRIN-BIROULET *et al.* 2007; LEWIS 2011).

Since one biomarker may not be sufficient for the assessing of diagnosis with high diagnostic accuracy, due to complexity of disease pathogenesis, there is also a need to search and develop the concept of a biomarker signature, in which a panel of biomarkers is assessed (VIENNOIS *et al.* 2015).

7. Aims

The general aim of this thesis was to analyze the biomarkers of gut-barrier damage and resulting inflammation that may improve the diagnostics of diseases associated with these pathogenetic mechanisms. These new biomarkers may improve the diagnostics and prediction of the disease course and outcome in diseases such as necrotizing enterocolitis and inflammatory bowel disease. These aims can be divided into following fields of interest:

1. To investigate the diagnostic value of serum markers of intestinal damage, ccCK18 and I-FABP in NEC diagnosis and their ability to distinguish NEC from sepsis in early stage of disease.

NEC is characterized by gut barrier damage, destruction of intestinal mucosa and transmural necrosis of the intestinal wall. Therefore, we searched for non-invasive test that will reveal this damage before it will be apparent on X-ray or ultrasound. Additionally, we searched for biomarkers capable not only to diagnose NEC, but also to distinguish it from sepsis during early phases of the diseases (COUFAL *et al.* 2016b).

2. To analyze the combination of urinary I-FABP, L-FABP, TFF-3 and SAA in diagnosis and disease course prediction in NEC at early stage of disease.

Since combination of biomarkers may improve NEC diagnosis over single biomarker, we combined analyses of several non-invasive biomarkers associated with gut-barrier and excessive inflammatory response with the aim to improve the NEC diagnosis and to predict the disease course already at the time of NEC suspicion (COUFAL *et al.* 2020).

3. To find out if I-FABP could be used as a biomarker for gut mucosal injury in neonates with gastroschisis (GS) and if it can predict the speed of patient recovery after the surgery.

We found previously that urinary I-FABP reflects the gut epithelium damage in NEC. Here, we analyzed the urinary I-FABP could be used as biomarker of intestinal epithelium damage in neonates after the surgery for GS and if it can predict the speed of their clinical recovery (KOKESOVA *et al.* 2019).

4. To gain an insight into the inflammatory bowel disease (IBD) pathophysiology and to find biomarker pattern specific for different forms of IBD using broad spectrum analysis.

Since the dysbiosis of gut microbiota, gut barrier failure and aberrant immune response are common pathogenic features also in the pathogenesis of inflammatory bowel disease, we performed the broad spectrum analysis of serum and peripheral blood mononuclear cells (PBMCs) biomarkers to find a specific biomarker signature and to gain an insight into not fully understood pathophysiology of ulcerative colitis, Crohn's disease and inflammatory bowel disease associated with primary sclerosing cholangitis (PSC-IBD) (COUFAL *et al.* 2019).

8. List of publication

8.1. Publications

The thesis was prepared on the basis of these publications:

Coufal S, Kokesova A, Tlaskalova-Hogenova H, Snajdauf J, Rygl M, Kverka M. Urinary Intestinal Fatty Acid-Binding Protein Can Distinguish Necrotizing Enterocolitis from Sepsis in Early Stage of the Disease. J. Immunol. Res. 2016; 2016: 5727312

Coufal S, Kokesova A, Tlaskalova-Hogenova H, Frybova B, Snajdauf J, Rygl M, Kverka M. Urinary I-FABP, L-FABP, TFF-3 and SAA can diagnose and predict the disease course in necrotizing enterocolitis at the early stage of disease. J. Immunol. Res. 2020; 2020: 3074313

Kokesova A, Coufal S, Frybova B, Kverka M, Rygl M. The intestinal fatty acid-binding protein as a marker for intestinal damage in gastroschisis. PLoS One 2019, 14 (1): e0210797

Coufal S, Galanova N, Bajer L, Gajdarova Z, Schierova D, Jiraskova Zakostelska Z, Kostovcikova K, Jackova Z, Stehlikova Z, Drastich P, Tlaskalova-Hogenova H, Kverka M. Inflammatory Bowel Disease Types Differ in Markers of Inflammation, Gut Barrier and in Specific Anti-Bacterial Response. Cells 2019, 8 (7): 719; 8070719

8.2. Other impacted publications

Kostovcikova K, Coufal S, Galanova N, Fajstova A, Hudcovic T, Kostovcik M, Prochazkova P, Jiraskova Zakostelska Z, Cermakova M, Sediva B, Kuzma M, Tlaskalova-Hogenova H, Kverka M.: Diet Rich in Animal Protein Promotes Pro-inflammatory Macrophage Response and Exacerbates Colitis in Mice. Front Immunol. 2019, 10: 919

Stehlikova Z, Kostovcikova K, Kverka M, Rossmann P, Dvorak J, Novosadova I, Kostovcik M, Coufal S, Srutkova D, Prochazkova P, Hudcovic T, Kozakova H, Stepankova R, Rob F, Juzlova K, Hercogova J, Tlaskalova-Hogenova H, Jiraskova Zakostelska Z.: Crucial Role of Microbiota in Experimental Psoriasis Revealed by a Gnotobiotic Mouse Model. Front Microbiol. 2019, 10: 236

Stehlikova Z, Tlaskal V, Galanova N, Roubalova R, Kreisinger J, Dvorak J, Prochazkova P, Kostovcikova K, Bartova J, Libanska M, Cermakova R, Schierova D, Fassmann A, Borilova Linhartova P, Coufal S, Kverka M, Izakovicova-Holla L, Petanova J, Tlaskalova-Hogenova H, Jiraskova Zakostelska Z.: Oral Microbiota Composition and Antimicrobial Antibody Response in Patients with Recurrent Aphthous Stomatitis. Microorganisms 2019, 7 (12): 636

Dotlacil V, Bronsky J, Hradsky O, Frybova B, Coufal S, Skaba R, Rygl M: The Impact of Anti-Tumor Necrosis Factor Alpha Therapy on Postoperative Complications in Pediatric Crohn's Disease. Eur J Pediatr Surg. 2020, 30 (1): 27-32

8.3. Other publication

Coufal Š, Kokešová A, Tlaskalová-Hogenová H, Kverka M. Role střevní bariéry, mikrobioty a imunitního systému v patogenezi nekrotizující enterokolitidy. Alergie 3/2016. [Article in Czech]

8.4 Chapter in book

Coufal S., Tlaskalova-Hogenova H., Kokesova A., Kverka M. Vývoj imunitního systému jedince: v Imunologie a imunopatologie lidské reprodukce. Mladá fronta, 2020 (in press). [Article in Czech]

I hereby confirm that the author of this thesis, Štěpán Coufal, has substantially contributed to the publications included in this thesis. He performed the majority of the experimental work and significantly contributed to the manuscript preparation in the case of his first-author publication.

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MUDr. Miloslav Kverka, Ph.D.

9. Results

9.1. Urinary Intestinal Fatty Acid-Bidning Protein Can Distinguish Necrotizing Enterocolitis from Sepsis in Early Stage of the Disease

Stepan Coufal, Alena Kokesova, Helena Tlaskalova-Hogenova, Jiri Snajdauf, Michal Rygl and Miloslav Kverka

Journal of Immunology Research 2016; 2016: 5727312

In this study we found elevated total CK18 and decreased ccCK18/CK18 ratio in infants who will develop NEC as compared with infants who will develop sepsis in first 12 hours from NEC suspicion. This suggest higher portion of necrotic cell death in infants who will later develop NEC at the time of NEC suspicion. These differences, however, did not reach statistically significance.

Infants who will later develop NEC had significantly higher levels of I-FABP in serum in first 12 hours from NEC suspicion in comparison with infants who will later develop sepsis ($p<0.05$). These high levels decreased significantly at the end of antibiotic therapy ($p<0.01$), reaching low levels, which are typical for healthy individuals.

In first 12 hours after NEC suspicion infants who will later develop NEC had significantly elevated levels of urinary I-FABP than infants who will later develop sepsis and healthy controls (both $p<0.001$). The non-invasive mean of urine collection allowed continual monitoring of urinary I-FABP showing decrease of urinary I-FABP during successful NEC treatment ($p<0.05$). Urinary I-FABP was capable to distinguish infants who will later develop NEC or sepsis or healthy controls in the first 12 hours after suspicion for NEC. Furthermore, we found significantly higher urinary I-FABP in patients who will develop stage III NEC as compared to those who will develop stage II ($p<0.05$). We did not find any significant differences in serum and urinary I-FABP between spontaneous and surgery related NEC ($p=0.60$; $p=0.80$ respectively).

Urinary I-FABP has high sensitivity (81%) and specificity (100%) and can even distinguish surgery related NEC from sepsis in patients after surgery for congenital intestinal malformation. The addition of urinary I-FABP examination to the imaging methods (X-ray, ultrasound) increases the sensitivity and negative predictive value for

NEC to 91% and 89%, respectively. This approach revealed 9 radiologically and ultrasonographically negative patients who later developed NEC.

Thus, urinary I-FABP can be used to distinguish NEC from neonatal sepsis, including postoperative one, better than currently used imagining methods.

My contribution: sample analyses, data analyses and interpretation, manuscript writing

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Research Article

Urinary Intestinal Fatty Acid-Binding Protein Can Distinguish Necrotizing Enterocolitis from Sepsis in Early Stage of the Disease

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Necrotizing enterocolitis (NEC) is severe disease of gastrointestinal tract, yet its early symptoms are nonspecific, easily interchangeable with sepsis. Therefore, reliable biomarkers for early diagnostics are needed in clinical practice. Here, we analyzed if markers of gut mucosa damage, caspase cleaved cytokeratin 18 (ccCK18) and intestinal fatty acid-binding protein (I-FABP), could be used for differential diagnostics of NEC at early stage of disease. We collected paired serum (at enrollment and week later) and urine (collected for two days in 6 h intervals) samples from 42 patients with suspected NEC. These patients were later divided into NEC (n = 24), including 13 after gastrointestinal surgery, and sepsis (n = 18) groups using standard criteria. Healthy infants (n = 12), without any previous gut surgery, served as controls. Both biomarkers were measured by a commercial ELISA assay. There were no statistically significant differences in serum ccCK18 between NEC and sepsis but NEC patients had significantly higher levels of serum and urinary I-FABP than either sepsis patients or healthy infants. Urinary I-FABP has high sensitivity (81%) and specificity (100%) and can even distinguish NEC from sepsis in patients after surgery. Urinary I-FABP can be used to distinguish NEC from neonatal sepsis, including postoperative one, better than abdominal X-ray.

1. Introduction

Necrotizing enterocolitis (NEC) is one of the most common gastrointestinal emergencies in the newborn infant. NEC occurs in 1–3 per 1000 live births, its mortality varies between 15 and 30% and surgical treatment is necessary in 20–40% [1, 2].

NEC accounts for long-term morbidity in survivors of neonatal intensive care and early recognition and proper treatment can improve clinical outcomes [3]. The diagnosis of NEC is based on the combination of clinical, laboratory, and radiologic findings, which are defined by modified Bell's staging criteria [4]. Rapid onset and nonspecific early signs are typical for NEC, so it can be often misdiagnosed as neonatal sepsis. On the other hand, there are only few specific signs, such as *pneumatosis intestinalis* on X-ray or gas in the portal vein on ultrasonography, but these findings appear rather late in the course of the disease so their absence must be interpreted with extreme caution [5]. So there is a strong need for identification of new biomarkers, suitable for early diagnosis of NEC, which would give the opportunity for early intervention.

The best biomarkers should reflect the major steps in early disease pathogenesis. Several defensive mechanisms of innate immune system, such as NK cell response or antimicrobial peptide production, are defective in preterm children, which lead to susceptibility to various microbial infections and mucosal barrier damage [6, 7]. NEC is characterized by

destruction of the mucosal layer and by transmural necrosis of the intestinal wall [8]; therefore we start to search for noninvasive test that will reflect this destruction before it is apparent on X-ray. During the enterocyte damage, the cytokeratin 18 (CK18) is released to the circulation. If the cells died preferentially by apoptosis, increased proportion of the caspase cleaved form of CK18 (ccCK18) could be found in the circulation [9]. Interestingly, in animal model of NEC the excessive apoptosis was suggested as a major form of gut epithelium death even before typical necrosis took over [10]. This makes the ccCK18 an interesting target for early NEC diagnosis. Intestinal fatty acid-binding protein (I-FABP) is also released to the circulation during the enterocyte death. This protein constitutes up to 2% of the cytoplasmic protein content in the mature enterocyte [11]. Therefore I-FABP, as a marker of intestinal cell damage, is increased in serum of patients with NEC and sepsis and even in otherwise healthy people after abdominal surgery, trauma, or alcohol consumption [12–15]. Thanks to its small size (14-15 kDa), I-FABP can quickly pass through the kidney to urine [16], which gives an opportunity to measure it noninvasively in urine.

The aim of this study was to investigate the diagnostic value of serum markers of intestinal damage, ccCK18 and I-FABP, in their ability to distinguish NEC from sepsis in early stage of disease.

2. Material and Methods

2.1. Patients. We enrolled 42 candidate patients with suspected NEC and 12 healthy infants as controls. All of them were recruited from the patients admitted to the Department of Pediatric Surgery of Motol University Hospital, Prague, Czech Republic, between April 2012 and December 2014 (Table 1). The inclusion criteria were stage IA of NEC according to the modified Bell's staging criteria, which are characterized by temperature instability, apnea, lethargy, increased gastric residuals, abdominal distension, and occult blood in stool. These patients were later divided into NEC (n = 24), including infants who developed surgery-related NEC (n = 13) after the surgery for congenital intestinal malformation (gastroschisis, volvulus, intestinal atresia, anorectal atresia, and Hirschsprung's disease) and sepsis (n = 18) using standard criteria for NEC (*pneumatosis intestinalis* on X-ray or presence of gas in portal vein or in peritoneal cavity) or sepsis (suggestive clinical signs, laboratory examination results, and positive blood culture) as published previously [17, 18]. Two patients with sepsis were later excluded from the study, because only one sample was obtained for each of them. All these infants were treated with antibiotics for 7 or more days, according to the recent recommendations [17, 18]. There were no significant differences among groups, except for the birth weight in children suffering from NEC compared with control infants (2.4 ± 0.89 kg versus 3.1 ± 0.90 kg; $p < 0.05$). The study was approved by the Ethics Committee of the Motol University Hospital, and written informed consent was obtained from parents of all infants included in this study.

2.2. Sample Collection and Processing. First serum sample was taken at enrollment to the study and the second was taken 7–10 days later, at the last day of antibiotic treatment. Urine samples were collected for two days in 6-hour intervals starting at the time of enrollment to the study. In infants who developed NEC after the surgery for congenital gastrointestinal malformation, the urine was collected in 6 h intervals starting just after the surgery to evaluate the biomarker dynamics in infants before the suspicion of NEC. These samples were analyzed retrospectively in those that developed symptoms. The urine was collected either from urine bag connected to an indwelling catheter or from cotton wool swab placed in the diaper and squeezed through a syringe barrel into a collection tube. The urinary creatinine was measured in each sample immediately after sampling and samples for biomarker analysis were frozen at −20°C.

Table 1: Patients' demographics. The data are expressed as number of cases (%) or mean ± standard deviation.

	NEC	Sepsis	Control
Number of infants	24	16	12
NEC stage II	11 (45.8%)	—	—
NEC stage III	13 (54.2%)	—	—
Sex, female	7 (29.2%)	6 (37.5%)	6 (50.0%)
Gestational age (weeks)	35.5 ± 3.3	35.9 ± 4.2	37.8 ± 2.7
Birth weight (kg)	2.4 ± 0.9	2.7 ± 0.9	3.1 ± 0.9
Delivery by cesarean section	14 (58.3%)	5 (31.3%)	6 (50.0%)
Birth asphyxia	10 (41.7%)	5 (31.3%)	2 (16.7%)
Congenital heart disease	4 (16.7%)	0 (0.0%)	0 (0.0%)
Postnatal age at disease evaluation (day)	18.3 ± 30.5	19.9 ± 31.8	13.6 ± 11.4

2.3. Enzyme-Linked Immunosorbent Assay (ELISA). The degree and form of enterocyte death was analyzed by measuring the concentration of total cytokeratin 18 (CK18) and its caspase cleaved form (ccCK18) in serum by M65 or M30 Apoptosense® ELISA (both Peviva, Stockholm, Sweden), respectively. The assay was performed according to the manufacturer's instruction and the concentration of ccCK18 and total CK18 in serum is presented as U/L.

The concentration of I-FABP was measured by Human I-FABP ELISA (Hycult Biotech, Uden, Netherlands), which is certified for both serum and urine. The assay was performed according to the manufacturer's instruction and the I-FABP concentration in serum is presented as ng/mL. To eliminate fluctuation in urine excretion, the urinary I-FABP was normalized to urinary creatinine and it is presented as pg/nmol of creatinine.

2.4. Statistical Analysis. The variables were tested for normality by Shapiro-Wilcoxon normality test and the differences between studied groups were analyzed by either Student's t-test or Mann-Whitney test. Continuous variables are presented as mean ± standard deviations (SD) and dichotomous

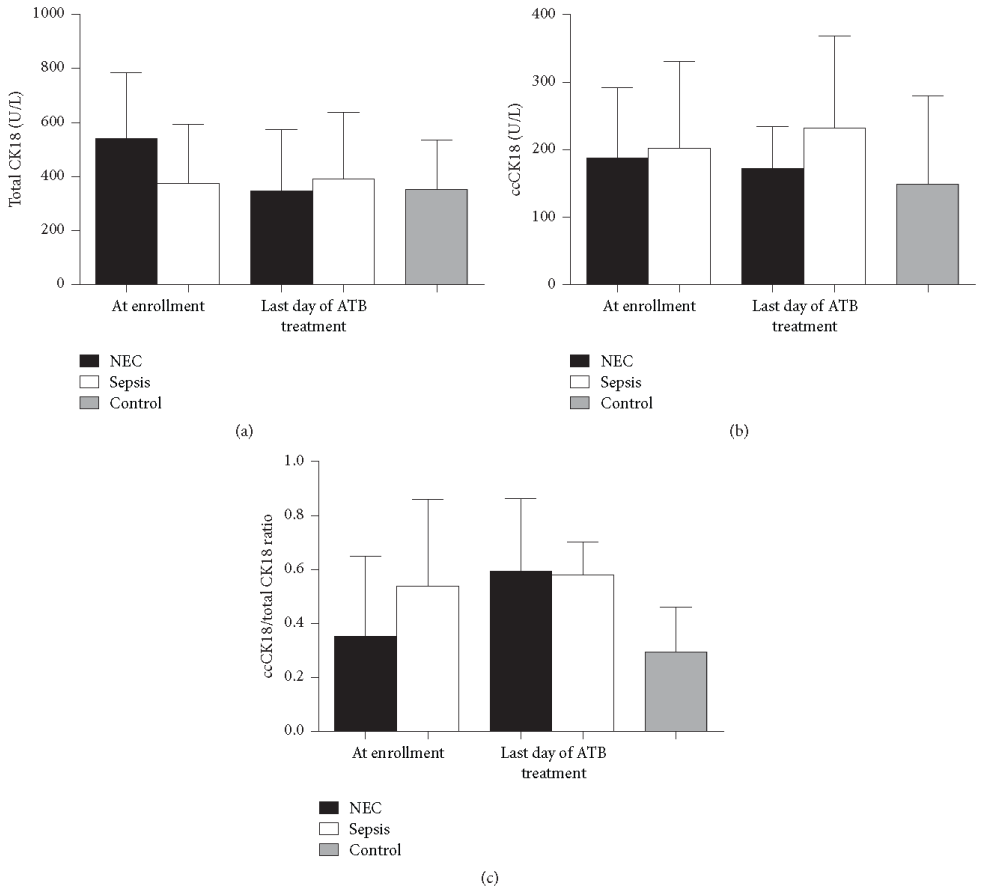

Figure 1: The analysis of cytokeratin 18 (CK18) in serum. (a) The differences between NEC and sepsis in caspase cleaved CK18 (ccCK18) (p = 0.7606, Mann-Whitney test), (b) total CK18 (p = 0.2317, Mann-Whitney test), and (c) ccCK18/total CK18 ratio (p = 0.1783, Mann-Whitney test), at the time of enrollment and last day of antibiotic (ATB) treatment.

variables as percentages. The cutoff levels were calculated as the mean of the group plus 2 SD. Receiving operating characteristic (ROC) analyses were constructed to assess the performance of I-FABP as a predictor of impending NEC and sensitivity, specificity, likelihood ratio, positive predictive value, and negative predictive value were calculated to show diagnostic utility of this approach. Statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software, GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Serum Cytokeratin 18. There were no statistically significant differences in ccCK18 or total CK18 between NEC and sepsis (Figures 1(a) and 1(b)). At the time of enrollment, there was slightly elevated total CK18 and slightly decreased ccCK18/CK18 ratio in NEC as compared to sepsis suggesting higher proportion of necrotic cell death in NEC patients (Figure 1(c)). These differences were, however, not statistically significant.

3.2. Serum I-FABP. At the time of enrollment, patients who developed NEC had significantly higher concentrations of I-FABP than patients who developed sepsis (Figure 2(a)). At the end of antibiotic therapy, the levels of I-FABP in NEC patients decreased significantly, reaching low levels typical for patients with sepsis or healthy controls (Figure 2(b)). The ROC curve analysis revealed that serum I-FABP is suitable biomarker for distinguishing NEC from sepsis (LR+ = 4.75 LR− = 0.67 and optimal cutoff = 4.1 ng/mL) (Figure 2(c)).

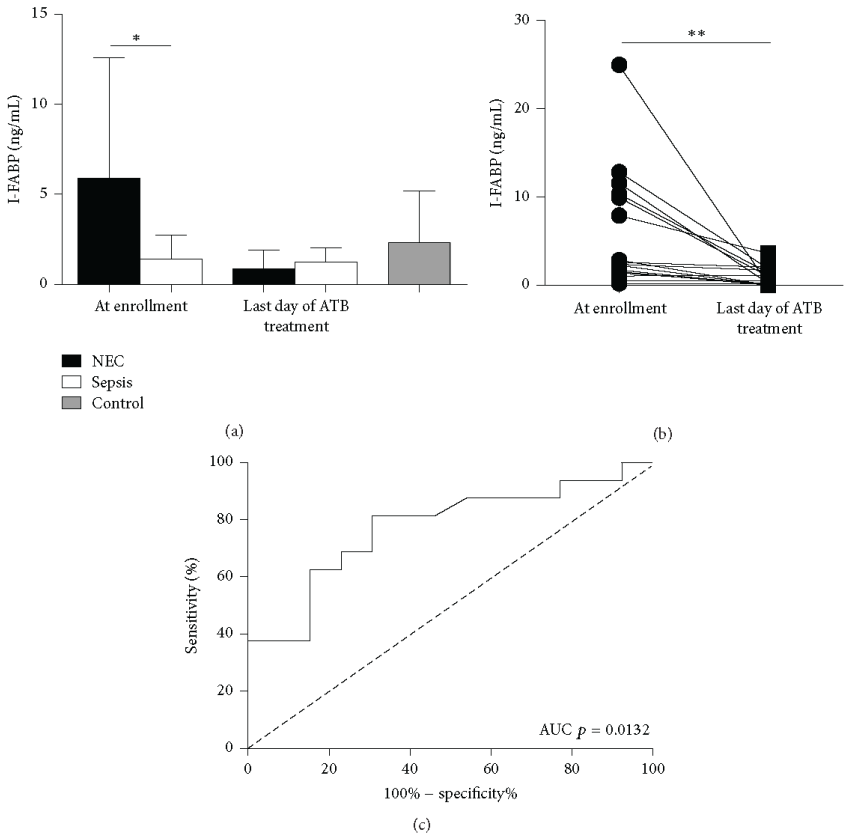

Figure 2: The analysis of I-FABP in serum. (a) The comparison of I-FABP for patients with NEC and sepsis or healthy patients at the time of enrollment ($^{*}p < 0.05$, unpaired Student's t-test with Welch's correction), (b) the comparison of I-FABP in patients with NEC at the time of enrollment and last day of ATB treatment ($^{**}p < 0.01$, Paired Student's t-test), and (c) the ROC curve analysis using I-FABP levels in NEC and sepsis group at the time of admission.

