The role of the intestinal permeability in the progression of nonalcoholic fatty liver disease in obese children

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Abstract. Many studies in recent years have revealed increased intestinal permeability in the non-alcoholic fatty liver disease (NAFLD) development and progression to nonalcoholic steatohepatitis (NASH) and liver fibrosis. The prevalence, course, and diagnostic criteria of pediatric NAFLD were considered in the article. The role of increased intestinal permeability in the pathogenesis of NAFLD has been demonstrated. Attention was paid to the structure of the intestinal barrier and possible methods for its permeability examination. Current studies of intestinal permeability in NAFLD in adults and children, which confirm its key role in the progression of NAFLD, were reviewed. A literature search was conducted in electronic databases Scopus, MedLine, EMBASE, Pubmed, Google Scholar, etc.

Keywords: intestinal permeability; non-alcoholic fatty liver disease; obesity; children; review

Introduction
In recent decades, nonalcoholic fatty liver disease (NAFLD) has become the most common liver disease in both children and adults. According to epidemiological studies, NAFLD is found in 10% of the general pediatric population, its prevalence rate reaches 41.6% among obese children [1–5]. Although the early stages of NAFLD are reversible by diet and lifestyle modifications, the detection of such stages is hampered by the lack of specific symptoms and imperfection of non-invasive diagnostic methods. At present, the mechanisms of NAFLD development and progression remain unclear [6].

Terminology
Pediatric NAFLD, as defined by the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN), is a chronic hepatic steatosis in children under 18 years of age associated with insulin resistance, central or generalized obesity, dyslipidemia characterized by high triacylglyceride and low high density lipoproteins levels, which is not secondary to genetic, metabolic, infectious diseases, protein-energy deficiency and the use of steatogenic drugs or ethanol [7]. Based on the histological picture, NAFLD can be divided into simple steatosis, defined as hepatic steatosis without signs of inflammation, and NASH, which is manifested by steatosis with lobular inflammation and hepatocellular damage with or without fibrosis [7].

Epidemiology of NAFLD
Over the past 20 years the prevalence of pediatric NAFLD has more than doubled [3]. This escalation is connected with the increasing prevalence of obesity among children making NAFLD the most common cause of liver disease [1–3]. The overall average NAFLD prevalence in the world ranges from 2.5% (UK) to 12.5% (Italy) among children in the general population and from 29.4% to 41.6% in obese children according to various studies [2–4, 8]. The risk of NAFLD development in obese school age children is higher than in adults [3, 5]. The prevalence of NAFLD also depends on ethnicity [3]. The risk of NAFLD development increases in 4 times in Latin Americans [4] compared to non-Hispanic adolescents [1, 8]. White and Asian obese children also have a higher NAFLD prevalence than African American children [2]. NAFLD is observed twice as often in boys than in girls [2, 3].

The course of the disease
Almost a quarter of the liver steatosis patients develop inflammation that progress to nonalcoholic steatohepatitis (NASH) and leads to the liver fibrosis and cirrhosis [9]. Like
other chronic liver diseases, NAFLD is often asymptomatic. This is the reason that 15% of NAFLD children have 3 or higher stages of liver fibrosis already at the first visit to the doctor [10]. Also the severity of the disease in children is usually higher compared to adults [11].

Thus, the problem of NAFLD is caused on the one hand by the rapid increase in the prevalence of the disease on the obesity background and on the other hand by the severity of the disease consequences, such as NASH, liver fibrosis and cirrhosis [3].

**Diagnosis**

According to the latest NASPGHAN guideline (2017), determination of the alanine aminotransferase (ALT) activity and the detection of sonographic signs of steatosis such as ultrasound attenuation are considered suitable for NAFLD screening in obese children [7]. The recommended cut-off value of ALT in overweight and obese children is 22 IU/l for girls and 26 IU/l for boys. Prolonged (more than 3 months) hypertransaminemia with ALT more than twice than recommended cut-off value is considered as a sign of liver damage and a reason for a detailed examination. ALT levels usually do not reflect the severity of NAFLD. However, NASH is more common in children with ALT ≥ 80 U/l compared to children with ALT < 80 U/l (41% vs. 21%, respectively) [7]. The current standard for determining the presence and severity of NAFLD, including the presence of NASH, alternative or concomitant diagnoses exclusion is considered liver biopsy [7].