There were no significant differences between spontaneous and surgery-related NEC ($p = 0.60$, data not shown).

3.3. Analysis of I-FABP in Urine. During the first twelve hours after enrollment, patients who developed NEC had significantly higher concentrations of I-FABP then either those who developed sepsis or healthy infants (Figure 3(a)). There were no significant differences between spontaneous and surgery-related NEC ($p = 0.80$, data not shown). The levels of urinary I-FABP in patients with sepsis were indistinguishable from these found in control infants. The continuous sampling of urine showed that urinary I-FABP levels decrease during the therapy (Figure 3(b)). Urinary I-FABP is, therefore, capable of distinguishing NEC from either sepsis (Figure 3(c)) or healthy infants (Figure 3(d)). All control infants had the level of I-FABP under the calculated cutoff level 2.52 pg/nmol creatinine. Although the levels of urinary I-FABP were significantly higher in patients with stage III NEC than in patients with stage II NEC (data not shown) in the first twelve hours after enrollment, it cannot distinguish between surgical (stage IIIB) and medical (stages IIIA and below) NEC (AUC $p = 0.18$).

3.4. Diagnostic Performance of I-FABP ELISA. At the time of enrollment, analysis of urinary I-FABP had higher sensitivity and higher negative predictive value for NEC than either *pneumatosis intestinalis* on X-ray or gas in portal vein on ultrasound (Table 2). When urinary I-FABP analysis was combined with imagine methods the sensitivity and negative predictive values for NEC at the time of first symptoms increased to 91% and 89%, respectively. This approach revealed 9 (33%) radiologically and ultrasonographically negative patients who later developed NEC.

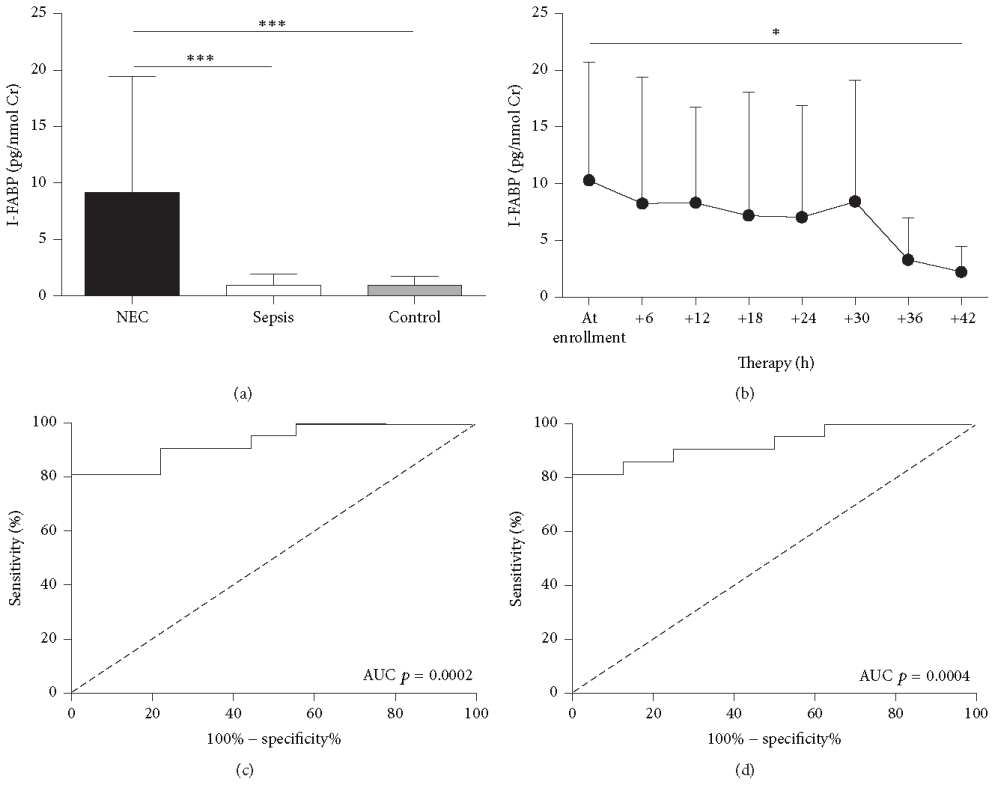

Figure 3: The analysis of I-FABP levels in urine. (a) The I-FABP levels in first twelve hours from the time of enrollment (***$p < 0.001$, Mann-Whitney test) and (b) the dynamics of I-FABP in urine in NEC patients (*$p < 0.05$, paired Student's t-test). The ROC curve analysis using I-FABP levels in (c) NEC and sepsis or (d) NEC and controls.

Table 2: Diagnostic performance of standard X-ray (gold standard), ultrasound, and serum or urinary I-FABP as analyzed in this study. Imagine methods refer collectively to the X-ray and ultrasound (USG).

NEC × disease	Imagine methods		Serum I-FABP		Urinary I-FABP		Imagine methods + urinary I-FABP
	X-ray	USG	NEC versus sepsis	NEC versus control	NEC versus sepsis	NEC versus control	
Sensitivity	41%	29%	38%	31%	81%	81%	91%
Specificity	100%	100%	92%	100%	100%	100%	100%
Positive predictive value	100%	100%	86%	100%	100%	100%	100%
Negative predictive value	53%	48%	55%	31%	69%	67%	89%

3.5. Urinary I-FABP in Diagnosis of Surgery-Related NEC. Patients who developed NEC after the surgery for congenital intestinal malformation showed rapid increase in urinary I-FABP from the time point of 12 h after surgery to the time of suspected NEC (Figure 4). This increase was not present in infants who later developed sepsis.

4. Discussion

Necrotizing enterocolitis is one of the most severe diseases of infant's gastrointestinal tract (GIT). It is characterized by unexpected onset and very rapid progression with the risk of gut perforation and the infant's death. The clinical signs are

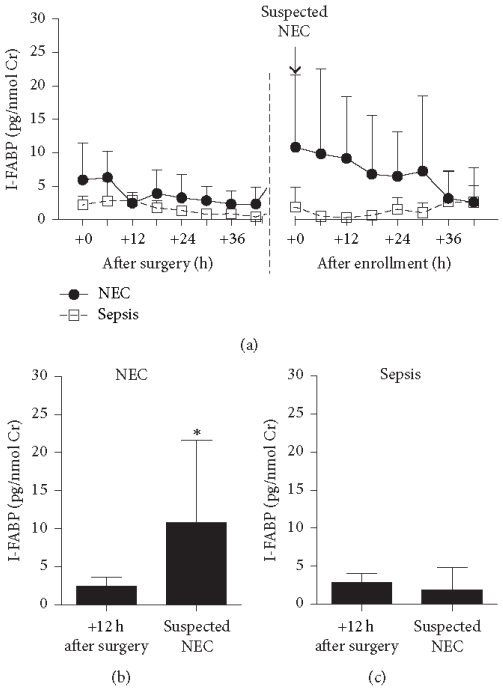

FIGURE 4: The urinary I-FABP in patients that developed NEC or sepsis after surgery for congenital intestinal malformation. (a) The dynamics of urinary I-FABP. Change in urinary I-FABP between time 12 h after surgery and time of suspected NEC ($^*p < 0.05$, Wilcoxon matched-pairs signed-rank test) in (b) NEC and (c) sepsis patients.

in the early stage nonspecific and easily interchangeable with other GIT disorders or sepsis.

The diagnosis of NEC is based on presence of clinical symptoms (i.e., abdominal distension and blood in stool), radiologic or sonographic findings of *pneumatosis intestinalis* or gas in portal vein, and in most severe cases presence of gas in peritoneal cavity. Unfortunately, both imagine methods have very low sensitivity and negative predictive value and uncover mainly advanced cases of NEC [19]. Therefore, many NEC cases are missed at the initial submission to the hospital and the newborns with suspected NEC being subjected to harmful effects of ionizing radiation by numerous abdominal X-rays [20]. Early diagnosis of NEC allows more efficient intervention, consisting of cessation of enteral feeding, administration of broad spectrum antibiotics, and supportive care, which has major impact on the disease prognosis [21–23].

Since the gut barrier disruption has crucial role in the early steps of NEC pathogenesis, we analyzed markers of gut barrier disruption, ccCK18 or I-FABP in serum and urine, as possible early biomarkers for NEC and its distinction from sepsis. Although coagulative necrosis of the gut wall is the major hallmark of NEC, it may be preceded with excessive apoptosis of gut epithelial cells, as reported before in animal model of NEC [10]. Therefore, we measured ccCK18 and CK18 as a marker of necrosis and apoptosis of gut epithelium. We did not find any significant differences in ccCK18 and total CK18 concentrations between NEC and sepsis groups. We found only slightly increased proportion of epithelium necrosis at enrollment in infants with NEC, which reflects the major histopathologic feature of the disease [8, 24, 25], but this difference was not statistically significant.

Increased serum concentration of I-FABP as possible biomarker of gut epithelium damage during NEC was first noted in animal model [26]. Later studies in humans found that serum I-FABP can distinguish infants with NEC from healthy preterm infants and that I-FABP levels correlate with NEC severity [27, 28]. Recent meta-analysis concluded that serum I-FABP is a valid biomarker for NEC that can significantly decrease the high false negative rates of current diagnostic procedures [29]. But since increase in I-FABP was described also in sepsis [30], we decided to use serum I-FABP to distinguish NEC from sepsis. We found that I-FABP levels were significantly higher in patients with NEC than those with sepsis at the time of recruitment to the study but normalized after the successful treatment. Moreover, we found that I-FABP levels can distinguish NEC stage II and NEC stage III in the first 12 h after the suspicion of NEC, although it cannot make more clinically relevant distinction between medical and surgical NEC. Nevertheless, recent study demonstrated that length of bowel resection in surgical NEC correlates with I-FABP levels at disease onset, suggesting that the I-FABP levels mirror the degree of intestinal damage [31].

Thanks to its small molecule, I-FABP can be easily measured in urine, thus sparing the infant from unnecessary blood draw. The noninvasive means of its collection is advantageous, because the invasive diagnostic procedures may contribute to adverse neurocognitive outcome [32]. The previous studies showed that urinary I-FABP could be used to distinguish patients with NEC from healthy newborns [16, 33, 34]. In this study we found that I-FABP was significantly higher in infants who developed NEC than in either those who developed sepsis or control infants. Furthermore, the levels of I-FABP in infants with sepsis and control infants were similar. Currently, the gold standard for NEC diagnosis is abdominal X-ray, which has very low sensitivity and negative predictive value [19]. Urinary I-FABP reached 100% positive predictive value, which is similar as that reached by diagnostic gold standard. More importantly, it revealed 9 (33%) radiologically and ultrasonographically negative patients that later developed NEC, thus reaching sensitivity of 81% as compared to either healthy controls or sepsis patients. When standard imagine methods during the admission to the hospital were supplemented with urinary I-FABP analysis, it significantly raised the sensitivity (91%) and negative predictive value (89%) of such combined diagnostic approach, while keeping the high specificity and positive predictive value (both 100%).

Both NEC and sepsis are common complications of surgery for congenital intestinal malformation. The development of NEC is in these cases probably driven by decreased mesenteric blood flow to the intestine. On the other hand, any breach of abdominal wall in infants has some risk of sepsis; its incidence is 8% for laparotomy without enterotomy or 20% for laparotomy with enterotomy [35]. And since even uncomplicated surgery may lead to increase in urinary I-FABP [12, 36, 37], we measured its levels in patients with surgery-related NEC and sepsis. We found that the I-FABP concentration in urine significantly increased in the time of suspected NEC but only in patients who developed NEC later, but not in those who developed sepsis. Therefore, the diagnostic power of urinary I-FABP was not hindered by previous surgery.

5. Conclusions

These results show that urinary I-FABP can be used to distinguish NEC from neonatal sepsis, including postoperative one, better than current gold standard (abdominal X-ray). This is very important for the clinician at the moment when the clinical symptoms of NEC are nonspecific and when NEC can be confused with neonatal sepsis. The addition of this harmless and noninvasive examination to the standard X-ray significantly increases the sensitivity and negative predictive value of such approach in the NEC diagnosis.

Abbreviations

NEC: Necrotizing enterocolitis
I-FABP: Intestinal fatty acid-binding protein
CK18: Cytokeratin 18
ccCK18: Caspase cleaved cytokeratin 18
USG: Ultrasonography
SD: Standard deviation
ROC: Receiving operating characteristic
PPV: Positive predictive value
NPV: Negative predictive value
AUC: Area under the curve
ELISA: Enzyme-linked immunosorbent assay
GIT: Gastrointestinal tract
LI: Likelihood ratio.

Competing Interests

The authors declare no potential competing interests.

Acknowledgments

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9.2. Urinary I-FABP, L-FABP, TFF-3 and SAA can diagnose and predict the disease course in necrotizing enterocolitis at the early stage of disease

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In this study we found that infants who will later develop NEC had significantly higher urinary I-FABP, L-FABP, TFF-3 and SAA in comparison with healthy infants in first six hours after NEC suspicion ($p<0.01$). In first six hours after NEC suspicion had infants who will later develop NEC significantly higher levels of urinary I-FABP and L-FABP than infants who will later develop sepsis ($p<0.01$, $p<0.05$, respectively). Urinary SAA was significantly higher in both, infants who will later develop NEC or sepsis as compared to healthy infants in first six hours after NEC suspicion. While urinary I-FABP was capable to distinguish infants who will later develop NEC from those who will develop sepsis (AUC=0.831), urinary SAA was capable to distinguish infants who will later develop sepsis from healthy controls (AUC=0.898). The combination of both, urinary I-FABP and SAA then allowed discrimination of infants who will later develop NEC from healthy controls (AUC=0.941).

We did not find any significant differences in the levels of these biomarkers in spontaneous and surgery related NEC. But we found higher level of urinary I-FABP and L-FABP in patients who will later develop NEC in postoperative period after surgery for congenital intestinal malformation as compared to those who will later develop sepsis. Furthermore, we found steep increase in these biomarkers at the time of NEC suspicion, but only in patients who will later develop NEC.

Infants who will develop the most serious stage of NEC (stage IIIB NEC) had elevated urinary SAA and TFF-3 as compared to infants who will later develop stage IIA, IIB, IIIA of NEC. The urinary SAA was the strongest factor for distinguishing between stage IIIB and the other stages of NEC (AUC=0.779). Further, we found that the combination of urinary TFF-3, I-FABP and SAA can predict *pneumatosis intestinalis* (AUC=0.819) and the combination of urinary I-FABP, L-FABP and SAA can predict not only gas in portal vein (AUC=0.727), but also the length of hospitalization in NEC patients in first six hours

after the NEC suspicion. The short achievement of full enteral feeding could be predicted by low levels of urinary SAA (AUC=0.810).

Thus, these biomarkers may be useful not only in the early, non-invasive diagnostics but also in the subsequent NEC management.

My contribution: sample analyses, data analyses and interpretation, manuscript writing

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Research Article

Urinary I-FABP, L-FABP, TFF-3, and SAA Can Diagnose and Predict the Disease Course in Necrotizing Enterocolitis at the Early Stage of Disease

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Necrotizing enterocolitis (NEC) is a severe gastrointestinal disease affecting mainly preterm newborns. It is characterized by unexpected onset and rapid progression with specific diagnostic signs as *pneumatosis intestinalis* or gas in the portal vein appearing later in the course of the disease. Therefore, we analyzed diagnostic and prognostic potential of the markers of early NEC pathogenesis, such as excessive inflammatory response (serum amyloid A (SAA)) and gut epithelium damage (intestinal and liver fatty acid-binding protein (I-FABP and L-FABP, respectively) and trefoil factor-3 (TFF-3)). We used ELISA to analyze these biomarkers in the urine of patients with suspected NEC, either spontaneous or surgery-related, or in infants without gut surgery (controls). Next, we compared their levels with the type of the disease (NEC or sepsis) and its severity. Already at the time of NEC suspicion, infants who developed NEC had significantly higher levels of all tested biomarkers than controls and higher levels of I-FABP and L-FABP than those who will later develop sepsis. Infants who will develop surgery-related NEC had higher levels of I-FABP and L-FABP than those who will develop sepsis already during the first 6 hours after the abdominal surgery. I-FABP was able to discriminate between infants who will develop NEC or sepsis and the SAA was able to discriminate between medical and surgical NEC. Moreover, the combination of TFF-3 with I-FABP and SAA could predict *pneumatosis intestinalis*, and the combination of I-FABP, L-FABP, and SAA could predict gas in the portal vein or long-term hospitalization and low SAA predicts early full enteral feeding. Thus, these biomarkers may be useful not only in the early, noninvasive diagnostics but also in the subsequent NEC management.

1. Introduction

Necrotizing enterocolitis (NEC) is a severe acute gastrointestinal disease affecting mainly preterm newborns. The pathophysiology of NEC remains poorly understood. The main risk factors of NEC are immaturity of gut barrier and immune system together with enteral feeding and abnormal microbial colonization of the gut. On the other hand, breastfeeding represents an important factor protecting from NEC [1, 2]. Thanks to its unique composition, mother's milk not only accelerates gut barrier maturation but also protects neonatal gut from infection. The former is achieved mainly by numerous growth factors and the later by antimicrobial factors (e.g., lactoferrin and lysozyme) and by secretory IgA, which could bind microbes in antigen-specific and nonspecific manner by Fab or glycans, respectively [3, 4].

With overall incidence of 1 to 3 cases per 1000 live births and mortality as high as 50% [5, 6], NEC is one of the leading causes of morbidity and mortality in neonatal intensive care units [7]. The early recognition and proper treatment can, however, improve the clinical outcomes [8]. The current diagnosis of NEC is based on the combination of clinical, laboratory, and radiologic or sonographic findings, which are defined by modified Bell's staging criteria [9]. But NEC has

often rapid onset and progression with nonspecific early signs, which may delay the NEC treatment by misdiagnosing it as neonatal sepsis or other medical emergency. While there are specific signs for NEC, such as *pneumatosis intestinalis* or gas in the portal vein, they appear rather later in the disease course and their absence must be interpreted with caution [10]. Therefore, the identification of specific biomarkers for early diagnosis of NEC is strongly needed.

The best biomarkers should be specific for the early steps in NEC pathogenesis. Since several studies demonstrated the advantages of biomarker combination in the NEC diagnosis, we combined analysis of several noninvasive markers of excessive inflammatory response and destruction of the gut mucosa, all typical features of NEC pathogenesis [11].

Previously, we showed that urinary intestinal fatty acid-binding protein (I-FABP) can distinguish NEC from sepsis in early stage of the disease [12]. Fatty acid-binding proteins (FABPs) are small (14-15 kDa) tissue-specific cytoplasmic proteins involved in the metabolisms of fatty acids. The I-FABP constitutes up to 2% of cytoplasmic protein content in the mature enterocyte. The liver fatty acid-binding protein (L-FABP) is expressed in similar pattern of tissue distribution along the duodenal-colonal axis, but with the higher tissue content than I-FABP (up to 40-fold). During the enterocyte death, both I-FABP and L-FABP are released into the circulation and thus can be used as a marker of intestinal damage [13, 14]. Both I-FABP and L-FABP are elevated in patients with NEC, but also with sepsis, after abdominal surgery or trauma [15–19].

The trefoil factor-3 (TFF-3) is expressed in intestinal tract and is associated with maintaining of mucosal barrier integrity, promotion of mucosal barrier restitution, gastrointestinal inflammation, and cell migration [20–23]. TFF-3 is elevated in patients with severe sepsis and inflammatory bowel diseases. TFF-3 levels correlate with the activity of ulcerative colitis localized to the colonic mucosa, suggesting the site-specific upregulation of TFF-3 in inflamed colonic mucosa [24–26]. Moreover, it was shown that TFF-3 was associated with protective effect on intestinal tract injury in animal model of NEC via protection of excessive apoptosis by the increase of Bcl-2 and reduction of caspase-3 and Bax expression [27].

Serum amyloid A (SAA) is an acute phase inflammatory protein. It increases in innate defence mechanisms in response to infection, inflammation, and trauma. The SAA was suggested to be more sensitive and specific marker of inflammation than C-reactive protein in various inflammatory conditions [28–30]. The SAA has been shown to be useful in diagnosis of various acute diseases, including neonatal sepsis and NEC. Moreover, it can determine the severity of the disease and response to therapy [31–33].

The aim of this study was to investigate the diagnostic potential of these gut-associated and inflammatory biomarkers in the early diagnostics of NEC, their association with clinically relevant and well-established disease-related parameters, and their capacity to predict the disease course. Thanks to their small size, all these biomarkers can pass through the kidney to urine, which gives unique opportunity to constant monitoring and noninvasive measurement.

Table 1: Patient's demographics. The data are expressed as number of cases (%) or mean ± standard deviation.

	NEC	Sepsis	Control
Number of infants	20	9	8
Spontaneous NEC	11 (55.0%)	—	—
Surgery-related NEC	9 (45.0%)	—	—
NEC stage II	11 (55.0%)	—	—
NEC stage III	9 (45.0%)	—	—
Sex, male	15 (75.0%)	4 (44.4%)	6 (75%)
Gestational age (weeks)	35.9 ± 3.4	36.7 ± 2.6	38.0 ± 2.8
Birth weight (kg)	2.4 ± 0.9	2.7 ± 0.8	3.2 ± 0.9
Delivery by cesarean section	12 (60.0%)	3 (33.3%)	3 (37.5%)
Birth asphyxia	8 (40.0%)	3 (33.3%)	0 (0.0%)
Congenital heart disease	4 (20.0%)	0 (0.0%)	0 (0.0%)
Age at diagnosis (days)	20.6 ± 33.0	10.1 ± 7.5	11.9 ± 12.6

2. Materials and Methods

2.1. Patients. In the study, we enrolled 29 patients with suspected NEC and 8 healthy infants without gut surgery and intestinal mucosa disruption as controls. All of them were recruited from the individuals admitted to the Department of Pediatric Surgery of Motol University Hospital, Prague, Czech Republic, between April 2012 and December 2014 (Table 1). The inclusion criteria were stage IA of NEC according to the modified Bell's staging criteria, which are characterized by temperature instability, lethargy, increased gastric residuals, abdominal distension, and occult blood in stool. The patients with suspected NEC were later divided into NEC group ($n = 20$) and sepsis group ($n = 9$) using the standard criteria for NEC (*pneumatosis intestinalis* on X-ray or presence of the gas in the portal vein) or sepsis (suggestive clinical signs, laboratory examination and positive blood culture) [34, 35]. Perinatal asphyxia was evaluated using the guidelines of the American Academy of Pediatrics (AAP) and American College of Obstetrics and Gynecology (ACOG) criteria as described previously [36]. Moreover, in infants who underwent surgery for congenital intestinal malformation (e.g., gastroschisis, volvulus, intestinal or anorectal atresia, and Hirschsprung's disease), urine was collected in 6 h intervals for 48 h after surgery and the levels of the selected biomarkers were compared between these who developed NEC and sepsis. There were no significant differences among groups, except for birth asphyxia in infants suffering from NEC compared with control infants. To analyze the capacity of these noninvasive biomarkers in the prediction of clinical outcome and recovery, we used clinical data from patients' health records and chose length of hospitalization, length of antibiotic (ATB) therapy, and time to full enteral feeding. The median number of the days was a cut off. Therefore, we identified "short" length of hospitalization as less than 19 days, "short" length of antibiotic therapy as less than 10 days, and "short" time to full enteral feeding as less than 9.5 days. The study was approved by the Ethics Committee of the Motol University Hospital, and written informed consent was obtained from parents of all infants included in this study.

Table 2: List of quantified biomarkers.

Biomarker	Abbreviation	Manufacturer	Cat. no
Intestinal fatty acid-binding protein	I-FABP	Hycult®Biotech	HK406
Liver fatty acid-binding protein	L-FABP	Hycult®Biotech	HK404
Trefoil factor-3	TFF-3	BioVendor	RD191160200R
Serum amyloid A	SAA	Hycult®Biotech	HK333

2.2. Sample Collection and Processing. Urine samples were collected for two days in 6-hours intervals starting at the time of enrolment to the study or already after the surgery for congenital intestinal malformation to evaluate the biomarker dynamics in infants before the suspicion of NEC. These samples were analyzed retrospectively in those who developed NEC or sepsis. The urine was collected either using urine bag connected to an indwelling catheter or from a cotton wool swab placed in the diaper and squeezed through a syringe barrel into a collection tube. The urinary creatinine was measured in each sample immediately after sampling and samples for biomarker analyses were frozen at −20°C.

2.3. Enzyme-Linked Immunosorbent Assay (ELISA). The concentrations of biomarkers were measured by ELISA (Table 2), which is certified for urine analysis by manufacturer. The assays were performed according to the manufacturer's instruction. To eliminate fluctuation in urine excretion, biomarkers were normalized to urinary creatinine and presented as pg/nmol of creatinine.

2.4. Statistical Analyses. The differences between studied groups were analyzed by nonparametric Mann-Whitney test. Continuous variables are presented as mean ± standard deviations (SD) and dichotomous variables as percentages. Statistical analyses were performed using GraphPad Prism statistical software (version 8.1.1, GraphPad Software, San Diego, CA, USA) and differences were considered statistically significant at $p < 0.05$. Regression analysis was performed in R and the effect of each biomarker on Akaike information criterion (AIC) was determined in the nnet package (ver. 7.3-12). Next, we performed both backward elimination and forward selection based on AIC to determine the best regression model to discriminate between the two states (e.g., disease type, stage, or outcome). Thus, we found minimum models with best prediction and performance capacity, which were used for construction of receiving operating characteristic (ROC) curves. The ROC curves and their area under curve (AUC) were calculated using ROCR package (ver. 1.0-7). Hierarchical clustering and heat map construction was performed using the Kendall distance calculating method in the ComplexHeatmap (ver 1.3) package for R [37].

3. Results

During the first 6 hours after the enrolment, infants who will later develop NEC (NEC group) had significantly higher urinary I-FABP, L-FABP, TFF-3, and SAA when compared with healthy infants and higher levels of I-FABP and L-FABP than those who will later develop sepsis (sepsis group) (Figure 1(a)). Similarly as in NEC, SAA was significantly higher in urine of patients with sepsis than that in healthy controls. Urinary I-FABP discriminated future NEC and sepsis, the SAA discriminated sepsis from healthy controls, and the combination of both discriminated NEC from healthy controls (Figure 1(b)). These effects are quite strong despite the fact that the cluster analysis of all these biomarkers did not find particularly strong clustering according to the presence, absence, or type of diagnosis (Figure 1(c)). There were no significant differences in these biomarkers between spontaneous and surgery-related NEC at the time of enrolment (data not shown). Patients who will develop NEC after the surgery for congenital intestinal malformation had higher levels of urinary I-FABP and L-FABP than those who will develop sepsis already in the first 6 hours after the surgery. Moreover, there was steep increase in urinary I-FABP and L-FABP around the time of NEC suspicion in infants who will later develop NEC (Supplementary figure 1).

Next, we analyzed the association of these biomarkers in the first 6 hours after the enrolment with clinically relevant and well-established disease-related parameters. We found that infants who will later develop stage IIIB NEC ("surgical"), which is associated with gut perforation, had elevated both SAA and TFF-3 in comparison with infants with stages IIA, IIB, and IIIA NEC ("medical") (Figure 2(a)). The SAA was capable to discriminate between medical and surgical NEC. The combinations of TFF-3 with I-FABP and SAA predicted well *pneumatosis intestinalis* and the combination of I-FABP with L-FABP and SAA predicted portal venous gas (Figures 2(b) and 2(c)). Perinatal asphyxia in NEC patients was associated with significantly higher levels of urinary SAA and TFF-3 and the TFF-3 alone was the best discriminating factor (Figure 2(d)).

Next, we analyzed the ability of these noninvasive biomarkers to predict clinical outcome in infants who will develop NEC. The urinary I-FABP and L-FABP were significantly higher in infants with long hospitalization (more than 19 days), and when combined with SAA, they can predict long hospitalization (Figure 3(a)). While L-FABP was associated with the long antibiotic (ATB) therapy in NEC infants, its ability to discriminate between short and long ATB therapy was rather low (AUC = 0.650) (Figure 3(b)). The urinary SAA was the best factor for prediction of the early full enteral feeding (more than 9.5 days) (Figure 3(c)).

4. Discussion

In our previous study, we found that both, serum and urinary I-FABP, can distinguish NEC from sepsis in the early stage of

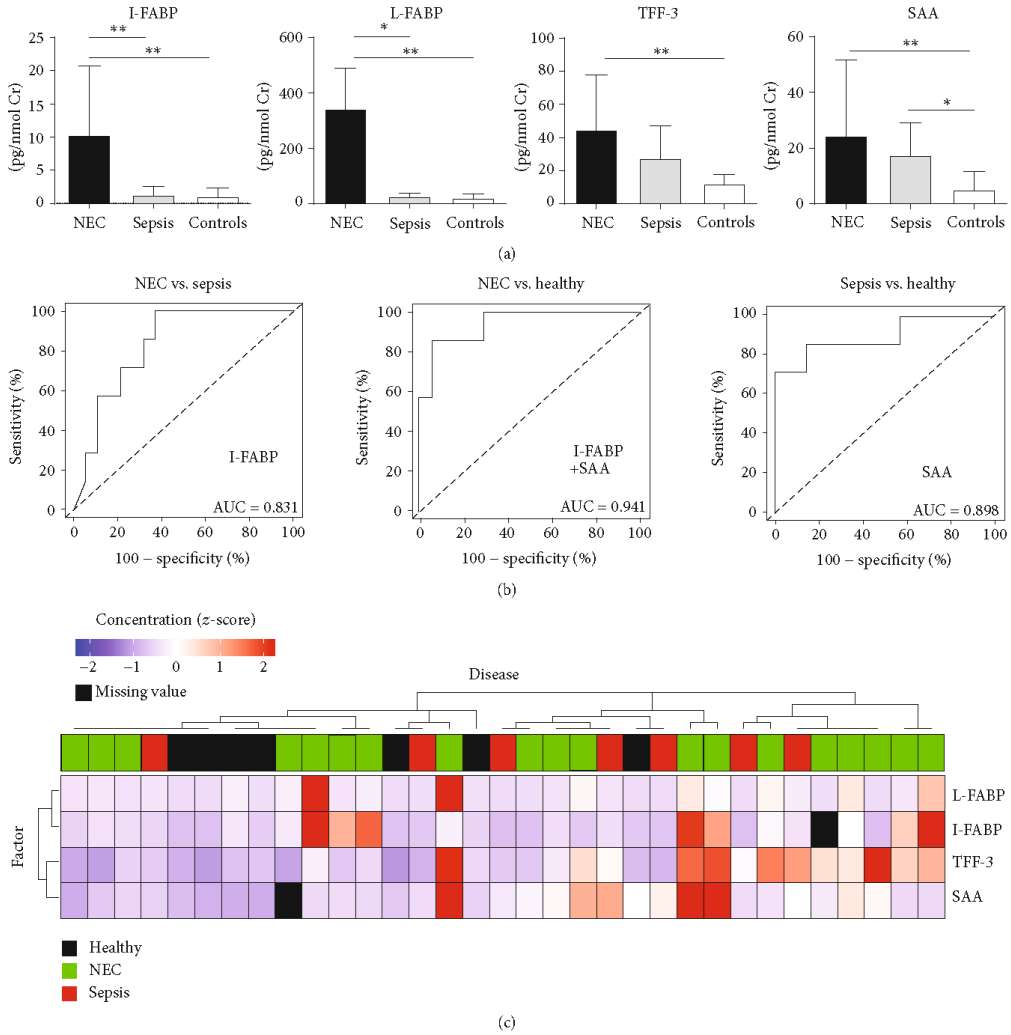

FIGURE 1: The analysis of I-FABP, L-FABP, TFF-3, and SAA in urine. (a) The comparison of I-FABP, L-FABP, TFF-3, and SAA for patients who will later develop NEC/sepsis or healthy infants in the first 6 hours from the enrolment ($^{*}p < 0.05$, $^{**}p < 0.01$; Mann-Whitney test). (b) Composite ROC curve analysis of the best model found by regression analysis. (c) Heat map and cluster analysis.

the disease, and the addition of I-FABP analysis to the diagnostic gold standard for NEC (X-ray and ultrasound) can significantly increase the sensitivity and negative predictive value [12]. Here, we combine urinary I-FABP with other gut-associated and inflammatory biomarkers with the aim to further improve the differential diagnosis of NEC and to get better insight into the NEC pathophysiology. While several previous studies analyzed these biomarkers in NEC patients, this is the first study analyzing the combination of all these biomarkers not only in early NEC diagnosis but also in association with clinically relevant and well-established disease-related parameters. Moreover, we tested their capacity to predict the disease course and outcome in a noninvasive way. The noninvasive means of urine collection is favourable because it minimizes the stress of the neonate from the repeated blood sampling [38].

In this study, we found that not only I-FABP but also L-FABP, SAA, and TFF-3 were significantly higher in the urine of infants who will later develop NEC in comparison with healthy infants already in the first 6 hours after the NEC suspicion. Their high levels suggest that gut mucosa damage and strong inflammatory response are detectable even before the

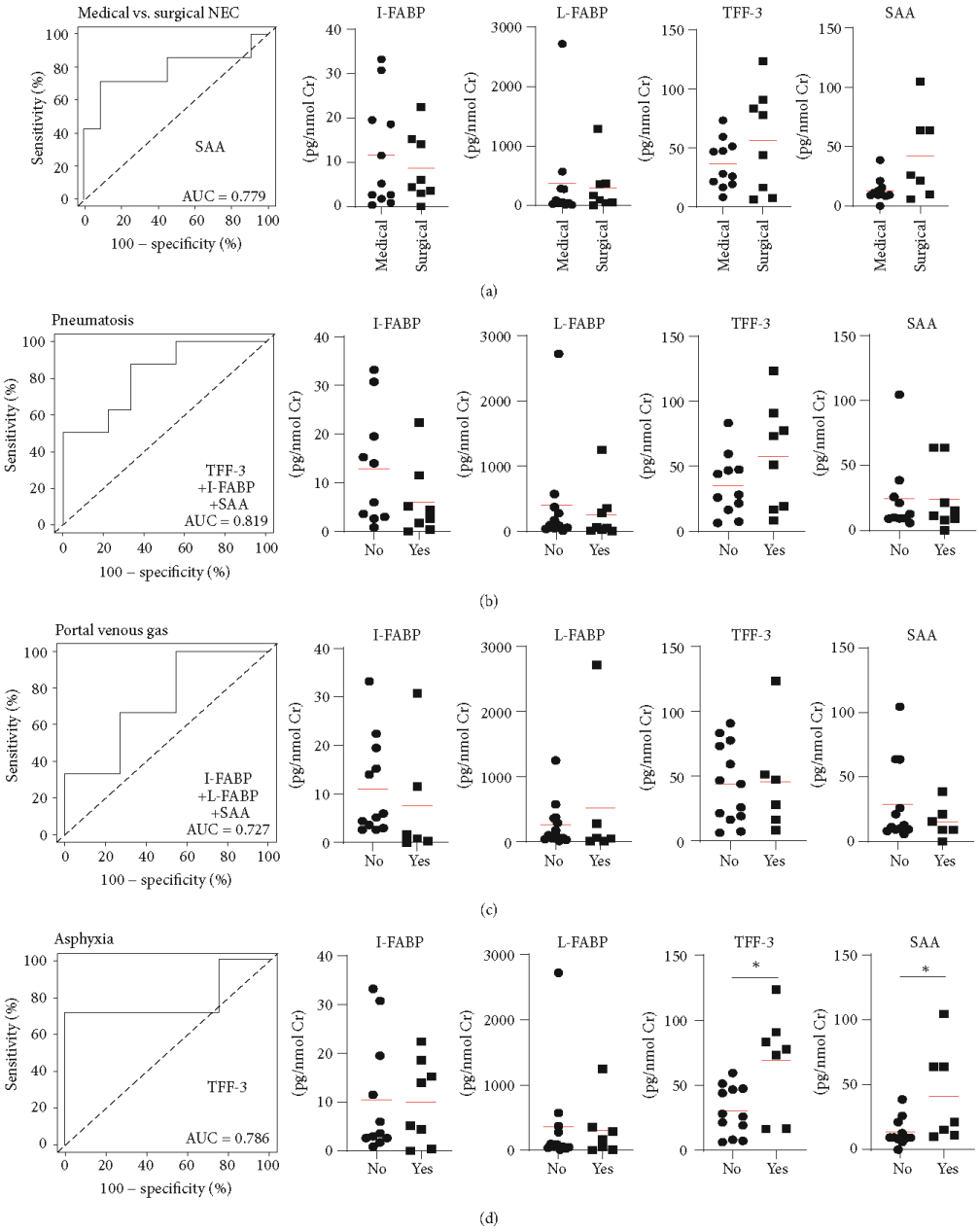

FIGURE 2: Biomarker patterns discriminating clinical stages. Composite ROC curve analysis of the best model found by regression analysis and quantitative plots of analyzed biomarkers ($^*p < 0.05$; Mann-Whitney test).

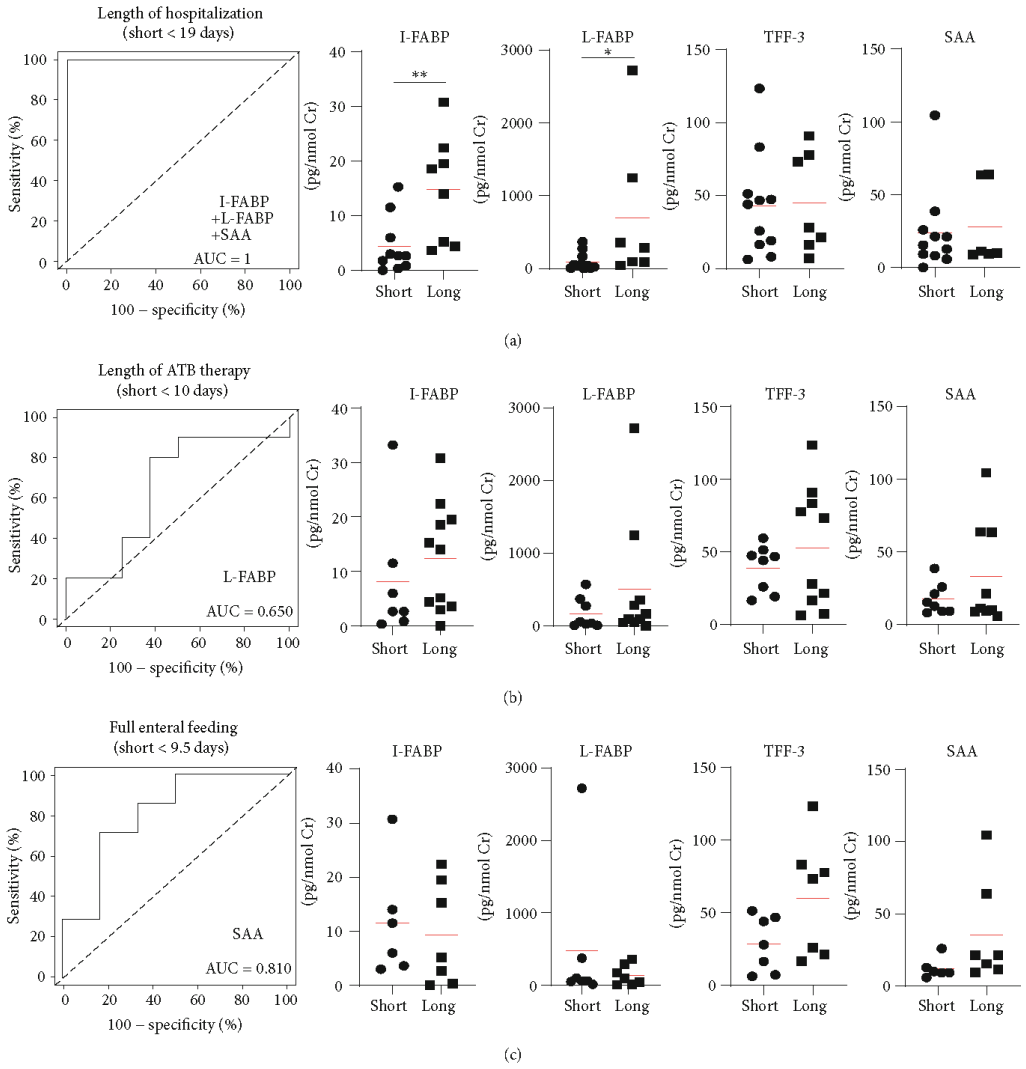

FIGURE 3: Biomarkers predicting clinical outcome in patients with NEC. Composite ROC curve analysis of the best model found by regression analysis and quantitative plots of analyzed biomarkers ($^*p < 0.05$, $^{**}p < 0.01$; Mann-Whitney test).

full spectrum of NEC symptoms is apparent. The increase of I-FABP and L-FABP in NEC infants suggests that they are both associated with the gut epithelium damage. This is in agreement with findings that L-FABP has similar pattern of tissue distribution with higher tissue content than I-FABP [13]. Therefore, L-FABP may be a more sensitive biomarker for detection of gut epithelium damage in early stage of NEC when it is released from the damaged enterocytes, in spite of its association with hepatocellular injury. Our results are thus in agreement with the previous findings, where both analytes were increased in plasma of NEC patients [14]. Among studied biomarkers, the urinary I-FABP was the strongest factor for distinguishing patients who will later develop NEC from those who will develop sepsis. This is in agreement with our previous results and further supports the importance of I-FABP in differential diagnostics of NEC [12]. Since even uncomplicated abdominal surgery could lead to the increase of I-FABP levels [17, 39, 40], we compared all tested biomarkers in patients with surgery-related NEC or sepsis. We found that infants who will develop NEC after the surgery

for congenital intestinal malformation had higher levels of urinary I-FABP and L-FABP already in the first 6 hours after the surgery than those who will develop sepsis. This was followed by a substantial decrease after 12 hours from the surgery. At the time of NEC suspicion, there was rapid increase in the levels of urinary I-FABP and L-FABP but only in patients who will later develop NEC. The levels of studied biomarkers in this study did not significantly differ between spontaneous and surgery-related NEC, and the levels of urinary I-FABP and L-FABP between sepsis and control group were comparable.

Our finding that high levels of urinary TFF-3 are associated with NEC is in agreement with previous findings, where the elevated plasma TFF-3 was associated with intestinal damage in NEC [41]. Increased TFF-3 in NEC may represent protective feedback mechanism triggered by the gut injury, which in turn promotes the mucosal barrier restitution [22, 27]. The high serum TFF-3 was described also in the association with the severity, multiple organ dysfunctions, and prognosis of sepsis patients [25, 42]. Thus, we can speculate that increased TFF-3 in sepsis could be a secondary phenomenon caused by the blood redistribution leading to the gut barrier dysfunction, which in turn can contribute to the development of uncontrolled systemic inflammatory response syndrome. We found that urinary SAA is increased in both NEC and sepsis, indicating the central role of the inflammatory response in both diseases. This is in agreement with other studies describing its increase in plasma during both NEC and sepsis [28–30]. Taken together, these results further stress the importance of harnessing our understanding of the biological processes for diagnostic purposes.

Next, we analyzed if these biomarkers are associated with clinically relevant and well-established disease-related parameters in NEC. We found that SAA not only distinguished infants who will develop NEC stage II and III, it can also distinguish infants who will develop the most severe stage associated with gut perforation—stage IIIB NEC ("surgical") from stage IIA, IIB, and IIIA NEC ("medical") at the time of NEC suspicion. This is in agreement with a previous study finding urinary SAA correlated with diseases severity [43]. We also found that patient with surgical NEC had higher levels of urinary TFF-3 than those with medical NEC, suggesting the association of TFF-3 levels with more severe intestinal damage in the case of surgical NEC. The combination of gut-associated biomarkers with urinary SAA was the strongest predictor of *pneumatosis intestinalis* (TFF-3, I-FABP, and SAA) and portal venous gas (I-FABP, L-FABP, and SAA) already in the first 6 hours after the enrolment. In our previous study, we showed that currently used gold-standard methods for NEC diagnosis have low sensitivity and negative predictive value. This may cause the late diagnosis of NEC, which is accompanied with poor outcome or risk of death [44–46]. Moreover, infants with suspected NEC are subjected to harmful effects of ionizing radiation by numerous abdominal X-rays [47]. Our new results provide further insight into the early stages of NEC pathophysiology and the association of these biomarkers with clinically relevant and well-established disease-related parameters. Moreover, identification of patients who will develop severe NEC already at the moment of NEC suspicion is of great value for frontline neonatologist and surgeon in monitoring and management of NEC, especially in patients who may benefit from early surgical intervention.

The hypoxic ischemia was described as a one of the risk factor in NEC pathogenesis [8]. It may lead to the acute phase inflammatory response in neonate, development of neuronal damage, and tissue necrosis with devastating clinical outcome [48–50]. We found that among infants who developed NEC, these who were exposed to perinatal asphyxia have significantly higher levels of urinary TFF-3 and SAA than those who were not. Our results are thus in agreement with previous studies considering the SAA as a possible marker for ischemia-related inflammation closely associated with ischemic injuries [49, 51]. The high levels of TFF-3 were previously described in animal model of perinatal asphyxia, where the elevated TFF-3 was considered as the reaction to the tissue injury repair [52].

If these biomarkers reflect the pathogenesis and disease severity, they may predict outcome of the disease. Indeed, we found that higher levels of I-FABP, L-FABP, and SAA predict shorter hospitalization already in the first 6 hours from NEC suspicion. This is in agreement with previous observation that serial I-FABP measurement can predict development of complicated disease and that SAA in serum is useful tool for determining the disease severity and response to therapy in infants with NEC [32, 53]. We found similar trend in length of ATB therapy (L-FABP) and late achievement of full enteral feeding (SAA), both being signs of more severe disease. However, the number of individuals in this study was probably too low to find subtle differences in these parameters and bigger cohorts may be needed. The morbidity and long-term health outcomes among NEC survivors are highly influenced by the pathological stage of NEC and the extent of intestinal damage. The cost associated with both medically and surgically treated NEC is substantially higher than that of matched controls and poses high economic burden for the healthcare [46]. Moreover, there are long-term effects in NEC survivors (e.g., the short bowel syndrome, prolonged administration of parenteral nutrition, and growth and neurodevelopmental impairment) that require further management [54–56]. Our results showed that the gut mucosa damage and inflammatory biomarkers and their combination could be helpful not only in the diagnosis of infants who will develop NEC but also in the prediction of the disease course and outcome and thus can also have treatment and policy implications in management of NEC.

5. Conclusion

Development of NEC, its severity, and its consequences can be predicted using the panel of pathogenesis-relevant biomarkers before the symptoms became clinically apparent. The early diagnosis and prediction are useful for the management of NEC by the frontline clinicians.

Abbreviations

NEC: Necrotizing enterocolitis

I-FABP: Intestinal fatty acid-binding protein
L-FABP: Liver fatty acid-binding protein
SAA: Serum amyloid A
TFF-3: Trefoil factor-3
ROC: Receiver operating characteristic
AUC: Area under curve
ELISA: Enzyme-linked immunosorbent assay
GIT: Gastrointestinal tract.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interests.

Acknowledgments

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Supplementary Materials

Supplementary figure 1: biomarker dynamics in surgery-related NEC or sepsis. (A) Dynamics of biomarkers 48 hours after abdominal surgery and 48 hours after NEC suspicion. (B) Biomarkers level in the first 6 hours after the surgery for congenital intestinal malformation ($^{**}p < 0.01$; Mann-Whitney test). *(Supplementary Materials)*

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9.3. The Intestinal Fatty Acid-Binding Protein as a Marker for Intestinal Damage in Gastroschisis

Alena Kokesova, Stepan Coufal, Barbora Frybova, Miloslav Kverka, Michal Rygl

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We found that newborns with simple or complex gastroschisis (GS) had higher levels of urinary I-FABP in the postoperative period in comparison with their appropriate control group. The urinary I-FABP reached maximum level in first six hours after the surgery intervention. All complex GS patients were accompanied with small intestinal atresia. Therefore, we selected the patients with small intestinal atresia to establish the appropriate control group for complex GS. Interestingly, in these two groups there was also second peak in urinary I-FABP in 36 hours after the surgery. The levels of urinary I-FABP in the control subjects for simple GS were generally low.