**Pathogenesis of NAFLD**

According to the “multiple hit theory” liver tissue is affected by a number of factors that act sequentially or in combination, namely genetic and exogenous factors (high-fat and high-carbohydrate diet, sedentary lifestyle, smoking, etc.), which lead to intestinal dysbiosis, intestinal barrier disruption, increased endotoxemia, immunological (increased pro-inflammatory cytokines) and metabolic changes (insulin resistance, carbohydrate metabolism disturbances, hyper- and dyslipidemia) [9, 12–14]. As a result, low-grade inflammation is formed, which, in turn, causes inflammation and destruction of hepatocytes (NASH) with progression to liver fibrosis.

The accumulation of hepatocellular lipids occurs when there is an imbalance in the regulation of lipid accumulation in the liver. Processes that contribute to the formation of hepatic steatosis include: 1) lipolysis in adipose tissue caused by insulin resistance, which increases the flow of free fatty acids (FFA) to the liver; 2) hepatocellular lipogenesis; 3) excessive intake of FFA and cholesterol in the composition of chylomicrons absorbed in the small intestine from food. On the contrary, the processes that reduce the fat content in hepatocytes include: 1) beta-oxidation of FFA in mitochondria, peroxisomes with the formation of triglycerides (TG); 2) export of very low density lipoproteins (VLDL) from hepatocytes to the bloodstream [15].

A key factor in the development of NAFLD is insulin resistance, which on the one hand induces the breakdown of TG in adipose tissue by activating the hydrolase activity of specific enzymes and increases the flow of FFA to the liver and their accumulation in hepatocytes. Insulin resistance makes adipose tissue resistant to the antilipolytic effect of insulin, which leads to the breakdown of TG and the final formation of FFA and glycerin. On the other hand, hyperinsulinemia promotes increased lipogenesis de novo in hepatocytes due to the transcription factor sterol-regulatory element binding protein 1c (SREBP 1c). Substrates derived from glycolysis (acetyl-CoA) also initiate a multistage process of synthesis of FFA, which are then converted into TG. The accumulation of saturated FFAs, such as palmitic acid and stearate, in hepatocytes causes a lipotoxic effect, which in turn leads to organelle dysfunction (mitochondria and endoplasmic reticulum (EP)) and, eventually, to hepatocyte necrosis. EP stress is also associated with chronic inflammation due to excessive synthesis of reactive oxygen species (ROS) and activation of nuclear factor kappa B (NF-κB), c-Jun N-terminal kinase (JNK) pathway, leading to increased secretion of tumor necrosis factor alpha (TNFα). Saturated FFA, in addition to lipotoxicity, promotes inflammation of hepatocytes by activating TLR and increasing the production of pro-inflammatory cytokines [15, 16] (Fig. 1).

Hepatocellular inflammation and necrosis of hepatocytes occur secondarily due to activation of the immune system [15, 17–20], as well as due to oxidative stress, which is observed as a result of lipotoxicity [6, 15, 21]. Prolonged hepatocyte damage activates hepatic stellate cells, which produce type I collagen and secrete profibrogenic cytokines and inhibitors of matrix-degrading enzymes (tissue matrix metalloproteinase inhibitor), causing extracellular matrix production leading to liver fibrosis (Fig. 1).