The patients with complex GS had higher urinary I-FABP levels than patients with simple GS. Furthermore, we found that high level of urinary I-FABP in patients with complex GS was associated with subsequent surgery for mechanical ileus. There was no significant difference in the levels of I-FABP between the type of abdominal wall closure (primary closure or stepwise reconstruction). Although, there are clear differences in outcome between simple and complex GS in achievement full enteral feeding ($p<0.01$) and the length of hospitalization ($p<0.05$), urinary I-FABP was not capable to predict early start of minimal enteral feeding, full enteral feeding or length of hospitalization.

Thus, urinary I-FABP reflects the damage of intestinal mucosal in patients with gastroschisis but it has only a limited predictive value for patients' outcome.

My contribution: sample analyses, data analyses and interpretation.

PLOS ONE

RESEARCH ARTICLE

The intestinal fatty acid-binding protein as a marker for intestinal damage in gastroschisis

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Abstract

Background/Purpose

We analyzed the capacity of urinary Intestinal fatty acid-binding protein (I-FABP) to quantify the degree of mucosal injury in neonates with gastroschisis (GS) and to predict the speed of their clinical recovery after surgery.

Methods

In this prospective study, we collected urine during the first 48h after surgery from neonates operated between 2012 and 2015 for GS. Neonates with surgery that did not include gut mucosa served as controls for simple GS and neonates with surgery for intestinal atresia served as control for complex GS patients. The I-FABP levels were analyzed by ELISA.

Results

Urinary I-FABP after the surgery is significantly higher in GS newborns than in control group; I-FABP in complex GS is higher than in simple GS. I-FABP can predict subsequent operation for ileus in patients with complex GS. Both ways of abdominal wall closure (i.e. primary closure and stepwise reconstruction) led to similar levels of I-FABP. None of the static I-FABP values was useful for the outcome prediction. The steep decrease in I-FABP after the surgery is associated with faster recovery, but it cannot predict early start of minimal enteral feeding, full enteral feeding or length of hospitalization.

Conclusion

Urinary I-FABP reflects the mucosal damage in gastroschisis but it has only a limited predictive value for patients' outcome.

collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Abbreviations: GS, Gastroschisis; I-FABP, Intestinal fatty acid-binding protein; MEF, Minimal enteral feeding; FEF, Full enteral feeding; LOH, Length of hospitalization; PC, Primary closure; SR, Stepwise reconstruction.

Introduction

Gastroschisis (GS) is a congenital anomaly of the abdominal wall, which results in extrusion of abdominal viscera from the abdominal cavity. The prevalence of GS is increasing; currently reaching 4.9 per 10,000 live births [1]. Although survival in GS exceeds 90%, some babies experience significant morbidity, which is largely determined by the severity of prenatal and postnatal bowel injury [2].

It is still unclear how the structural changes correlate with malabsorption and small bowel dysmotility in GS and therefore with patient outcomes [3]. The extensive injury of intestinal mucosa could be a major source of complications both during the surgery and during the postoperative period and may have a major impact on patient recovery. A biomarker capable of quantifying the condition of intestinal mucosa after closure of the abdominal wall and during the acute post-surgery period is needed in clinical practice. I-FABP (Intestinal fatty acid-binding protein) is present in the cytoplasm of mature enterocytes in the small and large intestine and is released as soon as the cell membrane integrity is compromised, thus reflecting the extent of gut damage. It is used as a biomarker of mucosal injury and other diseases affecting the intestine [4]. Serum I-FABP correlates with the severity of villous atrophy in coeliac disease and with Crohn's disease activity [5, 6]. It is increased after abdominal surgery or infection and can be used as a biomarker of acute gut mucosa damage in necrotizing enterocolitis (NEC) or distinguish it from neonatal sepsis [7–9].

The aim of this prospective study is to find out if I-FABP could be used as a biomarker for mucosal injury in neonates after the GS surgery and if it can predict the speed of their clinical recovery.

Materials and methods

Population under study

Thirty-two patients with GS were recruited from the patients admitted to the Neonatal Intensive Care Unit, Department of Pediatric Surgery of University Hospital Motol, Prague, between 2012 and 2015. Two patients were excluded from the study because they died within the first 48 hours of life without passing any urine. One died of fulminant septic shock and one died of multiorgan failure resulting from prenatally acquired massive intestinal necrosis. Their samples and follow up were therefore not complete for later analysis. Out of our study group, 26 were born in our center and 4 were born in peripheral hospitals and transferred to our department immediately after delivery. All patients were operated within first 6 hours of life regardless of the place of birth.

Categorization of GS as simple or complex was based on the absence or presence of intestinal atresia, stenosis, perforation, necrosis or volvulus at birth, as suggested by Molik et al. [10]. All of our complex GS patients had small intestinal atresia, in 2 patients was intestinal atresia found during second revision, so these two individuals were reclassified as complex GS.

All subjects of this study were operated under general anesthesia in the operating theatre. A primary operative abdominal wall repair was attempted in all our patients. Primary closure was performed by an interrupted absorbable suture with attention to preserving the umbilicus. Gore-Tex silo (1mm Dual Gore-Tex patch) was used when primary closure was not possible. The decision was made by attending surgeon when viscera could not be primarily reduced without danger of impeding venous return, increase of peak inspiratory pressures and increase of abdominal pressure. The final closure and silo removal was performed at 18 (12–29) (median (range)) day of life.

Total parenteral nutrition was started within 24 hours of life via central venous catheter and performed in compliance with the current recommendation [11]. The nutritional regimen for all patients included mixture of amino acids (Primene 10%, Baxter Czech, Prague, Czech Republic), fat (Smoflipid 20%, Fresenius Kabi AB, Uppsala, Sweden) and dextrose, supplemented with the vitamins and trace-elements (Soluvit ® N, Vitalipid® N Infant, Peditrace®, Fresenius Kabi AB, Uppsala, Sweden).

Enteral feeding was introduced once postoperative ileus had been resolved and the decision was made by attending neonatologist. Neonates received mother's milk or bank milk continuously, via a nasogastric tube. Feeding was increased gradually according to tolerance and measurement of gastric residua. After initiation of feeding we followed minimal enteral feeding protocol as follows. Breast milk was given via nasogastric tube continuously, starting at 0.3–0.5 ml/kg/h. If it was well tolerated, the feeding was increased by 7–12 ml/kg daily. If neonate didn't tolerate the daily increase, the dose was restored to the last tolerated amount or stopped entirely.

To exclude the confounding factor of surgery, we established a different control group for either type of GS—newborns who underwent surgery without intestinal mucosa disruption (S1 Table) served as controls for simple GS. All of our complex GS patients had small intestinal atresia, so control group for complex GS sustained from patients operated for small intestinal atresia, who underwent as well as complex GS patients resection of atretic part of the intestine and then end-to end anastomosis. There were 12 newborns in the control group for simple GS and 6 newborns in the control group for complex GS.

To analyze the capacity of urinary I-FABP to predict the speed of the postoperative recovery, we used clinical data from patients' health records and chose time to minimal enteral feeding of 20 ml/kg/day (MEF), time to full enteral feeding (FEF) and length of hospitalization (LOH). The median number of days was a cut off. Therefore, we defined "early" MEF as less than 13 days, "early" FEF as less than 19 days and "short" LOH as less than 31 days. The detailed demographics of studied population is summarized in Table 1.

The study was approved by the Ethics committee of the University Hospital Motol, and written informed consent was obtained from parents of all children included in this study.

Sample collection and processing

Urine samples were collected for 48 hours in 6 hours' intervals, starting during the first 6h after the surgery. Samples were collected from urine bag connected to an indwelling catheter. The urinary creatinine was measured in each sample in our hospital biochemistry laboratory immediately after sampling and samples for I-FABP analysis were frozen at -20˚C until the analysis.

Table 1. The demographic data of the study group.

	GS simple (n = 25)	Control (n = 12)	P	GS complex (n = 5)	Control (n = 6)	P
Delivered by C-section, n (%)	10 (40.0)	3 (25)	0.46	3 (60)	2 (33.3)	0.58
Male, n (%)	13 (52.0)	8 (66.6)	0.49	2 (40)	3 (50)	1.0
Median gestation age in weeks (range)	36 (32–38)	39 (33–41)	< 0.01	34 (33–37)	39 (37–40)	< 0.01
Median birth weight in grams (range)	2400 (1490–3650)	2925 (1800–4200)	< 0.05	2400 (1475–2790)	3160 (2510–3445)	< 0.01
Prenatal diagnosis, n (%)	22 (88.0)	-	-	5 (100)	-	-
Treated with primary closure, n (%)	20 (80.0)	-	-	4 (80)	-	-
Treated with stepwise reconstruction, n (%)	5 (20.0)	-	-	1 (20)	-	-
Median hospital stay in days (range)	26.0 (10–83)	6.5 (3–25)	< 0.001	72.0 (28–93)	18.0 (10–28)	< 0.01

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Enzyme-Linked Immunosorbent Assay (ELISA)

Urinary I-FABP was measured by Human I-FABP ELISA (Hycult Biotech, Uden, The Netherlands). The assay was performed according to the manufacturer's instruction. To eliminate fluctuation in urine excretion, the urinary I-FABP was normalized to urinary creatinine and is presented as pg/nmol of creatinine.

Statistical analysis

The continuous variables were tested for normality by D'Agostino-Pearson normality test. The differences between GS and control group were analyzed either by Mann-Whitney test (continuous variables) or by Fisher's exact test (dichotomous variables). I-FABP levels in complex and simple GS were compared with each other and with these in controls using Kruskal-Wallis test with Dunn's post test. I-FABP was correlated with the time to MEF, FEF and with LOH using Spearman's rank correlation coefficient (r). Continuous variables are presented as median (range), dichotomous variables as the number of cases (percentages), and p values < 0.05 were considered statistically significant.

Correlation matrix was created using Spearman's rank correlation coefficient (r), and p values were divided by the number of analyzed values to prevent the type I error (false positivity). Predictive value of the I-FABP for the MEF, FEF and LOH was analyzed using receiver operating characteristic (ROC) curves. Statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). Ordinary least squares (OLS) regression was used to model the relationship between MEF, FEF and LOH and I-FABP levels at different time points. Regression analysis was performed in R (ver. 3.3.2; R Foundation for Statistical Computing, Vienna, Austria) and Akaike information criterion (AIC) was determined in the MASS package (ver. 7.3–45). Next, we performed both backward elimination and forward selection based on AIC to determine the best regression model.

Results

Simple and complex GS patients have higher urinary I-FABP after the surgery than control subjects (Fig 1A and 1B). The I-FABP dynamics in simple and complex GS don't differ. In both cases, I-FABP levels reach maximum in the first 6h after surgery (9.29 (0.59–58.56) pg/nmol for simple GS, 23.99 (16.59–41.90) pg/nmol for complex GS). Interestingly, I-FABP peaks 36 hours after the surgery in both patients with complex GS (15.57 (28.73–8.03) pg/nmol) and in controls for complex GS (4.20 (0.31–10.89) pg/nmol). The level of urinary I-FABP in controls for simple GS is generally low and without a distinct peak; in first 6 hours reaches 1.15 (0.06–3.41) pg/nmol.

In the first 48h after the surgery, the levels of I-FABP in patients with complex GS are higher than in patients with simple GS (Fig 1C). We did not find significant differences in I-FABP levels between patients treated with primary closure (PC) and stepwise reconstruction (SR), but I-FABP in both these groups was significantly higher than in controls (Fig 1D).

Urinary I-FABP during the first 6 hours after surgery is significantly higher in complex GS patients who will be later operated for mechanical ileus than in those operated only for silo removal (Fig 1E).

There is a clear difference in the outcome between complex and simple GS. Patients with complex GS have received FEF significantly later (median 59 vs. 17 days, $p = 0.0055$) and have significantly longer LOH than patients with simple GS (median 72 vs. 26 days, $p = 0.0120$). We used a linear regression model to analyze at which time point the urinary I-FABP has the highest capacity as a predictive biomarker for clinical outcome (MEF, FEF and LOH). Due to the clear differences in I-FABP dynamics, we included the changes in I-FABP as additional

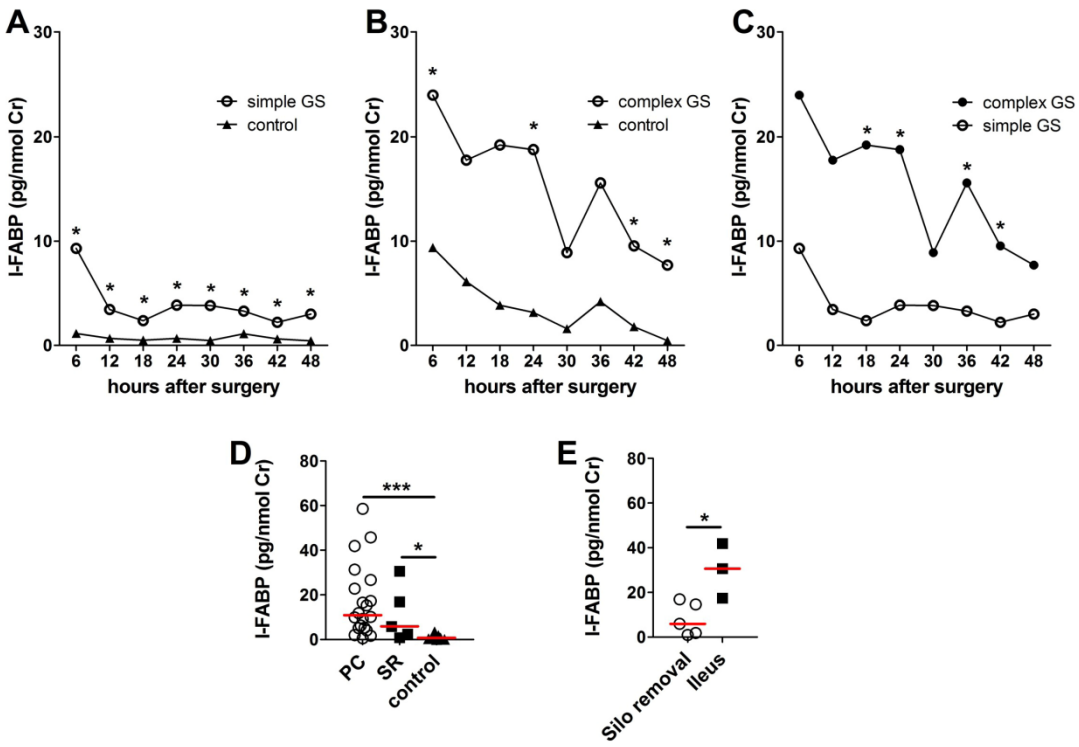

Fig 1. The analysis of urinary I-FABP. Urinary I-FABP after the surgery in simple GS (Fig 1A) and complex GS (Fig 1B) vs. controls. Comparison of I-FABP between simple and complex GS after the surgery (Fig 1C). I-FABP in groups treated with stepwise reconstruction (SR) or primary closure (PC) and in controls (Fig 1D). Urinary I-FABP during the first 6 hours after surgery (6h) in complex GS patients who will be later operated for mechanical ileus and in those operated only for silo removal (Fig 1E). Median $^{*}p<0.05$, $^{**}p<0.01$ and *** $p<0.001$.

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variables. None of the I-FABP levels measured at 6-hour intervals was a suitable predictor for the outcomes. However, three changes in I-FABP levels in time (decrease between 12 and 18 hours, decrease between 12 and 24 hours and increase between 24 and 30 hours) were found to be exceptionally good predictors (Table 2).

Table 2. Regression analysis outcome of the suitable models.

Model	12h-18h		12h-24h		24h-30h	
	Effect±SE	Adjusted R^2	Effect±SE	Adjusted R^2	Effect±SE	Adjusted R^2
MEF	-2.40±0.42	0.71***	-1.93±0.4014	0.63***	1.08±0.26	0.55**
FEF	-2.35±0.60	0.53**	-1.88±0.5387	0.46**	1.05±0.34	0.40**
LOH	-2.00±0.68	0.37*	-1.55±0.6109	0.29*	1.00±0.34	0.37*

*p<0.05

** p<0.01

*** p<0.001

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All these models provide an equivalent fit for LOH and FEF (Δ AIC < 3), but 12h-18h is equivalent to 12-24h (Δ AIC = 3) and gives slightly better fit than 24-30h (Δ AIC = 6). This was, however, not supported by ROC curve analysis, in which the decrease in I-FABP between 12-18h was not capable of predicting early MEF/FEF or short LOH, as defined for our dataset (Fig 2A). The levels of I-FABP at the time of surgery do not predict multiple surgeries, as analyzed by ROC curve analysis (S1 Fig).

To analyze the correlation of individual values and demographic characteristics of GS patients, we constructed a correlation matrix (S2 Table). We found a clear correlation among the levels of I-FABP at different time points, among all three recovery characteristics and between gestation length and birth weight. Another well-established connection is that patients with multiple surgeries are less likely to be operated with primary closure and have longer LOH. The raw data with appropriate metadata are in S3 Table.

Discussion

Disruption of intestinal mucosa causes major complications in patients with GS. The extent of this injury and its capacity to predict patient's recovery has not yet been sufficiently analyzed. Our study showed that I-FABP can serve as a biomarker for the gut mucosa damage after the closure of abdominal wall in GS.

I-FABP is a small cytoplasmic protein localized in epithelial cells of the small intestine [12], which is released into the circulation after enterocyte damage [13] and quickly passes into urine. Therefore, urinary I-FABP could be used as a non-invasive biomarker of acute gut mucosa damage in spontaneous and surgery-related necrotizing enterocolitis [7, 9, 14–16]. Since gut mucosa damage is a typical pathological feature of GS, we studied if urinary I-FABP could be also used as a biomarker to predict a patient's outcome.

We found that I-FABP is higher in patients with complex GS as compared to simple GS, which is consistent with significantly more severe mucosal damage in complex GS. However, traumatization to the gut during intestinal incision leads to a quick increase in plasmatic I-FABP as well [8], so it is unclear if this is an effect of more extensive damage during complex GS or just an effect of extensive surgery. All complex GS patients in this study had intestinal atresia, so they underwent intestinal wall incision, intestinal resection and intestinal wall suture. To control for the confounding factor of surgery and general anesthesia, we established a different control group for each type of GS—newborns who underwent surgery without intestinal mucosa disruption served as controls for simple GS and those operated for intestinal atresia with the same surgical technique were selected as controls for complex GS. These control groups were well matched for all characteristics except for those inherently associated with GS itself (gestational age and birth weight) and GS severity (hospital stay). We were not able to find any relevant information on the effect of gestational age and birth weight on I-FABP in the literature. None of these factors, however, correlated with I-FABP levels (S2 Table), so they do not seem to be crucial. Nevertheless, overall immaturity may influence the recovery regardless of gut damage, thus presenting a potential confounding factor. We found that while surgical damage to the gut mucosa increases the urinary I-FABP, its levels are significantly higher in both types of GS when compared to the relevant control group. Our results also showed that anesthesia, cold stress, volume therapy during surgery and general postoperative care do not influence I-FABP. Moreover, the I-FABP levels in controls for simple GS are not markedly higher than those in generally healthy newborns [7]. These results clearly show that damage to the gut mucosa in GS is not just a result of mucosal integrity disruption during surgery.

In this study, both ways of abdominal wall closure (i.e. primary closure and stepwise reconstruction) led to similar levels of I-FABP, suggesting that neither approach leads to more severe

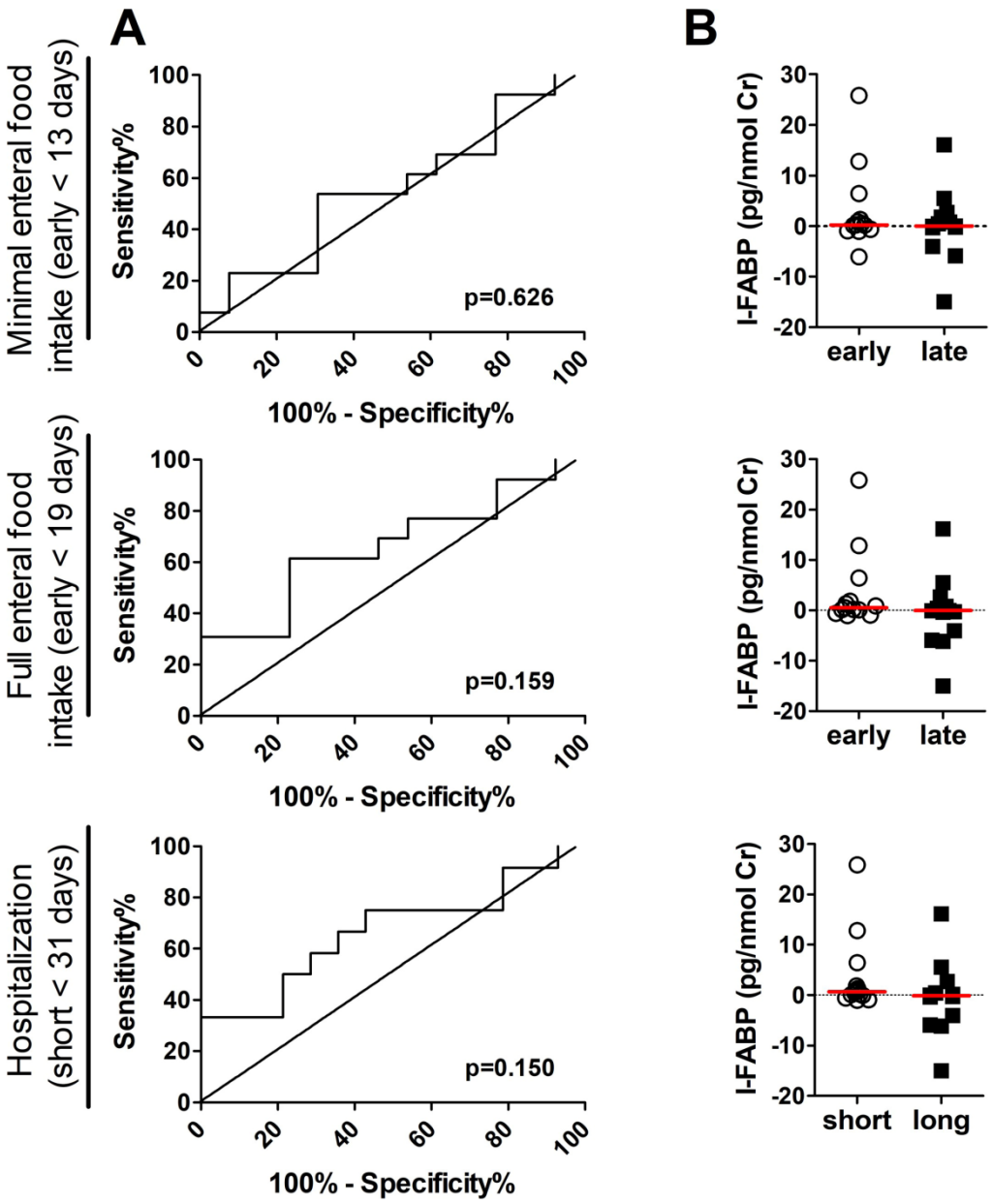
A
B
Minimal enteral food intake (early < 13 days)
Full enteral food intake (early < 19 days)
Hospitalization (short < 31 days)
Sensitivity%
100% - Specificity%
p=0.626
p=0.159
p=0.150
I-FABP (pg/nmol Cr)
early
late
short
long

Fig 2. The analysis of predictive capacity of I-FABP for clinical outcome. The decrease in urinary I-FABP between 12 and 18h after the surgery (ΔI-FABP 12-18h) does not distinguish between early and late start of minimal/full enteral feeding or short and long hospitalization as measured by ROC curve analysis (Fig 2A) or conventional statistics (Fig 2B).

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damage to the gut mucosa. In a recent meta-analysis, Kunz et al. compared short term outcomes of primary closure (PC) versus stepwise reconstruction (SR), finding that SR is associated with improved outcomes only if the method is selected randomly. Conversely, when the method is selected by the surgeon, PC is associated with improved outcomes [17]. This is probably caused by the fact that patients receiving silo are also more likely to be prone to worse clinical outcomes [18].

We found that while I-FABP quickly decreases in GS after the surgery, in patients with complex GS and controls with intestinal atresia, it increases again with a distinct peak at 30–36 hours after the surgery. This suggests that this delayed release of I-FABP after the small intestine surgery is either caused by protracted stricture of the circulation, which combines higher intestinal damage and delayed I-FABP release, or that it is a result of re-perfusion damage to the intestine [4]. This is in agreement with studies on animal models of GS, showing that increased intra-abdominal pressure leads to gut mucosa damage, possibly via oxidative stress and an increase in apoptotic activity of enterocytes [19, 20].

Interestingly, I-FABP is significantly higher in neonates with complex GS that were later operated for mechanical ileus compared to those operated just for silo removal, which further supports the hypothesis about continuous gut damage. These data, however, need to be interpreted with caution, because the number of neonates in both subgroups is low.

We found that the patient's recovery (MEF, FEF and LOH) is significantly faster in patients with simple GS than in those with complex GS. Several markers have been used to determine the patient outcome after GS surgery. Most of them focus on the sonographic findings on the fetal gut during prenatal examination. Dilated stomach of the fetus with gastroschisis is associated with higher neonatal death rate, volvulus, delayed enteral feeding and longer hospital stay postnatally [21]. Intraabdominal bowel dilation of multiple intestinal loops predicts not only earlier delivery, but also postnatal bowel complications in neonates with gastroschisis [22]. Not only the extent of the intraabdominal bowel dilation, but also its early appearance is associated with poor prognosis [23]. Prenatal bowel dilatation is associated with increased morbidity in patients with simple GS [24–26]. Measurement of the intraabdominal pressure (IAP) during surgery for gastroschisis may help to select optimal surgical technique and shorten the hospital stay [27]. Since we have just started with routine measurements of IAP during this study, we do not have data to correlate this potential biomarker with I-FABP levels.

We hypothesized that disruption of intestinal mucosal layer could be a major cause of complications in patients with GS and that the higher degree of gut mucosa damage predisposes to slower post-operative recovery. Since there are no guidelines for the division into early MEF/FEF and short LOH, we decided to use an unbiased approach and divide our dataset into two halves. Unfortunately, none of the measured levels of I-FABP could distinguish between these categories, although the decrease in I-FABP between 12 and 18h (or 12 and 24h) post-surgery explained well the variation of all three measured outcomes in regression analysis (Table 2).

We found that urinary I-FABP during the first 6 hours after surgery was significantly higher in complex GS patients who would be later operated for mechanical ileus than in those operated only for silo removal. There were, however, not enough patients with complex GS re-operated for ileus in our dataset to allow appropriate statistical analysis. The main advantage of single-center studies of rare diseases is the absence of variation between centers, but it comes at a cost of a small study population. This means that the statistical analysis is

underpowered and notable to properly assess some important facets of the disease. This is the main reason why we could not compare individual surgery protocols and why we could define only the broadest disease stages (simple GS vs. complex GS) and basic procedures (PC vs. SR).

Conclusions

Urinary I-FABP is a marker for intestinal mucosa damage in GS. Patients with complex GS have significantly higher levels of I-FABP and their recovery takes longer than in patients with simple GS. I-FABP fails to predict early MEF/FEF or shorter LOH, so it is not suitable for prediction of these parameters in clinical settings. Its capacity to predict subsequent operation for ileus in patients with complex GS needs to be interpreted with caution until a larger cohort of these patients is analyzed.

Supporting information

S1 Fig. The analysis of predictive capacity of I-FABP for multiple surgeries.
(TIF)

S1 Table. Type of surgery in controls for simple GS.
(DOCX)

S2 Table. Correlation matrix of individual values and demographic characteristics of GS patients.
(XLSX)

S3 Table. The raw data of studied GS and control patients.
(XLSX)

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9.4. Inflammatory Bowel Disease Types Differ in Markers of Inflammation, Gut Barrier and in Specific Anti-Bacterial Response

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Using protein array and subsequently using ELISA we found typical patterns of serum biomarkers that could distinguish CD, UC and healthy controls. Further, we analyzed the predictive value of these serum biomarkers. We found that proteins of matrix metalloproteinase system (MMP-9, MMP-14, TIMP-1) were the strongest factors discriminating IBD patients from healthy controls. Patients suffering from UC had decreased levels of osteoprotegerin in comparison with CD and PSC-IBD. The osteoprotegerin was not only the strongest factor for distinguishing of patients with UC and PSC-IBD (AUC=0.916), but in the combination with I-FABP, CXCR-1 and TIMP-1 it can also distinguish the patients suffering from UC and CD (AUC=0.924). We found that low levels of transforming growth factor-β1 (TGF-β1) was associated with disease relapse and when combined with TFF-3, MMP-9 and lipopolysaccharide binding protein (LBP) it can discriminate IBD patients according the colitis activity (AUC=0.909).

Although, IBD patients respond mostly similarly to selected commensal bacteria as healthy controls, the patients suffering from CD have lower antibody response, with significant decrease in IgA to *Faecalibacterium* and *Bacteroidetes* than healthy controls (40% of CD patients have undetectable IgA response to *Faecalibacteirum*). Furthermore, we found increase T cells response to these bacteria in CD patients.

Thus, these results stress the importance of the gut barrier function and immune response to commensal bacteria and point at the specific differences in pathogenesis of CD, UC and PSC-IBD.

My contribution: sample analyses, data analyses and interpretation, manuscript writing

cells

MDPI

Article

Inflammatory Bowel Disease Types Differ in Markers of Inflammation, Gut Barrier and in Specific Anti-Bacterial Response

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Abstract: Crohn's disease (CD), ulcerative colitis (UC) and inflammatory bowel disease (IBD) associated with primary sclerosing cholangitis (PSC-IBD), share three major pathogenetic mechanisms of inflammatory bowel disease (IBD)-gut dysbiosis, gut barrier failure and immune system dysregulation. While clinical differences among them are well known, the underlying mechanisms are less explored. To gain an insight into the IBD pathogenesis and to find a specific biomarker pattern for each of them, we used protein array, ELISA and flow cytometry to analyze serum biomarkers and specific anti-microbial B and T cell responses to the gut commensals. We found that decrease in matrix metalloproteinase (MMP)-9 and increase in MMP-14 are the strongest factors discriminating IBD patients from healthy subjects and that PSC-IBD patients have higher levels of Mannan-binding lectin, tissue inhibitor of metalloproteinases 1 (TIMP-1), CD14 and osteoprotegerin than patients with UC. Moreover, we found that low transforming growth factor-β1 (TGF-β1) is associated with disease relapse and low osteoprotegerin with anti-tumor necrosis factor-alpha (TNF-α) therapy. Patients with CD have significantly decreased antibody and increased T cell response mainly to genera *Eubacterium*, *Faecalibacterium* and *Bacteroides*. These results stress the importance of the gut barrier function and immune response to commensal bacteria and point at the specific differences in pathogenesis of PSC-IBD, UC and CD.

Keywords: inflammatory bowel disease; biomarkers; gut barrier; microbiota; antibodies; T cells

1. Introduction

Inflammatory bowel diseases (IBD), i.e., Crohn's disease (CD) and ulcerative colitis (UC), are severe chronic inflammatory illnesses of the gastrointestinal tract, affecting more than 0.3% of the people in many countries [1]. Although their etiology and pathogenesis is not fully understood, it is generally accepted that the inflammation results from an aberrant immune response to antigens of resident gut microbiota in genetically susceptible individuals [2]. Moreover, primary sclerosing cholangitis (PSC), chronic liver disorder characterized by inflammation and stenosis of the bile ducts, with concomitant IBD (PSC-IBD) has recently emerged as another form of IBD [3]. Despite the well-established headlines in IBD therapy, discontinuation of pharmacological intervention due to the inefficiency or adverse events is still common in all types of IBD therapy [4–6]. Ability to predict the disease relapses and

complications or suggest the ideal therapy for a particular patient during the time of diagnosis is a worthy goal of IBD diagnostics. Taking into account the complex and intertwined pathogenesis together with distinct forms of IBD, studies of those mechanisms may yield suitable biomarkers. Three major mechanisms are involved in IBD pathogenesis, gut microbiota dysbiosis, gut barrier failure and dysregulation of the immune system.

In humans, gut microbiota represents a complex ecosystem that consists of more than 1000 species of bacteria, five genera of Archaea, 66 genera of fungi and an ill-defined number of viruses, mostly bacteriophages [7–9]. The gnotobiotic (i.e., germ-free or artificially colonized animals) studies clearly showed that without this complex ecosystem, the immune system and many other physiological functions would never reach their full potential [10]. While gut microbiota cannot induce intestinal inflammation on its own [11], imbalances in intestinal microbiota (i.e., dysbiosis), or the presence of commensal bacteria with increased virulence in IBD patients, could cause excessive anti-microbial immune response [12–15]. It is still unclear, however, if these microbial perturbances in IBD are cause, consequence or just a confounding factor [16].

The gut barrier is a complex apparatus consisting of a mucus layer, a tightly connected epithelium supported by mucosal immune cells and their products that protect an organism's integrity [17]. Disruption of this barrier (defects of the epithelial continuity) increases its permeability allowing the excessive contact of the luminal antigens with the immune cells, which is one of the key steps in pathogenesis of IBD and several other diseases [18]. The gut barrier disruption is responsible for many IBD symptoms even during the mucosal healing [19]. Both UC and CD patients with an active disease have severe impairment of this barrier on multiple levels [20,21]. Several noninvasive biomarkers of gut barrier failure were suggested. Since both Intestinal and Liver Fatty Acid-Binding Proteins (I-FABP, L-FABP) reflect gut epithelium damage, they were previously successfully used as early biomarkers for severe neonatal emergencies, such as necrotizing enterocolitis or gastroschisis [22–24]. Because the matrix metalloproteinase (MMP) system has an important role in the gut barrier remodeling, both fecal and serum MMP-9 levels were suggested as promising biomarkers of gut barrier health [25,26].

The impairment of host–microbe interactions in IBD pathogenesis is supported by genome-wide association studies, which identified an association of IBD with multiple polymorphisms in genes encoding regulation of immune processes including the recognition, processing and killing of microorganisms [27,28]. Disruption of regulatory T cell functions and impairment of the mucosal immune response to normal microbiota play a crucial role in the pathogenesis of chronic intestinal inflammation [29]. Although the typical T helper (Th)1 and Th17 response is associated with CD pathogenesis, the presence of antibodies to some microbial constituents in sera of patients suggests a much broader spectrum of immune reactions in IBD [30].

The diagnosis of IBD and its clinical staging is still based mainly on the patient's history and medical examination, where endoscopy plays a major part. Several serological tests were proposed to improve the IBD diagnostics and some of them showed promising predictive value. The anti-Saccharomyces cerevisiae antibodies (ASCAs) reacting to the mannan protein in the *Saccharomyces cerevisiae* are significantly increased and highly specific for CD patients even if they have clinical remission and the perinuclear anti-neutrophil cytoplasmic antibodies (pANCAs) are increased in UC patients [31–33]. Apart from ASCA, serum antibodies to other microbial antigens were not only a source of potential biomarkers for IBD diagnosis and differential diagnosis, but also suggested the importance of anti-microbial response in IBD pathogenesis. These biomarkers included *Escherichia coli* outer membrane porin C (anti-OmpC), anti-flagellin (anti-Cbir1) [34] and the anti-I2 component of *Pseudomonas fluorescens* (anti-I2) [35]. Other biomarkers, such as serum and fecal calprotectin, fecal lactoferrin, S100A12, Lipocalin-2, showed promising results in relapse prediction. However, the utility of these markers in daily clinical practice is still rather low [36–38].

In this study, we performed broad analysis of serum and peripheral blood mononuclear cells (PBMCs) biomarkers, including chemokines, cytokines, specific antibodies and specific anti-microbial

T cell reactivity to gain an insight into the IBD pathogenesis and to find biomarker pattern specific for each form of IBD.

2. Materials and Methods

2.1. Study Population

All individuals were recruited from the patients admitted to the Hepatogastroenterology Department of the Institute for Clinical and Experimental Medicine (IKEM), Prague, Czech Republic, between May 2015 and December 2018. In total, we enrolled 119 patients with different forms of IBD; CD, UC and PSC-IBD and 28 healthy individuals served as controls (HC) (Table 1). Since CD can involve different parts of gastrointestinal tract, all IBD patients had colonic involvement to minimize the variability. Serum was aliquoted and stored at −20 °C until analyses.

Table 1. Clinical characteristics of the study participants. CD: Crohn's disease; HC: healthy control; PSC: primary sclerosing cholangitis; UC: ulcerative colitis.

	HC n = 28	PSC n = 47	UC n = 52	CD n = 20
Age (mean ±SD; years)	42.5 ± 10.5	38.0 ± 11.6	39.7 ± 9.8	33.5 ± 7.8
Sex (% of males)	53.6; 15/13	74.5; 35/12	53.9; 28/24	45.0; 9/11
Activity (% of active)	0.0	14.0	26.9	20.0
Extent of intestinal inflammation				
none (%; n)	100.0; 28	12.8; 6	0.0; 0	0.0; 0
partial (%; n)	0.0; 0	10.6; 5	38.4; 20	45.0; 9
pancolitis (%; n)	0.0; 0	72.3; 34	61.5; 32	50.0; 10
Therapy				
Mesalazine (5-ASA) (%; n)	0.0; 0	70.2; 33	92.3; 48	85.0; 17
Glucocorticoids (%; n)	0.0; 0	38.3; 18	21.2; 11	15.0; 3
Azathioprine (AZA) (%; n)	0.0; 0	31.9; 15	40.4; 21	35.0; 7
Anti-TNF-α (%; n)	0.0; 0	0.0; 0	38.5; 20	45.0; 9
E. coli Nissle 1917 (%; n)	0.0; 0	8.5; 4	23.1; 12	20.0; 4

2.2. Antibody Array Assay for Serum Biomarkers

A training set of 18 samples, six each of HC, UC and CD, was assayed for the relative amount of 507 human proteins using RayBio Label-Based (L-Series) Human Antibody Array L-507 according to the manufacturer's protocol (RayBiotech, Peachtree Corners, GA, USA). The target proteins included cytokines, chemokines, adipokines, growth factors, angiogenic factors, proteases, soluble receptors and soluble adhesion molecules. The signals were scanned at a wavelength of 532 nm using GeneTAC UC4 Microarray Scanner (Genomic Solution, United Kingdom; resolution, 10 µm), and the resulting image was analyzed and processed in AG Scan software (ver. 18.7. 2007, The GenoToul bioinformatics, France) [39]. To compare the median fluorescence intensity (MFI) values, we subtracted the background staining and normalized the data to the positive control MFI average for all arrays, and then transformed to z-scores for each protein. The classifiers for HC, UC and CD were analyzed by nearest shrunken centroid method by Prediction Analysis of Microarrays (PAM; ver. 1.56) package for R (ver. 3.5.2; R Foundation for Statistical Computing, Vienna, Austria) [40].

2.3. ELISA for Serum Biomarkers

Next, we selected several biomarkers found by microarray profiling and several other, proposed markers and quantified them in the serum by ELISA (Table 2). Due to the limited amount of sample, not all samples were analyzed for all biomarkers.

Table 2. List of biomarkers quantified in sera of inflammatory bowel disease (IBD) patients and healthy subjects.

Biomarker	Abbreviation	Manufacturer	Cat. No
Endocrine-Gland-derived Vascular Endothelial Growth Factor *	EG-VEGF	R&D systems	DY1209
Interleukin-8 receptor, alpha *	CXCR1/IL8RA	LifeSpan BioScience	LS-F11255
Osteoprotegerin	OPG	R&D systems	DY805
Tomoregulin 1	TMEFF1	LifeSpan BioScience	LS-F52730
Insulin-like Growth Factor 2 *	IGF2	R&D systems	DY292
Transforming Growth Factor-β1 *	TGF-β1	R&D systems	DY240
TROY protein	TNFRSF19	LifeSpan BioScience	LS-F39966
Roundabout Guidance Receptor 4 *	ROBO4	RayBiotech	ELH-ROBO4
Matrix Metalloproteinase 9	MMP-9	R&D systems	DY911
Matrix Metalloproteinase 14	MMP-14	R&D systems	DY918
Tissue Inhibitor of Metalloproteinases 1	TIMP-1	R&D systems	DY970
Mannan-Binding Lectin	MBL	R&D systems	DY2307
Soluble CD14	CD14	R&D systems	DY383
Lipopolysaccharide-Binding Protein	LBP	R&D systems	DY870
Trefoil Factor 3	TFF-3	R&D systems	DY4407
Endotoxin-Core Antibody IgM	EndoCab	MyBiosource	MBS9352896
Serum Amyloid A	SAA	HyCult Biotech	HK333
Pre-haptoglobin 2	Zonulin	MyBiosource	MBS2880564
D-amino-acid oxidase	DAAO	MyBiosource	MBS2886321
Intestinal Fatty Acid-Binding Protein	I-FABP	HyCult Biotech	HK406
Liver Fatty Acid-Binding Protein	L-FABP	HyCult Biotech	HK404

* Identified by the array.

2.4. Bacterial Antigen Preparation

We selected typical representatives of healthy Czech gut microbiota, using data from our previous study [41]. Different bacteria were cultured in their respective optimal media for 24 h at 37 °C (Table S1). Fresh bacterial culture was centrifuged, pellets were washed in sterile water (B. Braun Medical, Prague, Czech Republic) and inactivated by French press (French Pressure Cell Press Model FA-078, SLM Instruments) at 1500 psig, the pressurizing procedure was repeated three times. Samples were freeze dried in a lyophilizer (Lyovac GT 2, Leybold Heraeus) and stored in aliquots at -28 °C until analyses. The following bacterial strains were used for PBMCs stimulation or for antigen coating in indirect ELISA: *Lactobacillus plantarum*, *Bifidobacterium adolescentis*, *Blautia coccoides*, *Roseburia intestinalis*, *Eubacterium rectale*, *Faecalibacterium prausnitzii*, *Ruminococcus flavefaciens*, *Bacteroides thetaiotaomicron*, *Prevotella ruminicola* and *Escherichia coli*.

2.5. Peripheral Blood Mononuclear Cells (PBMC)

Human Peripheral Blood Mononuclear Cells (PBMCs) were isolated by Ficoll-Paque Plus (GE-Healthcare; Cat# 17-1440-03) density gradient centrifugation (740 × *g*, 30 min, room temperature (RT), brake OFF) from heparinized blood and stored at −150 °C using freeze-thaw method optimized for maximum viability [42]. Briefly, after the collection the cells were washed in sterile pre-warmed phosphate-buffered saline (PBS) (330 × *g*, 10 min, RT), re-suspended in pre-warmed Roswell Park Memorial Institute (RPMI) medium (Sigma-Aldrich; Cat# R0883), counted and re-suspended at 20×10^6 /mL live cells in RPMI containing 60% Fetal calf serum (FCS; Biochrom GmbH, Germany; Cat# S 0115). Next, the equal volume of pre-warmed mixture of 80% FCS and 20% DMSO (Sigma-Aldrich; Cat# D 2650) was gently added drop wise with gentle shaking after each drop to equalize the cryopreservant. After the 5 min incubation (RT), cells were aliquoted to cryovials and gently frozen in a Mr. Frosty Freezing Container (Thermo Fisher Scientific; Cat# 5100-0001) at −80 °C. After 12–48 h,

cells were transferred to −150 °C for long term storage until analyses. For thawing cryotubes were placed in a 37 °C water bath for 8 min, then transferred to a 15 mL tube, diluted drop wise with 8 mL of pre-warmed RPMI medium, centrifuged (300 × *g*, 5 min, RT) and the supernatant was discarded. After another washing step, the cells were counted using Trypan blue exclusion and diluted to 2×10^6 /mL live cells. Cells were then transferred to sterile 96U-well tissue culture plate (TPP, Trasadingen, Switzerland; Cat# 92197) at 100 µL/well in complete RPMI medium containing 10% FCS, 1% antibiotic-antimycotic solution (Sigma-Aldrich; Cat# P 0781) and 1% L-glutamine solution (Sigma-Aldrich; Cat# 1.00289) and placed into the humidified incubator (37 °C, 5% CO_2) for 2 h before the stimulation. Next, 100 µL/well of the stimulus was added and the cells were incubated under similar conditions for another 14 h. The final concentration of microbial lysate was 10 µg/mL and 1 µg/mL of *Staphylococcus aureus* toxin B (SEB; Sigma-Aldrich; Cat# S 4881) served as a positive control.

2.6. Indirect Enzyme-Linked Immunosorbent Assays (ELISA)

The serum concentrations of anti-bacterial antibodies in Immunoglobulin M (IgM), Immunoglobulin G (IgG) and Immunoglobulin A (IgA) isotypes were analyzed by in-house developed indirect ELISA. Bacterial lysates, were dissolved in phosphate buffered saline (PBS) and incubated at 0.1 mg/mL (*Prevotella*, *Ruminococcus* and *Bacteroides*), 0.5 mg/mL (*Faecalibacterium*) or 1 mg/mL overnight in the 96F-well plate (NUNC Maxisorp; Cat# 442404). Optimal concentration of the coated lysates was extensively tested with sera of HC and IBD patients, but there were no major differences between 0.1 and 5 mg/mL for most lysates. Next, plates were washed with 1xPBS containing 0.05% Tween® 20 (Merck KGaA, Darmstadt, Germany). Each well was blocked with 1% Bovine Serum Albumin (BSA; Merck) for 1 h. After the washing procedure, patient serum samples were applied in appropriate dilution. After 2 h of incubation plates were washed and corresponding secondary antibody (Peroxidase-conjugated AffinniPure F(ab')2 fragment goat anti-human Fc fragment specific; Jackson ImmunoResearch Laboratories, Inc., Ely, UK; Cat# 109-036-170, 109-036-011 or 109-036-129) was added and incubated 1 h in the dark. After washing, substrate solution was added and plates were incubated for 5 min in the dark. Absorbance was measured at 450 nm and 650 nm by spectrophotometer (Multiskan Ascent Plate Reader 96/384, MTX Lab Systems). Selected serum sample was used on all plates to serve as a standard. Its serial dilutions were used for the antibody response quantification with optical density (OD) at 1:200 defined as 1000 arbitrary units (AU).

2.7. Flow Cytometry Analysis (FACS)

Cells were stained with the Fixable Viability Dye eFlour 780 (eBioscience, San Diego, CA, USA; Cat# 65-0865-18) and following fluorescently labeled monoclonal antibodies: Fluorescein isothiocyanate (FITC) anti-human CD3 Antibody (UCHT1; Biolegend, San Diego, CA, USA; Cat# 300452), Qdot 605 anti-human CD4 Antibody (S3.5; Invitrogen, Carlsbad, CA, USA; Cat# Q 1008), Alexa Flour 700 anti-human CD8 Antibody (SK1; Biolegend; Cat# 344724), Brilliant Violet 711 anti-human Interleukin (IL)-17A Antibody (BL168; Biolegend; Cat# 521327), Allophycocyanin (APC) anti-human IL-4 Antibody (8DE-8; eBioscience, Cat# 17-7049-81), Brilliant Violet 510 anti-human Tumor necrosis factor-alpha (TNF-α) Antibody (Biolegend; Cat# 502949), Phycoerythrin (PE) anti-human Interferon-gamma (IFN-γ) Antibody (4S.B3; eBioscience; Cat# 12-7319-81), PE-Cyanine7 IL-2 Antibody (MQ1-17H12; eBioscience; Cat# 25-7029-41), Brilliant Violet 421 anti-human CD154 (24-31; Biolegend; Cat# 310 824).

For intracellular cytokine staining, cells were stimulated for 14 h with Staphylococcal enterotoxin B from *Staphylococcus aureus* (final concentration 1 µg/mL; Merck) or with corresponding bacterial lysates (final concentration 10 µg/mL). Brefeldin A (final concentration 3 µg/mL, eBioscience) and Monensin (final concentration 2 µM, eBioscience) were added and after 4 h the cells were stained with Fixable Viability Dye, fixed with Intracellular (IC) Fixation Buffer (Invitrogen), and stained for cytokines in Permeabilization Buffer (Invitrogen). Gating on CD154 (CD40 ligand) was combined with intracellular cytokine analysis to focus on activated memory T helper cells as described previously [43].

Human Fc-γ receptor (FcR) Binding Inhibitor Purified (eBioscience) was used for inhibition of the non-specific FcR-mediated binding of monoclonal antibodies. UltraComp eBeads Compensation Beads (Invitrogen) were used for compensations. Cells were analyzed on FACS LSR II (BD Biosciences, San Jose, CA, USA). Data were analyzed with FlowJo (version 7.2.5., Tree Star, Inc., Ashland, OR, USA).