Many preclinical and clinical studies suggest that altered intestinal microbiome and intestinal permeability play a key role in the occurrence of steatosis, inflammation and liver fibrosis in NAFLD [2, 3, 6, 15, 16, 21, 23–28]. There is a bidirectional relationship between the intestine and the liver. The liver supplies bile acids, IgA and antimicrobial peptides (cathelicidins, defensins, hepatic-expressed antimicrobial peptide 2 (LEAP-2), hepcidin) through the biliary tract, and intestinal microflora metabolites (secondary bile acids, short-chain fatty acids, ethanol) and endotoxins (lipopolysaccharide, peptidoglycan, flagellin) are transported from the intestine to the liver through the intestinal barrier and portal vein [22, 36, 40, 65] (Fig. 1). One of the suggested mechanisms linking dysbiosis to NAFLD is impaired intestinal permeability and elevated levels of circulating endotoxins [24, 26, 28, 31], which causes an inflammatory and fibrogenic response of the liver, triggering a cascade of the above-mentioned immune responses with TLR4- and TLR9-activation [26, 32]. Bacterial translocation has been shown to increase the expression of specific receptors localized on the surface of hepatocytes, such as TLR, which are associated with a pro-inflammatory reaction mediated by TNFa, interleukin-1β (IL-1β) and interferons [17–20, 32].

**Intestinal barrier structure**

The intestinal barrier is a structure that prevents intestinal bacteria and their toxins from the translocation into the bloodstream. This structure consists of intestinal microbiota, the mucosal layer that covers the surface of the intestinal epithelium, the intestinal epithelial cells connected by tight
The first level of the intestinal barrier is the resident microbiota, which consists of hundreds of trillions of microorganisms whose genome is 10 times larger than the human genome and whose weight is about 1–2 kg. The intestinal microbiota plays a key role in the processing of nutrients and vitamins, transformation of primary bile acids, and prevention of the pathogenic microorganisms adhesion to the intestinal mucosa [26, 39].

The second level of the intestinal barrier is intestinal mucus, namely glycosylated proteins secreted by goblet cells of the intestinal epithelium. This barrier represents the extracellular layer that covers the intestinal epithelium, consists of digestive secretions, antimicrobial peptides and cytokines, separates the intestinal microbiome and the intestinal epithelial cells. The thickness of the mucus layer increases from the stomach to the colon. The microbiota colonizes the outer layer of mucus and uses nutrients from the mucus [16, 40]. The inner layer of mucus which is quite static and direct contact with enterocytes does not

Figure 1 — Gut permeability, bacterial translocation, and TLR signaling in NAFLD/NASH [60]
contains bacteria because of antimicrobial peptides and proteins enrichment. Paneth cells secrete antimicrobial peptides (AMPs) into the internal mucus, and enteronecrine cells produce secretory IgA (sIgA) to protect against commensal bacteria contributing the formation of a biochemical barrier. This layer promotes the absorption of water and nutrients [16, 29].

The third level of the intestinal barrier is determined by the gastrointestinal tract motility and digestive secretion. These two factors prevent the spread and ensure the timely elimination of pathogenic microorganisms from the intestinal lumen. The main fluids are gastric acid and bile, which have antimicrobial properties [16, 38, 40].

The fourth level of the intestinal barrier is presented with the single layer of intestinal epithelium, which includes enterocytes, goblet cells, Tuft cells (with chemosensory function) and Paneth cells (which produce antimicrobial peptides) [16, 38, 40]. This cellular barrier has certain physical, electrical and chemical properties contributing to impermeability to most substances [41]. The intercellular space is closed by a specific junctional complexes, composed of tight junctions (TJ), adherens junctions, desmosomes, and gap junctions [41] (Fig. 3).

More than 40 proteins contribute to the functioning of junctional complexes: Claudines, occludines, junctional adhesion molecule (JAM), scaffolding and adapter proteins, such as zonula occludens (ZO)-1, -2, and -3, E-cadherins and nectins [16, 29, 38, 41]. The cytoskeleton connects tight junctions, facilitating the active and passive transport of substances across the intestinal barrier. TJ regulates the passive flow of solutes and water through the paracellular pathway and acts as a filter that selects the size and charge of molecules [16, 26, 41]. There are two routes of transport through the epithelial wall: the transport of large substances and molecules (proteins and bacterial components) is transcellular by foam or exocytosis, while small molecules and substances are transported paracellularly (actively or passively) through TJ. These processes control the movement of molecules across the intestinal barrier [29, 40].