2.8. Ethics Statement

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Institute of Clinical and Experimental Medicine and Thomayer Hospital (G 14-08-45).

2.9. Statistical Analysis

Non-parametric Kruskal–Wallis test with Dunn's multiple comparison test was used to compare multiple experimental groups and the Mann–Whitney test was used to compare two experimental groups. Non-parametric paired Friedman test with Dunn's multiple comparison test was used to compare the CD154 expression after the cultivation with different microbial antigens with CD154 expression in non-stimulated sample. The data are presented as the median ±95% confidence interval and differences were considered statistically significant at $p \leq 0.05$. GraphPad Prism statistical software (version 8.1.1, GraphPad Software, San Diego, CA, USA) was used for analyses.

Regression analysis was performed in R and the effect of each biomarker on Akaike information criterion (AIC) was determined in the nnet package (ver. 7.3-12). Next, we performed both backward elimination and forward selection based on AIC to determine the best regression model to discriminate between the two states. The composite receiver operating characteristic (ROC) curves were constructed and their area under the curve (AUC) was calculated using ROCR package (ver. 1.0-7).

Hierarchical clustering was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and the heatmap.plus (ver. 1.3) package for R.

3. Results

3.1. Healthy Subjects, CD and UC Patients Each Have a Distinct Cytokine Signature in Human Serum

Using the nearest shrunken centroid method, we searched for typical patterns of serum proteins that could differentiate between HC, CD and UC (Figure 1A). Subsequent cluster analysis of these proteins showed good separation of HC from IBD patients and their separation between UC and CD (Figure 1B). When the training set of patients (HC = 9, UC = 9 and CD = 10) was analyzed by ELISA, the ability to classify was only marginal, with osteoprotegerin (OPG) having the strongest effect. Both tomoregulin 1 (TMEFF1) and roundabout guidance receptor 4 (ROBO4) had only a negligible role on the HC vs. IBD classification (AUC = 0.785) and OPG was the strongest discriminating factor. In these cases, ELISA was in agreement with the protein array, with OPG being increased in CD and endocrine-gland-derived vascular endothelial growth factor (EG-VEGF) in UC, but neither difference was statistically significant.

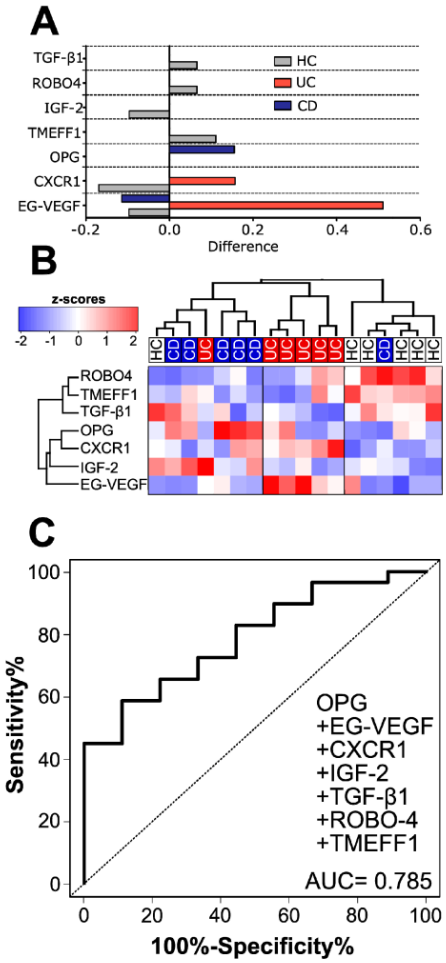

Figure 1. HC can be easily distinguished from IBD by only seven proteins, but separation of UC and CD is not as clear: (**A**) Shrunken differences for the seven differently abundant proteins in sera; (**B**) Heat map and cluster analysis of the chosen proteins. "HC" healthy controls, "UC" ulcerative colitis, "CD" Crohn's disease.; (**C**) Composite receiver operating characteristic (ROC) curve for the seven proteins analyzed by ELISA with the training set of samples HC (n = 10) and IBD patients consisting of UC (n = 9), CD (n = 10) and PSC-IBD (n = 10).

3.2. Validation of Microarray Data by ELISA

Next, we analyzed the predictive value of these potential serum biomarkers using ELISA. Moreover, we selected other serum biomarkers of the gut barrier (MMP-9, MMP-14, tissue inhibitor of metalloproteinases 1 (TIMP-1), zonulin, I-FABP, L-FABP and trefoil factor 3 (TFF-3)) or inflammation (mannan-binding lectin (MBL), CD14, lipopolysaccharide-binding protein (LBP), EndoCab, serum amyloid A (SAA), D-amino-acid oxidase (DAAO) and TNF receptor superfamily member 19 (TNFRSF19) that may be involved as well. This analysis was performed in a larger cohort of patients including patients with UC, CD and PSC-IBD. We focused on the analysis of disease type, presence of the complications, disease activity, extent and localization. Except for some patient's samples, both interleukin-8 receptor, alpha (CXCR1) and TNFRSF19 were below the detection limit of 195 ng/mL and

781 pg/mL, respectively. First, we found that many of these biomarkers are significantly increased in PSC-IBD patients. Except for the significant increase in TIMP-1 in CD, none of the other groups differed significantly from healthy controls (Figure S1), but the proteins from the MMP system (MMP-9, MMP-14 and TIMP-1) were the strongest discriminating factor in all forms of IBD (Figure 2B,C). Decreased serum concentration of OPG is typical for UC, as compared to CD and PSC-IBD (Figure 3), but while LBP was the strongest predictor of them all (AUC = 0.663), neither serum biomarker was capable to predict extent of colitis well (Figure S2).

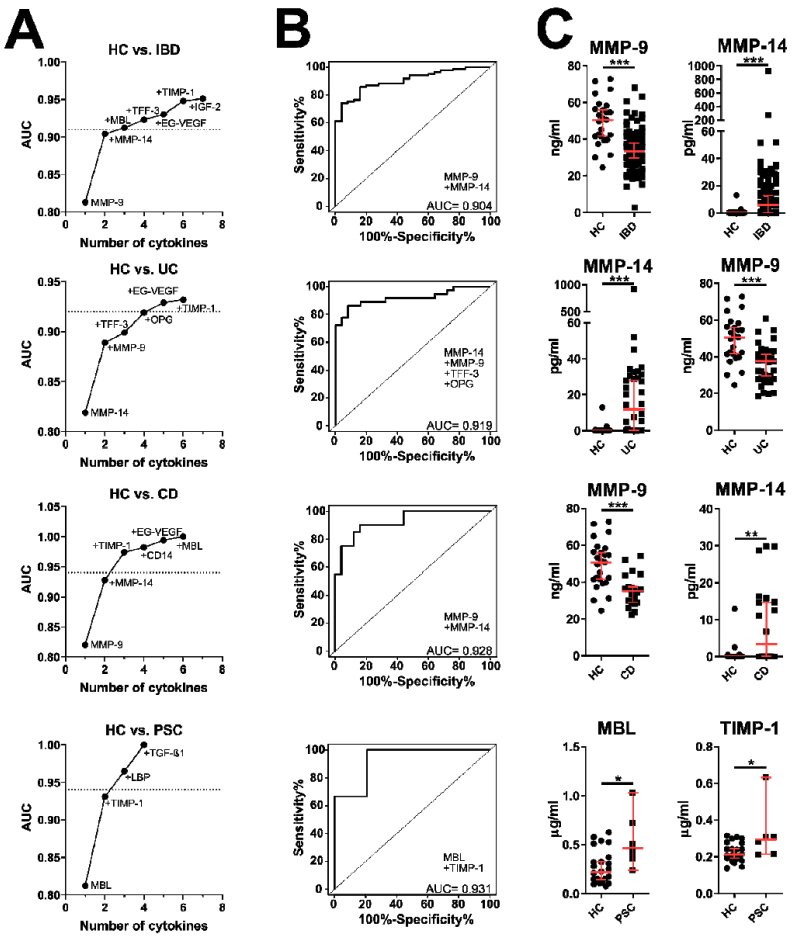

Figure 2. Cytokine patterns discriminating different forms of IBD from healthy controls. (**A**) Relative importance for each cytokine for the AUC increment within the best model found by regression analysis and (**B**) composite ROC curve analysis with the reliable discriminating power (AUC > 0.9). (**C**) Quantitative plot of the two most efficient discriminating factors analyzed by Mann Whitney test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Full quantitative comparison across all types of IBD is in Figure S1. Healthy controls (HC, $n = 25$), IBD patients ($n = 85$), UC patients ($n = 36$), CD patients ($n = 20$), PSC patients without concomitant IBD (PSC; $n = 6$).

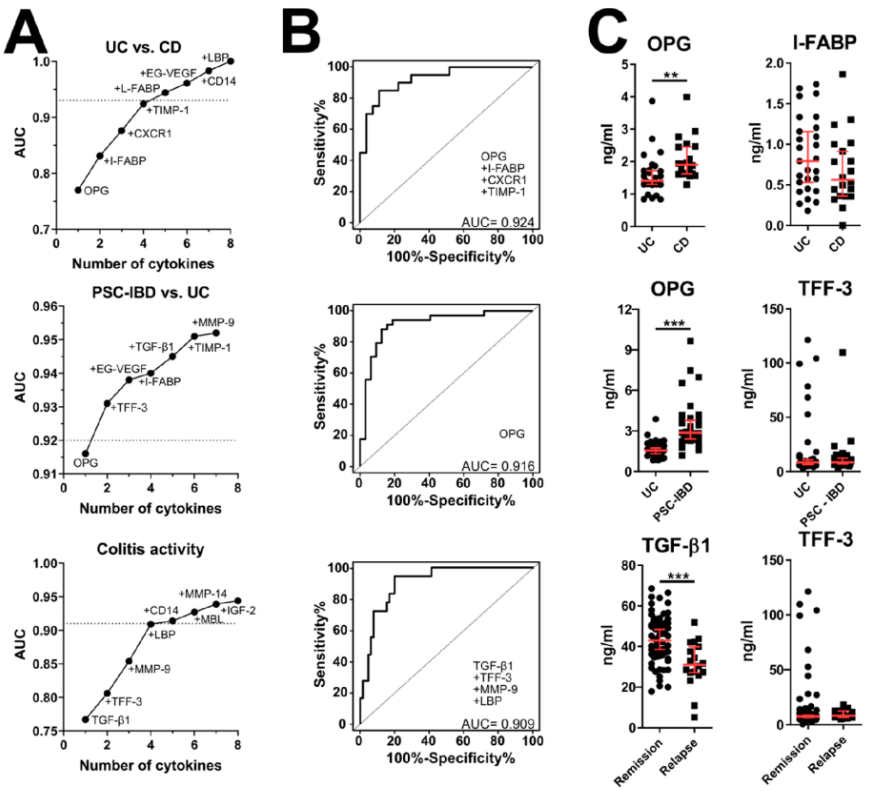

Figure 3. Significant differences in serum biomarkers between different types of IBD and activity. (**A**) Relative importance for each cytokine for the AUC increment within the best model found by regression analysis and (**B**) composite ROC curve analysis with the reliable discriminating power (AUC > 0.9). (**C**) Quantitative plot of the two most efficient discriminating factors analyzed by Mann–Whitney test. ** $p < 0.01$, *** $p < 0.001$. HC ($n = 25$), UC patients ($n = 36$), CD patients ($n = 20$), PSC-IBD patients ($n = 32$), Remission ($n = 66$), Relapse ($n = 18$).

3.3. Serum Antibodies Against Bacteria

The importance of gut barrier functions and gut microbiota for the IBD pathogenesis suggested specific antibodies to gut commensal microbiota as suitable biomarkers. Therefore, we analyzed IgA, IgG and IgM antibodies specific to gut commensal bacteria in serum of IBD patients and healthy controls. We found that patients with IBD respond in similarly to most commensal bacteria as healthy controls, with few notable exceptions (Figure 4A). CD patients have generally lower antibody response, with significantly decreased IgA response to *Faecalibacterium* and *Bacteroides* as compared to healthy controls. In fact, 23% of patients with UC and 40% of patients with CD but none with the PSC-IBD have undetectable IgA response to *Faecalibacterium*. Further analysis showed clear positive correlation within isotypes, but not across them, suggesting that each of them acts independently and that gut commensals share antigenic determinants (Figure 4B). The latter case is supported by the fact that the strongest correlation is among least specific IgM and the weakest is among the generally more specific IgG.

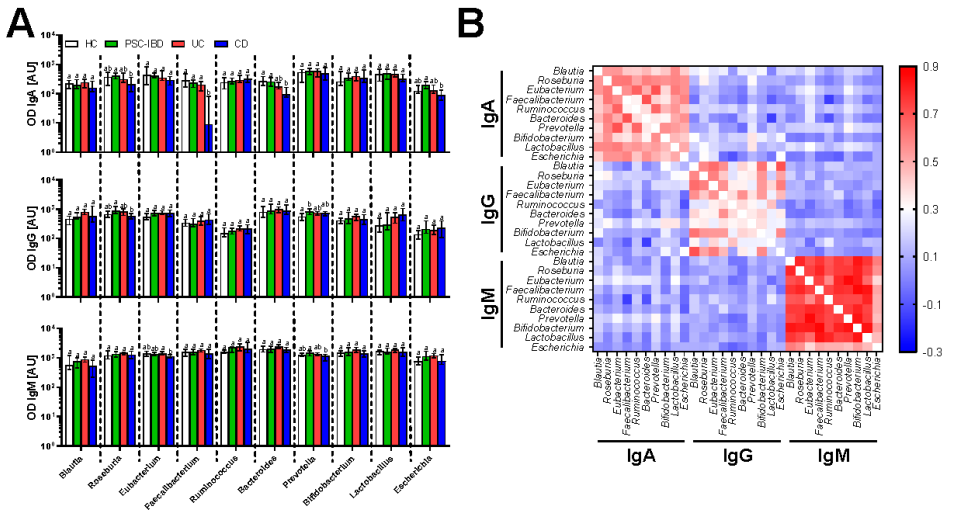

Figure 4. Differences in antibody response among patients with different forms of IBD and healthy controls. (**A**) Comparison of specific anti-bacterial antibody response. Different letters indicate statistically significant differences. (**B**) Correlation matrix showing Spearman's rank correlation coefficient. HC (n = 27), PSC-IBD (n = 41), UC (n = 52), CD (n = 20).

3.4. Circulating Gut Microbiota Reactive T-cells

While there is some specific pattern in CD154 expression on helper T cells among healthy controls and in different forms of IBD, PBMCs from all subjects reacted strongly to antigens from Clostridiales XIVa cluster, *Prevotella*, *Lactobacillus* and *Escherichia* in all subjects regardless of presence or absence of IBD. IBD patients have this spectrum of reactivity broadened to *Bifidobacteria* in patients with UC, *Faecalibacterium* in patients with PSC-IBD and CD and *Ruminococcus* in patients with CD (Figure S6). Circulating $CD4^{+}CD154^{+}$ T cells react to gut bacteria with production of several pro-inflammatory cytokines. However, their reactivity in patients with UC and PSC-IBD is generally similar to HC. T cells from CD patients react more strongly to antigens from *Roseburia*, *Eubacterium*, *Faecalibacterium* and *Bacteroides* (Figure 5). The potential for maximum cytokine production, after super-antigen stimulation, is similar for all tested groups in all cytokines but IL-17. Upon stimulated with SEB, PBMCs from CD patients have higher significantly proportion of IL-17+CD154+ T cells than HC.

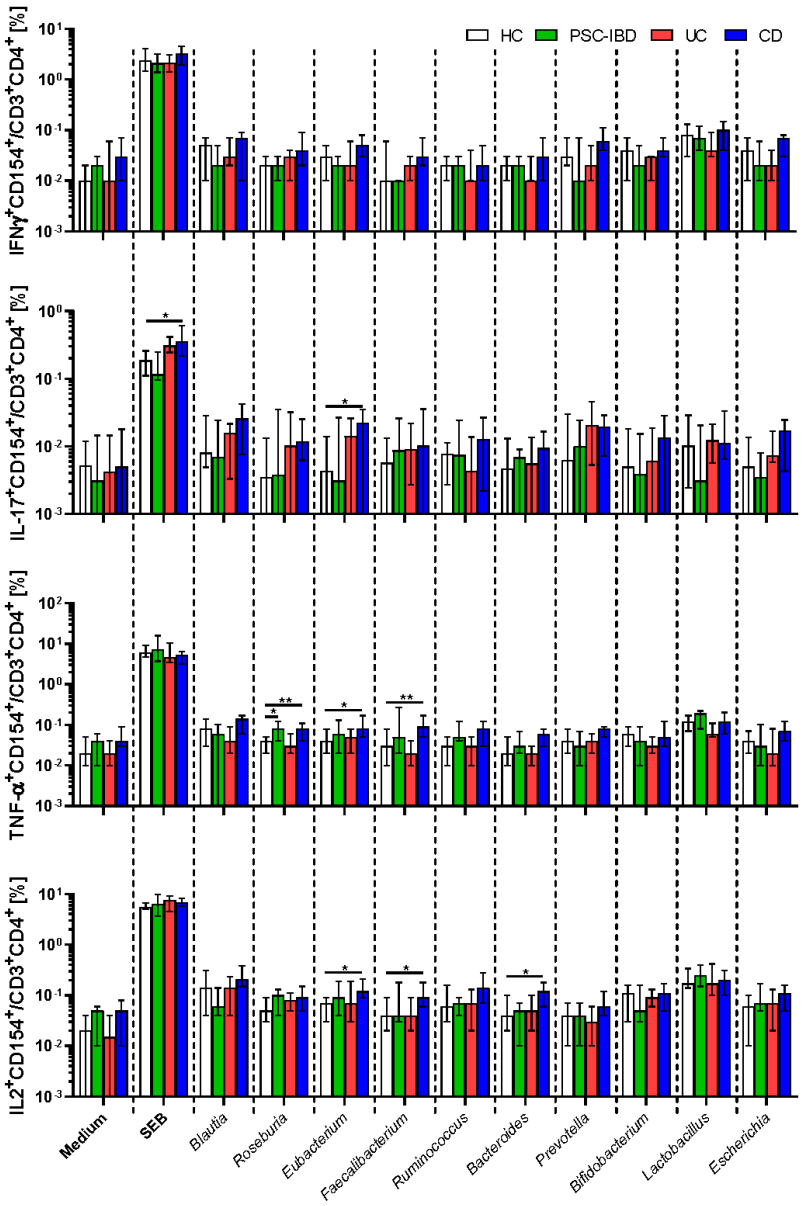

Figure 5. Circulating microbiota-reactive T cells react more strongly in CD patients than in any other form of IBD as analyzed by the Kruskal–Wallis test with Dunn's multiple comparison test vs. HC group. * $p < 0.05$, ** $p < 0.01$. HC ($n = 19$), PSC-IBD ($n = 9$), UC ($n = 15$), CD ($n = 17$).

3.5. *Effect of IBD Treatment*

Next, we analyzed how the therapy influences the serum biomarkers (Figure S3), antibodies (Figure S4) and anti-microbial T cell response (Figure S5). We found that the effect of different treatments was generally milder than the effect of disease type, but there were several factors that

clearly distinguished the effect of drugs. Mesalazine (5-ASA) increased MMP-14 and decreased IgM against *Bacteroides*, Azathioprine decreased the proportion of IFN-γ production by $CD154^+CD4^+$ T cells after their stimulation with *Roseburia* and oral *E. coli* Nissle 1917 (Mutaflor) increased serum IgG against *Bifidobacteria*. Most changes were, however, induced by anti-TNF-α treatment, which significantly decreased serum OPG, increased IFN-γ production from *Roseburia*- or *Escherichia*-treated $CD154^+CD4^+$ T cells and increased TNF-α production from *Escherichia* treated $CD154^+CD4^+$ T cells.

4. Discussion

Common pathogenetic mechanisms, gut microbiota dysbiosis, gut barrier failure and immune system dysregulation, link the different types of IBD. Yet there are clear clinical differences between CD, UC and PSC-IBD. Here, we found several serum markers that not only distinguish the major forms of IBD, but also mirror its activity or treatment. Moreover, we compared anti-microbial antibody and T cell responses to gut commensal bacteria prevalent in the Czech population, finding clear shifts in CD patients.

Using protein array, we found that out of the 507 serum proteins, high EG-VEGF and CXCR1 are strongly associated with UC and low EG-VEGF and high OPG are typical for CD. This is an interesting distinction between the two major forms of IBD, suggesting that they may differ in angiogenesis and inflammation regulation. Members of the VEGF family are not only key positive mediators of angiogenesis, but they also have a pro-inflammatory role in inflammatory diseases, including IBD [44–46]. While mainly linked to reproduction, EG-VEGF (Prokineticin 1) may mediate similar biological effects. In fact, human monocytes activated with EG-VEGF have elevated IL-12 and TNF-α and down-regulated IL-10 production in response to Lipopolysaccharide (LPS) [47]. This effect may decrease the triggering threshold in monocytes in the intestinal wall, thus worsening the inflammation when the gut barrier is breached in the vicinity of ulcers in patients with UC. CXCR-1 is a G protein-coupled receptor, which can recruit the neutrophils in the site of inflammation, induce their oxidative burst and degranulation, thus worsening the local inflammation [48,49]. This supports our findings since the neutrophils are major constituents of inflammatory infiltrate in UC [50]. OPG is not only a key factor in bone density regulation [51] but it also affects cell turnover, differentiation, death and survival [52]. Previous studies found elevated serum OPG in patients with IBD and showed that OPG correlated significantly with concentration of pro-inflammatory cytokines (e.g., TNF-α) suggesting that OPG production is influenced by cytokine milieu in chronic inflammation [53]. The increase in serum OPG in patients with IBD described by us and others [54] does not support the fact that IBD patients have generally worse bone mineral density than healthy controls [55]. While the negative effect of corticosteroid therapy on bone metabolism is well established [55], we did not find it mirrored in OPG levels in corticosteroid-treated patients. Nevertheless, we found a significant decrease in serum OPG in IBD patients on anti-TNF-α treatment. This may be caused by the feedback reaction of the organism to the anti-inflammatory treatment and efficient blockage of TNF-α. Moreover, this may explain the discrepancy in OPG with studies performed before the widespread use of TNF-α blockers. In our training cohort, we found OPG increased in CD, but not in UC patients. This may be due to the fact that protein array gives only relative quantification and that our UC cohort of patients for protein array consisted of only 6 patients, which may be too low. Therefore, we quantified this interesting factor with ELISA in the extended cohort of HC, UC and CD subjects. We found that the combination of OPG with six other proteins (EG-VEGF, CXCR-1, insulin-like growth factor 2 (IGF2), transforming growth factor-β1 (TGF-β1), ROBO4 and TMEFF1) can reasonably well distinguish healthy individuals from those with IBD, so we selected the strongest predictors and performed the analysis on the extended experimental set. Interestingly, OPG was not only the strongest factor distinguishing CD and UC, on its own (AUC = 0.916) it could easily distinguish UC and PSC-IBD patients, where its levels are even higher than in CD, despite the fact that all PSC-IBD patients showed UC-like features of inflammation. This suggests that inflammatory control in PSC-IBD is very different from UC. This stronger response

was not limited to the OPG, because we found multiple serum biomarkers (e.g., MMP-14, MBL, CD14, I-FABP, L-FABP, ROBO4) increased in patients with PSC-IBD.

The extracellular matrix and connective tissue of the gut wall in healthy subjects is constantly remodeled and repaired by the carefully regulated release of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs). The disturbance in the balance between synthesis and degradation of the extracellular matrix can result in typical features of IBD, such as ulcer formation, fibrosis or organ destruction [56]. In our dataset, decreased MMP-9 and increased MMP-14 were the strongest factors distinguishing IBD patients from healthy controls. Moreover, increased TIMP-1 was the second strongest factor distinguishing PSC patients without IBD from healthy controls and it was significantly increased in patients with PSC-IBD. However, the ability of TIMP-1 to distinguish between healthy and PSC patients may be influenced by the rarity of patients suffering from PSC without the concomitant IBD [3], so these results need to be verified on a larger cohort of patients. These findings clearly suggest the importance of the matrix metalloproteinase system in IBD pathogenesis. MMP-14 is a collagenase responsible for collagen degradation during re-modeling and for the activation of other enzymes and factors, thus triggering a proteolytic cascade or modulating important inflammatory factors [57]. The increase in MMP-14 we found in IBD patients may be a factor that mirrors the constant pathological remodeling of gut mucosa. MMP-9 is strongly expressed in inflamed mucosa during IBD [58] and several reports found increased serum MMP-9 as a marker for IBD activity in pediatric and adult IBD [59,60]. In our dataset, patients with IBD had significantly lower serum MMP-9 as compared to controls and there were neither significant differences in serum MMP-9 between patients with relapse and remission nor any correlation between MMP-9 and C-reactive protein (CRP). This discrepancy may be caused by the differences in the studied population, such as a high proportion of patients in remission and with the disease localized to the colon, since the disease localization may influence local and serum levels of MMPs [61]. Neither glucocorticoids nor TNF-α blockers influenced MMP-9 levels similarly as found in pediatric IBD patients by others [61].

Low serum TGF-β1 was the strongest factor associated with the active disease (relapse) as compared to quiescent disease (remission) and together with other factors (TFF-3, MMP-9 and LBP) was capable of distinguishing between these two conditions with high accuracy (AUC = 0.909). TGF-β is important cytokine for the maintenance of intestinal homeostasis through its immunoregulatory functions, gut barrier support and wound healing [62–64]. However, by promoting collagen III production by myofibroblasts it is partially responsible for typical CD complications, such as intestinal fibrosis, fistulae and strictures [65]. While our findings of elevated anti-inflammatory TGF-β1 in remission as compared to the active disease may be counterintuitive, one previous study already described similar findings in pediatric IBD [66]. This suggests that high TGF-β1 in remission may reflect the organism's successful effort to dampen the inflammation during IBD, which makes TGF-β1 an interesting potential candidate for relapse prediction.

Gut barrier failure and immune response to gut commensal microbiota are both hallmarks of IBD. The barrier failure leads to exposure of the immune cells in the gut mucosa to bacterial antigens, thus anti-bacterial immune response may serve as an indirect marker of chronic gut barrier failure. In order to measure which bacteria are targeted most in IBD as well as in HC, we selected 10 bacteria covering typical gut bacteria found in healthy Czech subjects [41]. We found that there are generally no differences between IBD patients and healthy controls except for patients suffering from CD that have generally lower antibody response against gut commensals. While the CD patients did not significantly differ from HC in IgM and IgG response, we found significantly decreased IgA response to *Faecalibacterium* and *Bacteroides* in CD patients compared with HC. In fact, 23% of UC patients and 40% of CD patients have undetectable IgA response to *Faecalibacterium*. In the normal healthy gut *F. prausnitzii* accounts for more than 5% of the total bacterial microbiota and is one of the most abundant commensal species [41,67], but it is markedly under-represented in the gut of patients with CD [68,69]. In fact, its low abundance on ileal mucosa or in feces predicts relapse in CD patients [69,70]. *F. prausnitzii* is able to produce not only anti-inflammatory molecules such as butyrate, but it can

modulate the host's immune response with a specific anti-inflammatory protein [71]. Our data suggest that the absence of *F. prausnitzii* during the inflammation in CD leads to a decrease in antibody response. We may speculate that significant decrease in IgA response to *Bacteroides* and non-significant decrease in *Blautia* and *Roseburia* may be caused by similar mechanisms, because many butyrate-producing bacteria, including *Blautia faecis*, *Roseburia inulinivorans* and *Bacteroides uniformis*, are significantly reduced in the gut of CD patients as compared to healthy controls [72]. There is, however, limitation to this assay, because many antigens are shared among species of a particular bacterial genus, while changes in abundances and or biological activities may be specific to a particular species or even isolate [73].

T cell response to microbiota plays an important role in IBD pathogenesis and commensal gut bacteria provide antigenic stimulation that can activate pathogenic T cells and lead to chronic intestinal inflammation [74]. Specific polarization of these cells is linked to the particular type of IBD, with Th1 and Th17 T cells are associated with CD and Th2 T cells are often associated with UC [75,76]. However, gut microbiota-specific cells are normally property of the memory T helper repertoire of PBMCs and do not necessarily indicate interaction between immune cells and the gut commensal microbiota. Nevertheless, the cytokine profile of these cells is changed during the intestinal inflammation [43]. Therefore, we used multi-color flow cytometry to analyze how differences in their cytokine profiles reflect different forms and states of IBD. We found that T helper cells from healthy subjects quickly up-regulate CD154 when stimulated with antigens from *Blautia*, *Roseburia*, *Prevotella*, *Lactobacillus* and *Escherichia* and that this spectrum of reactivity is generally broadened in patients with IBD. In our experiments, we focused on the response to bacteria found in the Czech population, so the reactivity to these particular microbes may not be universal worldwide.

When we focused on individual cytokines, we found only minor differences among the individual groups of IBD patients and HC, with a general increase in cytokine-producing memory T cells in CD group. Despite this trend, there were no significant differences in IFN-γ^+CD154$^+$CD4$^+$ T cells. Antigens from *Roseburia* had significant impact on memory T cells from PSC-IBD and CD as compared to HC and antigens from *Eubacterium*, *Faecalibacterium* and *Bacteroides* had significantly different impacts on memory T cells from PSC-IBD and CD as compared to HC. This suggests that while specific IgA response to *Roseburia*, *Faecalibacterium* and *Bacteroides* is decreased in patients with CD, their memory T cells react more strongly to these particular microbes. Unfortunately, in all bacteria lysate-stimulated samples the numbers of IL-4$^+$CD154$^+$CD4$^+$ T cells were so low, that we were not able to perform reliable analysis, and had to exclude it from analyses. Interestingly, we found a significantly higher proportion of IL-17$^+$CD154$^+$ cells in CD patients and a similar trend in UC patients, but not in PSC-IBD group, when stimulated with the super-antigen SEB. We did not observe any other differences in super-antigen-stimulated samples. This suggests that, unlike circulating memory T cells from patients with CD (and to a lesser degree in patients with UC), those from patients with PSC-IBD do not have an increased capacity to form Th17 cells.

Decreasing the inflammatory response by steroidal and non-steroidal anti-inflammatory drugs, biologicals and other immunomodulators is a cornerstone of IBD therapy and each type and severity of IBD requires an individual therapeutic approach. For example, anti-TNF-α is only rarely used in PSC-IBD patients and more severe cases of UC and CD are often treated with combination of drugs. This individual approach was present in our cohort as well, with 5-ASA used more in PSC-IBD patients than in UC patients and anti-TNF-α treatment is used only in UC and CD patients. The specificities of IBD pharmacotherapy limit the generalization of this study, but it does not make it irrelevant for clinical use, because the patients will be treated differently in the future. Therefore, we analyzed the effect of each therapeutic intervention in all patients with intestinal inflammation to find the therapy-specific biomarkers and to gauge the impact of the pharmacotherapy on the analyzed biomarkers. The effect was generally mild, with only a few notable exceptions. The significant increase in MMP-14 in patients treated with 5-ASA could be responsible for its general increase in IBD patients, because 82% of them were treated with 5-ASA. A similar effect could be partially responsible for the higher

levels of OPG in PSC-IBD as compared to UC, because anti-TNF-α was not used in any PSC-IBD patient. Moreover, we found an increase in IFN-γ production from *Roseburia*- or *Escherichia*-treated $CD154^+CD4^+$ T cells and increased TNF-α production from *Escherichia* treated $CD154^+CD4^+$ T cells in patients treated with anti-TNF-α. This may represent a biological background for rebound phenomenon when TNF-α blockers are excluded from the system and unchecked biological feedback increases the pro-inflammatory response. The opposite trend for Azathioprine, which acts directly on the cells, may reflect the biological background for the recently published meta-analysis finding the significant decrease in relapse rate when anti-TNF-α is discontinued under the screen of immune-modulators [77].

5. Conclusions

In this study we established the panels of biomarkers reflecting the specificities of pathogenesis of the different forms of IBD that may represent interesting future biomarkers. While the MMP system seems to be the strongest discriminator between healthy subjects and IBD patients, we identified markers reflecting colitis activity and anti-TNF-α treatment. Furthermore, we performed comprehensive screening of humoral and cellular adaptive immune response against gut commensal bacteria, finding several clear differences between healthy subjects and IBD patients, most notably CD. In general, these consisted of decreased IgA response to *Faecalibacterium* and *Bacteroides* with an increased T cell response to similar bacteria. These results stress the importance of gut barrier function and immune response to commensal bacteria in IBD pathogenesis and clearly show that PSC-IBD, UC and CD each represent a distinct form of IBD.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4409/8/7/719/s1. Table S1: Bacteria and cultivation conditions. Figure S1: Significant differences in serum biomarkers between PSC-IBD, UC and CD patients and healthy controls as analyzed by the Kruskal–Wallis test with Dunn's multiple comparison test. Different letters indicate statistically significant differences. Figure S2: Serum biomarkers do not describe the extent of colitis well. (A) Heat map and cluster analysis of the chosen proteins. (B) Composite ROC curve analysis for the biomarkers discriminating between partial and total colonic inflammation using the best model found by regression analysis. PSC-IBD (n = 29), UC (n = 34), CD (n = 19). Figure S3: Only a few serum biomarkers are influenced by IBD treatment, but their discriminating power is generally low. (A) Heat map and cluster analysis of the serum biomarkers. (B) Relative importance of each cytokine for the AUC increment within the best model found by regression analysis and quantitative plot of the strongest discriminating factor analyzed by the Mann–Whitney test. * $p < 0.05$, ** $p < 0.01$; other = anti-α4β7 PSC-IBD (n = 31), UC (n = 34), CD (n = 20). Figure S4: Serum antimicrobial antibodies are influenced by IBD treatment only to a limited degree. (A) Heat map and cluster analysis of the serum biomarkers. (B) Relative importance of each anti-microbial antibody for the AUC increment within the best model found by regression analysis and quantitative plot of the strongest discriminating factor analyzed by Mann Whitney test. * $p < 0.05$; other = anti-α4β7; PSC-IBD (n = 32), UC (n = 37), CD (n = 20). Figure S5: Anti-TNF-α treatment influences cellular antimicrobial response in IBD patients. (A) Heat map and cluster analysis of the serum biomarkers. (B) Quantitative plot of the strongest discriminating factors selected by correlation analysis as analyzed by the Mann–Whitney test. *** $p < 0.001$. PSC-IBD (n = 8), UC (n = 14), CD (n = 17), AZA treated (n = 13), AZA non-treated (n = 26), anti-TNF-α treated (n = 10), anti-TNF-α non-treated (n = 27). Figure S6: Response of memory T helper cells has a specific profile for each healthy control (n = 14) and PSC-IBD (n = 9), UC (n = 12) and CD (n = 13) patient as analyzed by non-parametric paired Friedman test with Dunn's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Figure S7: Gating strategy for flow cytometry using SEB-stimulated PBMCs

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cells

MDPI

Supplementary Materials:

Bacterium	Cultivation medium	Cultivation condition
Lactobacillus plantarum CCDM 182	MRS Broth for Lactobacilli (ATCC Medium No. 416)	anaerobic
Bifidobacterium adolescentis CCUG 18363	DSMZ medium No. 58. for Bifidobacteria	anaerobic
Blautia coccoides	Modified chopped meat medium (ATCC Medium No. 1490)	anaerobic
Roseburia intestinalis L1-82	Keister's Modified TYI-S-33 (ATCC Medium No. 2695)	anaerobic
Eubacterium rectale ATCC 33656	Chopped meat carbohydrates with rumen fluid (ATCC Medium No 1703)	anaerobic
Faecalibacterium prausnitzii A2-165	Modified YCFA medium (DSMZ medium No. 1611)	anaerobic
Ruminococcus flavefaciens DSM 25089	Medium for anerobes with 0.1% cellobiose (ATCC Medium No. 1365 E)	anaerobic
Bacteroides thetaiotaomicron VPI 5482	Modified chopped meat medium (ATCC Medium No. 1490)	anaerobic
Prevotella ruminicola M384	Chopped meat carbohydrates with rumen fluid (ATCC Medium No 1703)	anaerobic
Escherichia coli K6	Luria-Bertani broth (Merck, L3022)	aerobic

Table S1: Bacteria and cultivation conditions

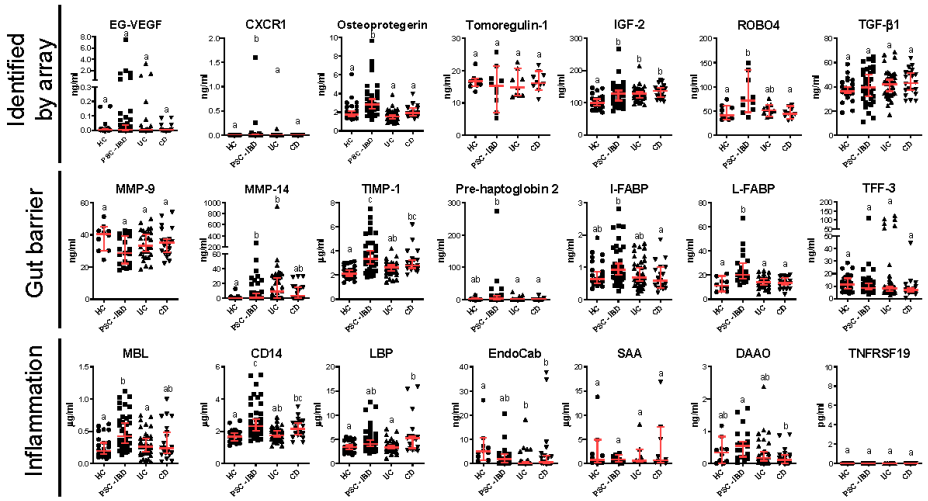

Figure S1: Significant differences in serum biomarkers between PSC-IBD, UC and CD patients and healthy controls as analyzed by Kruskal-Wallis test with Dunn's multiple comparison test. Different letters indicate statistical significant differences.

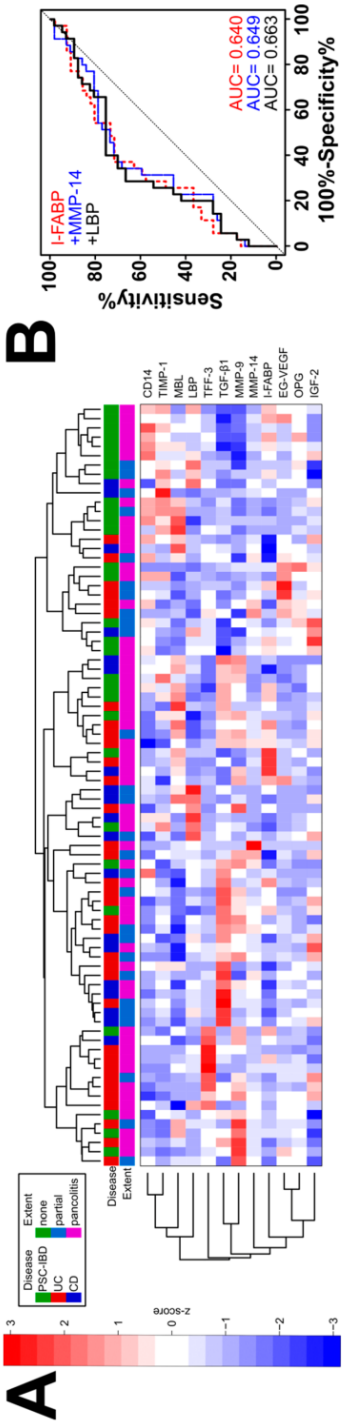

Figure S2: Serum biomarkers do not describe the extent of colitis well. (A) Heat map and cluster analysis of the chosen proteins. (B) Composite ROC curve analysis for the biomarkers discriminating between partial and total colonic inflammation using the best model found by regression analysis. PSC-IBD (n=29), UC (n=34), CD (n=19).

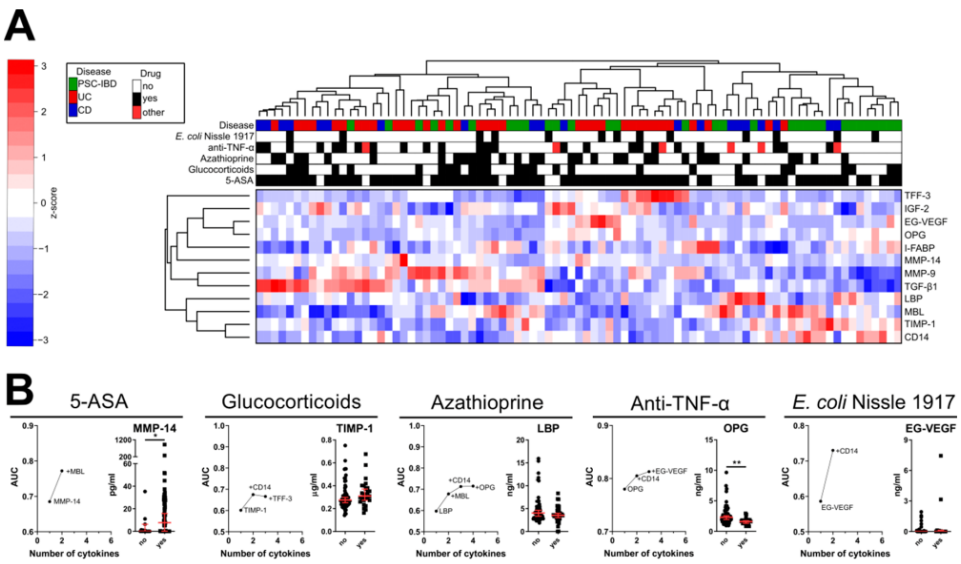

Figure S3: Only few serum biomarkers are influenced by IBD treatment, but their discriminating power is generally low. (A) Heat map and cluster analysis of the serum biomarkers. (B) Relative importance of each cytokine for the AUC increment within the best model found by regression analysis and quantitative plot of the strongest discriminating factor analyzed by Mann Whitney test. * $p<0.05$, ** $p<0.01$; other = anti-$\alpha 4\beta 7$ PSC-IBD (n=31), UC (n=34), CD (n=20).

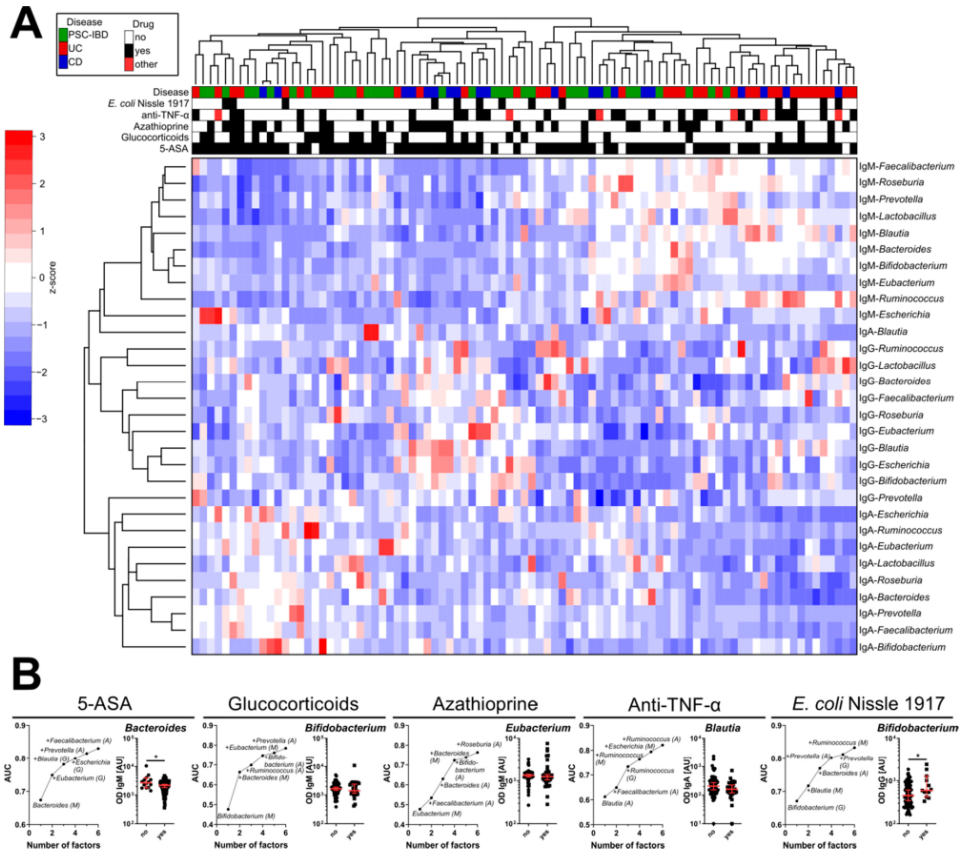

Figure S4: Serum antimicrobial antibodies are influenced by IBD treatment only to a limited degree. (A) Heat map and cluster analysis of the serum biomarkers. (B) Relative importance of each anti-microbial antibody for the AUC increment within the best model found by regression analysis and quantitative plot of the strongest discriminating factor analyzed by Mann Whitney test. * $p<0.05$; other = anti-$\alpha 4\beta 7$; PSC-IBD (n=32), UC (n=37), CD (n=20).