The functioning of this complex structure is directly influenced by many factors, including proinflammatory cytokines, TNFα, gamma-interferon signaling kinases and LPS mediated through activation of TLR-4 MyD88 on the epithelial membrane [16, 18, 42]. These factors reduce the expression of ZO-1 proteins in TJ [16, 29, 40], which allows LPS to enter the bloodstream and leads to low-grade

Figure 2 — Intestinal barrier components [29]
inflammation, including steatosis and insulin resistance [27, 29]. Molecular changes in intestinal TJ proteins, mainly ZO-1 and occludins, are the main mechanism contributing to increased intestinal permeability [27].

The fifth level of the intestinal barrier is the immunological barrier, which is provided by several antimicrobial peptides (i.e. defensins, cathelicidins, resistin-like molecules, bactericidal/permeability-increasing proteins, etc.) [16, 29, 38, 40]. The intestinal mucosal barrier is further reinforced by the population of intraepithelial (conventional and unconventional αβ and δχT-cells, mononuclear phagocytes) and lamina propria immune cells [16]. Intraepithelial lymphocytes are cytolytic, express type I cytokines, release antimicrobial peptides, so, along with mononuclear phagocytes that act as direct sensors of the intestinal lumen, these cells provide a first-line defense against infection and participate in the tolerance to food and microbial antigens [16, 29, 40]. Lamina propria immune cells which are highly specialized for recognition of microbial antigens or their metabolites play a second line of defense and promote tissue regeneration when the lamina propria is damaged [16].

The sixth level of the intestinal barrier is the intestinal-vascular barrier, similar to the blood-brain barrier [16, 40]. The so-called intestinal vascular unit includes the intestinal endothelium associated with pericytes, intestinal glial cells, and junctional complexes. This structure also prevents the translocation of bacteria and microbial components into the bloodstream [16, 25, 38, 40].

It should be noted that the translocation of a small number of microorganisms and their products to the mesenteric lymph nodes is an ongoing process. So, the immune system is constantly stimulated in order to form immune tolerance [15, 16] which allows to neutralize microorganisms without significant systemic inflammation. Small amounts of bacterial RNA and LPS can enter to the liver, where they are phagocytized by Kupffer cells [30]. However, the liver is usually free of bacteria, providing a next barrier for microorganisms that have penetrated the intestinal mucosa and avoided neutralization in the mesenteric lymph nodes [15, 16, 40].

Thus, summarizing the above, associated with LPS and proinflammatory factors TLR4-activation lead to the change in TJ structure and functioning, increasing intestinal permeability [16, 18, 29, 42]. The disrupted intestinal barrier contribute to the portal influx of pathogen-associated molecular patterns (PAMPs), such as LPS and metabolites of microorganisms, to the liver, activating a pro-inflammatory response.

Figure 3 — Intestinal epithelial cell junctional proteins [29]
cascade that exacerbates liver inflammation [15, 43]. These facts lead to the recognition of impaired intestinal permeability as a key component of the NAFLD pathogenesis, which is closely associated with increased TLR4 stimulation, increased synthesis of pro-inflammatory factors, insulin resistance, endotoxemia, hyperlipidemia and promote the free fatty acids accumulation, lipotoxicity, low-grade inflammation and hepatocytes damage. These data indicate an key role of intestinal permeability in the pathogenesis, development and progression of NAFLD.

Research overview

Several studies in humans and animals suggest that changes in intestinal microbiota composition, barrier function, and subsequently elevated bacterial endotoxin levels are critical in the development of NAFLD [16, 24–27]. Many preclinical and clinical studies indicate that changes in the intestinal barrier of NAFLD patients [20, 23, 29, 31] include components such as changes in the intestinal microbiota [18, 25, 29, 42], the quality and quantity of mucus, gastrointestinal peristalsis, damage of enterocytes (bile acids and endogenous ethanol), impaired TJ and intestinal immunity [16, 28, 29]. Some studies have shown an association between insulin resistance and increased intestinal permeability, namely TJ disruption [27, 29]. The influence of dietary factors (high fat diet) and bacterial products of gram-negative microflora on the functioning of TJ, changes in the structure of the mucous layer, have been demonstrated which in turn may increase intestinal permeability [15, 16, 25].