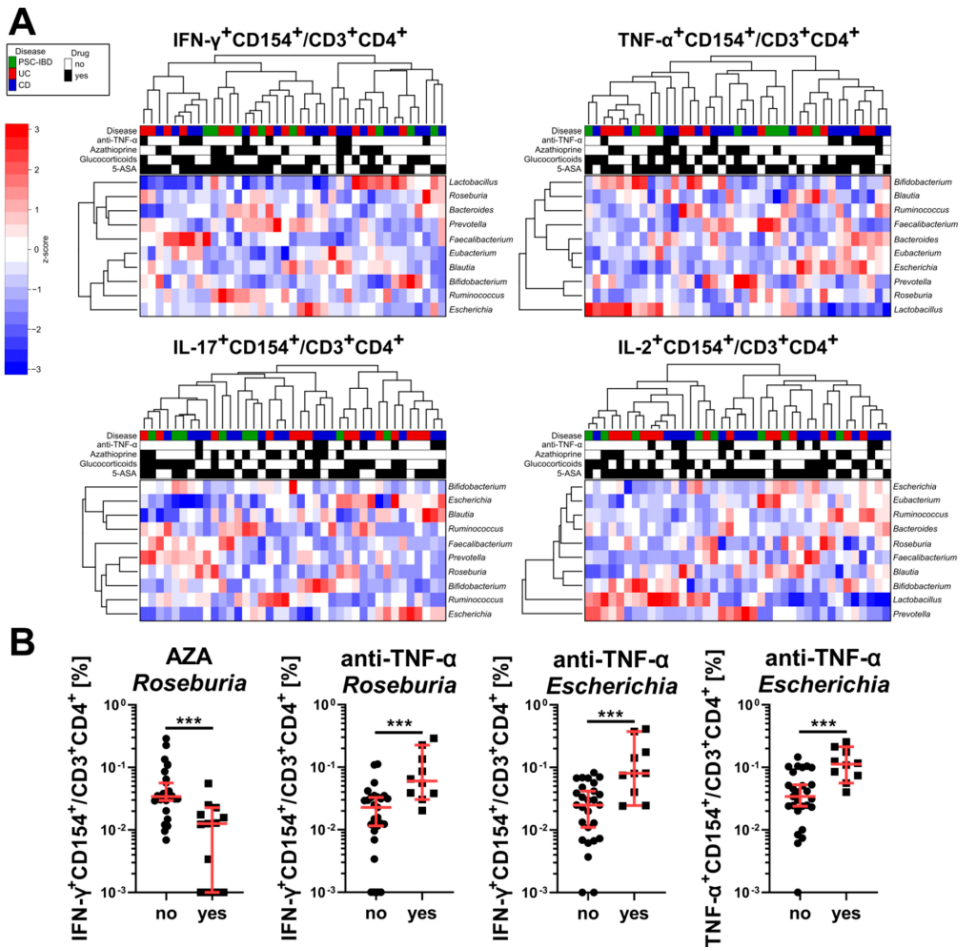

Figure S5: Anti-TNF-α treatment influences cellular antimicrobial response in IBD patients. (A) Heat map and cluster analysis of the serum biomarkers. (B) Quantitative plot of the strongest discriminating factors selected by correlation analysis as analyzed by Mann Whitney test. *** p<0.001. PSC-IBD (n=8), UC (n=14), CD (n=17), AZA treated (n=13), AZA non-treated (n=26), anti-TNF-α treated (n=10), anti-TNF-α non-treated (n=27)

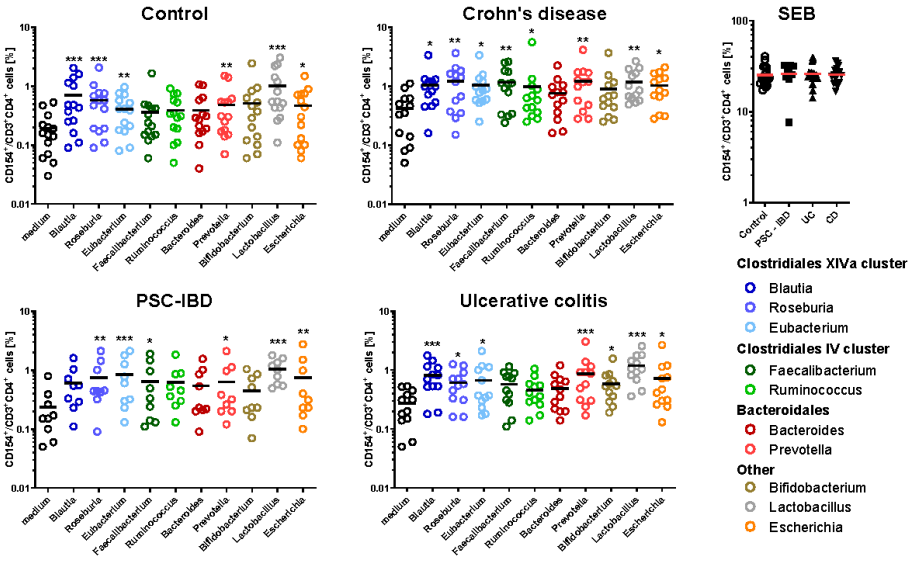

Figure S6: Response of memory T helper cells has a specific profile for each healthy controls (n=14) and PSC-IBD (n=9), UC (n=12) and CD (n=13) patients as analyzed by non-parametric paired Friedman test with Dunn's multiple comparison test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

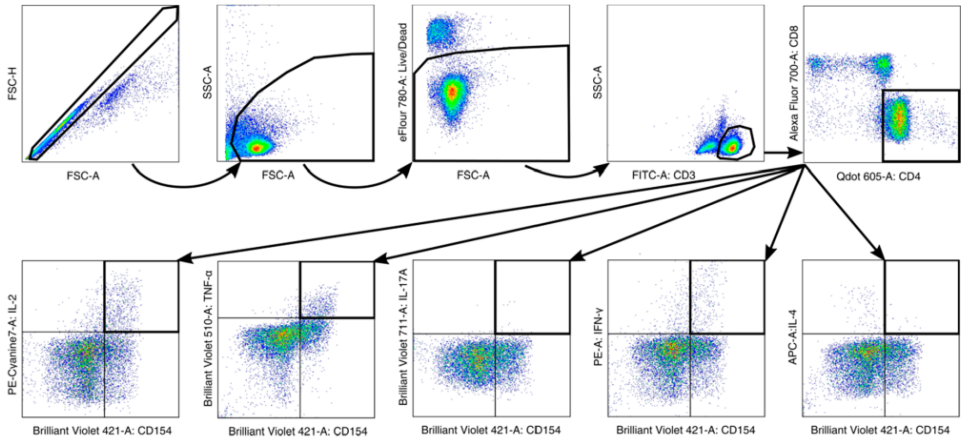

Figure S7: Gating strategy for flow cytometry using SEB-stimulated PBMCs

10. Discussion

The gut mucosa represents the interface between the host and the environment in the lumen of intestine. It has uneasy role to act as a selective barrier allowing the efficiently absorption of nutrients, electrolytes and water, while still maintains the effective defense against intraluminal toxins, antigens and enteric microbiota (GROSCHWITZ and HOGAN 2009). The gut barrier function is ensured by microbial (commensal microbiota), biochemical (humoral) and mechanical (physical) components. The gut barrier defense is mediated by the collaboration of innate and adaptive immune system. All these gut barrier components create in its mature form a functional and protective complex. Dysfunction and subsequent disruption of gut barrier is associated with various diseases and disorders. Necrotizing enterocolitis and inflammatory bowel disease are serious inflammatory intestinal diseases with several interesting parallels in their pathogenesis, involving disruption of gut microbiota, altered mucosal defense, inappropriate immune response and gut barrier damage (HARPAVAT *et al.* 2012). Since the gut barrier dysfunction and damage is common feature in the pathogenesis of both NEC and IBD, we analyzed the role of biomarkers associated with gut barrier and inflammatory response. Thus, we focused more on common pathogenetic feature of both diseases and tackled the specificities of these diseases by combining several potential biomarkers. This gave us several advantages, as we were able not only find new and useful diagnostic approaches, but we also increased our understanding of this important mechanism.

NEC is characterized by unexpected onset and very rapid progression with the risk of gut perforation and death. The clinical signs are non-specific during the early stage, which may delay the NEC treatment by misdiagnosing it as neonatal sepsis or other medical emergency. Thus, the current diagnosis of NEC is problematic and it is based on the combination of clinical (i.e. abdominal distension, blood in stool), laboratory and radiologic or sonographic findings of *pneumatosis intestinalis* or gas in portal vein, while only the radiologic or sonographic signs are specific for NEC. Unfortunately, these specific signs appear rather later in the disease course. Furthermore, X-ray and ultrasound have low sensitivity and negative predictive value and uncover mainly advanced stage of NEC (TAM *et al.* 2002). Early diagnosis of NEC would allow timely and efficient intervention, including cessation of enteral feeding, gastric decompression, therapy with broad-spectrum

antibiotics and supportive care, with the aim to prevent progression of disease, intestinal perforation and shock (GOMELLA *et al.* 2009; COUFAL *et al.* 2016b).

Destruction of mucosal layer and necrosis of gut wall are characteristic histopathological signs of NEC (BALLANCE *et al.* 1990). Therefore, we analyzed markers of gut barrier disruption, ccCK18 and I-FABP, as possible biomarkers for early NEC diagnosis, detectable before the specific signs for NEC will be apparent on X-ray or ultrasound.

Previously, it was shown that excessive apoptosis of gut epithelial cells may precede the necrotic damage of gut wall in animal model of NEC (JILLING *et al.* 2004; KHAILOVA *et al.* 2010). Therefore we analyzed the levels of both, caspase cleaved CK18 and total CK18 as a markers for apoptosis and necrosis of epithelial cells. We did not find higher rate of epithelial cell apoptosis in infants who will later develop NEC at the time of enrollment (at the time of NEC suspicion). At the time of enrollment we found only slightly increased rate of epithelial necrosis in infants who will later develop NEC, which reflects the major histopathologic feature of NEC (BALLANCE *et al.* 1990; COUFAL *et al.* 2016b).

The possible use of I-FABP as a biomarker for gut epithelium damage associated with NEC was first described in animal model (GOLLIN and MARKS 1993). Later, it was shown, that serum I-FABP is possible biomarker for distinguishing NEC from healthy preterm infants and furthermore, that its level correlates with the severity of disease (EDELSON *et al.* 1999; AYDEMIR *et al.* 2011). The serum I-FABP was later concluded as a biomarker for NEC allowing decrease the high false negative rates of currently used diagnostic procedures (CHENG *et al.* 2015). On the other hand, there was described also increase in I-FABP in sepsis. Therefore, we analyzed the serum I-FABP levels in patients who developed NEC or sepsis. We found the significantly higher levels of serum I-FABP in infants who will later develop NEC in comparison with infants who will later develop sepsis already at the time of NEC suspicion, but normalized during the successful treatment.

To measure I-FABP in urine may be feasible alternative, as urinary I-FABP can distinguish infants suffering from NEC from healthy infants (DERIKX *et al.* 2007; EVENNETT *et al.* 2010; MANNOIA *et al.* 2011). The noninvasive means of urine collection is advantageous, because it sparse the neonate from the stress of repeated blood sampling and thus it is unique way for constant monitoring (VINALL *et al.* 2014). In our study we found that

patients who will later develop NEC had significantly higher levels of urinary I-FABP in comparison with patients who will later develop sepsis already in first 12 hours from the NEC suspicion. The I-FABP in first twelve hours after NEC suspicion was also capable to distinguish NEC stage II and III. This suggests that I-FABP level reflects the degree of intestinal damage during NEC and it is in agreement with the finding that I-FABP level correlates with length of bowel resection in NEC requiring surgery intervention (surgical NEC) (HEIDA *et al.* 2014). There were no differences in I-FABP levels between patients who will develop sepsis and control infants (COUFAL *et al.* 2016b).

Currently X-ray and ultrasound diagnostic represent gold standard in NEC diagnosis, but they both have low sensitivity and negative predictive value (TAM *et al.* 2002). In fact, we showed that addition of the urinary I-FABP to the diagnostic procedure revealed 9 (33%) radiologically and ultrasonographically negative patients that later develop NEC. This markedly increased the sensitivity (91%) and negative predictive value (89%) of the diagnostic approach with 100% specificity and positive predictive value (COUFAL *et al.* 2016b).

Combination of multiple laboratory biomarkers may have several advantages. By combining analysis of several biomarkers reflecting different stages of disease pathogenesis we may be able to perform diagnostics in high throughput manner with good performance and moreover with the possibility to identify specific cases and stages of disease. Therefore, we decided to combine urinary I-FABP with other non-invasive gut-associated (L-FABP, TFF-3) and inflammatory biomarkers (SAA) with the aim to get better insight into pathophysiology of NEC and thus to further improve the differential NEC diagnostics and to predict the disease course and outcome. We found that not only urinary I-FABP but also urinary L-FABP, TFF3, SAA were significantly elevated in infants who will later develop NEC as compared to healthy controls in first 6 hours after the suspicion for NEC. This suggests the presence of gut epithelium damage and inflammatory response before the full spectrum of NEC symptoms is apparent. Among all biomarkers was the urinary I-FABP the strongest factor, which allowed discrimination of patients who will later develop NEC from those who will develop sepsis already in first 6 hours after the NEC suspicion. This supports our previous finding and further stress the importance of I-FABP in differential diagnostics of NEC. Because NEC and sepsis could be also a complication of surgery for congenital intestinal malformation, we analyzed if the urinary I-FABP and L-FABP could be used for discrimination of NEC and sepsis in this

case. We found that infants who will develop NEC in postoperative period after the surgery for congenital intestinal malformation had higher levels of urinary I-FABP and L-FABP as compared to infants who will develop sepsis. Furthermore, we found rapid increase in urinary I-FABP and L-FABP levels at the time of NEC suspicion but only in infants who will later develop NEC (COUFAL *et al.* 2016b, 2020).

Previously, we found that urinary I-FABP was capable to discriminate patients who will develop NEC stage II and NEC stage III already at the time of NEC suspicion. Similarly to I-FABP the urinary SAA was also capable to discriminate NEC stage II and NEC stage III, but it was capable to distinguish also clinically more relevant "surgical" stage NEC IIIB – the most severe NEC stage associated with gut perforation from "medical" NEC (stage IIA, IIB, IIIA) at the time of NEC suspicion (COUFAL *et al.* 2020). This finding was in agreement with previous study showing the correlation of SAA with disease severity (REISINGER *et al.* 2014). The patients with "surgical" NEC had also higher levels of urinary TFF-3 in comparison with patients with "medical" NEC, suggesting the association of TFF-3with severe intestinal damage in the case of surgical NEC. This could identify infants who will develop severe NEC and those who will require surgery already at the time of NEC suspicion, which is a great value for frontline neonatologists and surgeons.

Further we found, that the combination of gut-associated biomarkers with urinary SAA was strongest predictor of *pneumatosis intestinalis* (TFF-3, I-FABP, SAA) and portal venous gas (I-FABP, L-FABP, SAA), two typical features allowing the confirmation of NEC diagnosis, already in first 6 hours after NEC suspicion. The combination of I-FABP, L-FABP and SAA can also predict the length of hospitalization already in first 6 hours after NEC suspicion. These results are in agreement with studies showing that serial I-FABP measurement can predict the development of disease complication and serum SAA can determine the disease severity and response to therapy in patients suffering from NEC (ÇETINKAYA *et al.* 2010; SCHURINK *et al.* 2015). There was similar trend in the length of ATB therapy (L-FABP) and late achievement of full enteral feeding (SAA), all signs of the severe disease state (COUFAL *et al.* 2020).

Therefore, the combination of these biomarkers could be helpful not only in early diagnostics of NEC, but also in prediction of disease course and outcome. Thus, it can also have a treatment and policy implications in the management of NEC (COUFAL *et al.* 2020).

Not only immaturity, but also the congenital malformations associated with neonatal surgery intervention and intestinal mucosa damage are all risk factors for the development of NEC (BLANE *et al.* 1985; OLDHAM *et al.* 1988; JAYANTHI *et al.* 1998). Gastroschisis, a congenital anomaly of the abdominal wall, which results in extrusion of abdominal viscera from the abdominal cavity has become more prevalent over the last few decades (MELOV *et al.* 2018; KOKESOVA *et al.* 2019). Since the damage of intestinal mucosa is one of the pathological features of gastroschisis, which could be a major source of complication during the surgery intervention and also during the postoperative period, we analyzed the role of urinary I-FABP in the monitoring of intestinal mucosal damage in neonates after the surgery for simple and complex gastroschisis and if it could be used for prediction of their clinical recovery (ELEFTHERIADIS *et al.* 1996; CAGLAR *et al.* 2014; KOKESOVA *et al.* 2019). We found that urinary I-FABP could be used as a marker for intestinal mucosa damage also in neonates with gastroschisis. Patients with complex gastroschisis had significantly higher urinary I-FABP and they needed more time to recovery as compared to patients with simple gastroschisis. Furthermore, the high level of urinary I-FABP in patients with complex gastroschisis was associated with later surgery for mechanical ileus. However, although the I-FABP reflects the intestinal epithelium damage it was not capable to predict achievement of minimal enteral feeding, full enteral feeding or length of hospitalization (KOKESOVA *et al.* 2019). This suggests that there are additional sources of complications influencing the patient's recovery in addition to the intestinal damage.

Similarly as in NEC, gut microbiota dysbiosis, aberrant immune response and gut barrier failure are typical for pathogenesis of IBD. While there are clear clinical differences between IBD types, the mechanisms underlying their differences are less explored. Therefore, we performed broad spectrum analysis using protein array, ELISA and flow cytometry to analyze serum biomarkers and specific antimicrobial B and T cell response to gut commensals (COUFAL *et al.* 2019).

Using protein array with 507 proteins we performed broad range semi-quantitative analysis of training set of patients to identify the biomarkers signature and select the candidate biomarkers, which are strongly associated with two major forms of IBD. We found that high level of endocrine-gland-derived vascular endothelial growth factor (EG-VEGF) and interleukin-8 receptor, alpha (CXCR-1) was associated with UC, whereas low level of EG-VEGF and high osteoprotegerin (OPG) was associated with CD, suggesting that UC and

CD may differ in the regulation of angiogenesis and inflammation (COUFAL *et al.* 2019). Although, EG-VEGF is mainly associated with reproduction, previous studies showed its effect in human monocytes, which resulted in increase production of IL-12 and TNF-α while decrease production of IL-10 in response to lipopolysaccharide (LPS) (DORSCH *et al.* 2005). Thus, EG-VEGF may cause the decrease of the threshold of monocytes activation in gut wall leading to the exacerbation of inflammation during the breaching of gut barrier in ulcers vicinity in gut mucosa of patients suffering from UC (COUFAL *et al.* 2019). The CXCR-1 is receptor to IL-8, which plays important role in the recruitment of neutrophils in the site of inflammation and induces their activation (WU *et al.* 1996; SABROE *et al.* 2005). Our results are in agreement with the findings describing the neutrophils as major leukocytes present in inflammatory infiltrate in gut mucosa of patients with UC (MUTHAS *et al.* 2017). The OPG has important role in bone density regulation, cell turnover, differentiation, survival and death (SIMONET *et al.* 1997; LIU *et al.* 2015). Previous studies showed that OPG was increased in patients with IBD and its level correlated significantly with the pro-inflammatory cytokines, like TNF-α, suggesting that its production is influenced by pro-inflammatory cytokines (FRANCHIMONT *et al.* 2004). We found decreased OPG in patients with anti-TNF-α treatment, suggesting the presence of feedback response to the efficient blockage of TNF-α in the course of treatment (COUFAL *et al.* 2019).

Next, we use ELISA for biomarker quantification and to analyze their predictive value. Based on our previous findings and literature research we also selected several other relevant gut-associated and inflammatory serum biomarkers, and analyzed them on large cohorts of individuals. Apart from the obvious ability to distinguish healthy and diseased individuals and different forms of IBD, we analyzed their association with the presence of disease activity, extent, localization and treatment. We found that decreased OPG concentration is typical for UC in comparison with CD and PSC-IBD. The proteins of matrix metalloproteinase (MMP) system, which are involved in remodeling and repair of extracellular matrix and connective tissue of the intestinal wall, were the strongest discriminating factors in all forms of IBD from healthy controls. Our findings stress the important role of the MMP system in the IBD pathophysiology as was described previously (BAUGH *et al.* 1999; KOFLA-DLUBACZ *et al.* 2012; LAKATOS *et al.* 2012). The disturbance of the synthesis and degradation of extracellular matrix in intestinal wall can lead to the development of typical IBD features, such as ulcer formation, fibrosis or organ

destruction (KAPSORITAKIS *et al.* 2008). In contrast to other studies describing the high levels of MMP-9 in IBD and its association with IBD activity, we found significantly lower level of MMP-9 in IBD patients in comparison with controls. We did not find neither differences between patients in relapse or remission nor correlation between MMP-9 and inflammatory markers (e.g. CRP). This can be caused by the differences in the group of interest, like high proportion in remission and also disease localized in colon, because the localization of the disease may influence MMPs level (MÄKITALO *et al.* 2012). Further, we found, that low level of TGF-β was associated with relapse, while high level was associated with remission. Thus, TGF-β was the strongest factor associated with disease activity. TGF-β has important role in the maintenance of intestinal homeostasis via immunoregulatory function, gut barrier support and also by wound healing (CROWE *et al.* 2000; HAHM *et al.* 2001; HOWE *et al.* 2005). Thus, the high levels of TGF-β in remission may reflect the effort of the organisms to decrease the inflammatory response during IBD. Therefore, TGF-β could be interesting candidate for prediction of relapse during IBD. When we combined TGF-β with TFF-3, MMP-9 and LBP we can distinguish these two condition with high accuracy (AUC=0.909) (COUFAL *et al.* 2019).

Collectively, these results showed that gut-associated and inflammatory biomarkers are not only sufficient to distinguish major forms of IBD, but they also mirror its activity. Therefore, different pathogenic mechanisms are engaged in different forms and stages of IBD and these biomarkers represent interesting candidates for IBD diagnostics and outcome prediction (COUFAL *et al.* 2019).

The gut barrier failure leads to the exposure of immune cells in gut mucosa to bacterial antigens and thus the anti-bacterial immune response (anti-commensal antibodies) could be an indirect marker of chronic gut barrier failure. For this purpose, we selected 10 bacteria which cover typical gut bacteria found in healthy Czech subjects (BAJER *et al.* 2017). We did not find any significant differences in anti-commensal antibodies between IBD patients and healthy controls, except for patients suffering from CD. These patients had lower antibody response to gut commensal bacteria. Furthermore, we found significant decrease in IgA response to *Faecalibacterium* and *Bacteroides* in CD patients as compared to healthy controls (COUFAL *et al.* 2019). *F. prausnitzii* is one of the most abundant commensal species, which is able to modulate host's immune response by production of anti-inflammatory molecules like butyrate, but also via a specific anti-inflammatory protein (MIQUEL *et al.* 2013; QUÉVRAIN *et al.* 2016; BAJER *et al.* 2017). The low

abundance of *F. prausnitzii* was described in connection with CD patients (SOKOL *et al.* 2008, 2009; RAJCA *et al.* 2014). Thus, the decrease in antibody response to *F. prausnitzii* found in our study, suggests that the absence of *F. prausnitzii* leads to lesser stimulation and lower antibody response. We may speculate that gut barrier failure allow *F. prausnitzii* translocation in CD patients leading to strong immune response, which in turn may lead to *F. prausnitzii* elimination and the decreased IgA response to *F. prausnitzii* may thus be only secondary phenomenon. Similarly, in CD patients we found decrease in IgA response to *Blautia* and *Roseburia*, where belong other butyrate-producing bacteria (e.g. *Blautia faecis*, *Roseburia inulinivorans*) (TAKAHASHI *et al.* 2016; COUFAL *et al.* 2019). T cell response to microbiota has important role in the pathogenesis of IBD. The activation of pathogenic T cells by antigens of gut commensal bacteria can lead to chronic intestinal inflammation (IQBAL *et al.* 2002; FENG *et al.* 2010; CALDERÓN-GÓMEZ *et al.* 2016; COUFAL *et al.* 2019). But, gut microbiota-specific T cells are also common among the memory T helper repertoire of peripheral blood mononuclear cells and their presence does not always indicate interaction between immune cells and gut commensals, but their cytokine profile is changed during intestinal inflammation (HEGAZY *et al.* 2017). Thus, we analyzed the differences in cytokine profiles in different forms of IBD using multi-color flow cytometry. We found that circulating $CD4^+CD154^+$ T cells produced several pro-inflammatory cytokines after the stimulation with gut bacteria. While there were only minor differences in T cell response among the individual groups of IBD patients and healthy controls, the response to antigens from *Roseburia*, *Faecalibacterium* and *Bacteroides* was generally stronger in CD patients. Thus, while IgA response to *Roseburia*, *Faecalibacterium* and *Bacteroides* is in CD patients decreased, their memory T cells react more strongly to these bacteria. Thus, we found specific shifts in immune response to gut commensals in patients suffering from CD (COUFAL *et al.* 2019).

Our results showed that even healthy people have specific antimicrobial B and T cell response indicating permanent interaction between immune system and microbes. This interaction is skewed in IBD which leads to different signatures of antimicrobial responses. Thus, our results stress the importance of gut barrier function and immune response to commensal bacteria not only in pathogenesis of IBD, but also in the healthy people (COUFAL *et al.* 2019).

11. Conclusions

1.

We found that urinary I-FABP can distinguish NEC from neonatal sepsis, including the postoperative one, with better accuracy than currently used gold standard diagnostics. Thus the urinary I-FABP can help to clinicians at the moment when clinical symptoms of NEC are non-specific and NEC can be misdiagnosed with neonatal sepsis. Furthermore, the addition of urinary I-FABP examination to the standard imaging methods leads to increase of the sensitivity and negative predictive value of currently used diagnostic approaches (COUFAL *et al.* 2016b).

2.

We found that biomarkers associated with gut barrier function and inflammation and their combination can predict development, severity and outcome of NEC even before the symptoms of the disease are clinically apparent. Thus, these biomarkers allow early diagnosis and prediction of NEC and can be useful for the frontline neonatologist and surgeons to monitor and manage the NEC (COUFAL *et al.* 2020).

3.

Urinary I-FABP could be used as a marker for intestinal mucosa damage also in gastroschisis, where patients with complex gastroschisis had significantly higher levels of urinary I-FABP and required longer time for recovery than those with simple gastroschisis. The urinary I-FABP failed to predict time of achievement of minimal/full enteral feeding or shorter length of hospitalization. Therefore, it is not suitable for prediction of these outcomes. However, it is significantly higher in patients with complex gastroschisis, who will subsequently require surgery for ileus, as compared to those who would not, so it may be useful in predicting this severe complication in patients with complex gastroschisis. Before its use in clinical practice it is necessary to test on a larger cohort (KOKESOVA *et al.* 2019).

4.

We established the panels of biomarkers reflecting the specificities of pathogenesis of different forms of IBD and colitis activity, which may represent interesting future

candidates for IBD diagnostics and outcome prediction. We found that MMP system was the strongest discriminator between IBD patients and healthy subjects. Additionally, we performed the screening of specific antimicrobial humoral and cellular immune response against gut commensal bacteria. We found several differences between IBD patients and healthy subjects, most notably in patients suffering from CD. Collectively, these results show the importance of gut barrier function and immune response to gut commensal bacteria in the pathogenesis of IBD and support the notion that CD, UC and PSC-IBD represent a distinct form of IBD (COUFAL *et al.* 2019).

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