In recent years, many studies have demonstrated elevated blood endotoxin levels in adult patients with simple steatosis and NASH [15, 14, 27, 37], as well as in children with NAFLD [4, 8, 12, 33, 44]. Intestinal permeability correlates with the severity of NAFLD. In particular, intestinal permeability was increased both in children and adults with NASH compared to simple steatosis [19, 23, 45–48]. In the study of Miele et al. (2009) was found that increased intestinal permeability in patients with NAFLD was caused by disruption of intestinal TJ [35]. This was confirmed by a decrease in the expression of one of the major TJ ZO-1 proteins in the intestinal mucosa [27, 35]. These patients also had a threefold increase in the incidence of small intestine bacterial overgrowth syndrome (SIBO), which, along with increased intestinal permeability, correlated with the severity of hepatic steatosis [35].

In 2012, Volynets and his colleagues have shown in the adult NAFLD cohort that liver steatosis was associated with increased intestinal permeability, which was assessed by the lactulose/mannitol test and indirect measurement of serum endotoxin levels [49]. Similarly, in a pediatric NAFLD cohort, the lactulose/mannitol test confirmed the presence of increased intestinal permeability, which correlated with the severity of NAFLD [50, 51] and was significantly higher in patients with NASH [23, 46]. Serum endotoxin levels were also elevated in patients with NAFLD and correlated with histological markers of liver inflammation [12, 19, 23, 26, 33, 45, 47, 52]. LPS-binding protein levels were also elevated in another cohort of severely obese and NAFLD patients that correlated with the severity of liver damage [12, 44]. Increased intestinal permeability has been demonstrated in patients with NAFLD in many subsequent studies [23, 25, 36, 45, 49]. Several studies have also suggested that endotoxins increase intestinal permeability by activating TLR4/MyD88 pathways [17–20].

Also in the studies of L. Pacifico et al. (2014) [52], Cakir et al. (2017) [54] and Loffredo et al. (2017) [55] were demonstrated that the level of zonulin was probably higher in the group of NAFLD patients and had a positive correlation with the level of insulin. Zonulin levels have a positive correlation with the severity of steatosis, but not with NASH in these studies [52, 54, 55]. Olfit M. Handy et al. (2017) [47], Chwist et al. (2014) [48] and Chiara Rosso et al. (2020) [56] have demonstrated a positive correlation between serum zonulin level and progressive liver necrosis histological features in NAFLD adults. Valentina Giorgio, Luca Miele et al. (2014) have shown a positive correlation between increased intestinal permeability and liver steatosis, as well as a significant increase in intestinal permeability in children with NASH compared with simple steatosis [23]. Levels of bacterial endotoxin, lipopolysaccharide-binding protein (LBP) and proinflammatory markers such as interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) were significantly higher in children with NAFLD [12, 44, 33]. Guercio Nuzio et al. (2017) have demonstrated a significant increase in intestinal permeability in children with NAFLD depending on the stage of liver damage [57].

Evidence supporting the importance of dietary factors for the pathogenesis of NAFLD has also been accumulated over the last decade in experimental and clinical studies [12, 24, 25, 27, 29, 53]. Some studies have clearly shown that endotoxin level was constantly elevated in a mouse model of high-fat diet induced NAFLD [24, 58]. High sucrose and high fat diet also led to increased levels of circulating LPS in parallel with a significant liver fat accumulation and a significant decrease in the expression of occludin in rats [58]. These animals developed a typical metabolic syndrome and NAFLD [24]. Another study reported that fructose-induced NAFLD was associated with SIBO and increased intestinal permeability, contributing to Kupffer cells activation and liver damage in mice [20]. Inactivation of TLR4 as well as the introduction of prebiotics and probiotics modulating the intestinal microbiota improves hepatic steatosis caused by fructose [20, 27]. The results of Kento Imajo et al. (2012) study suggest that prolonged exposure to low-dose LPS causes markedly increased expression of TLR4, TNFα, and IL-6 mRNA in mice fed a high-fat diet, leading to liver inflammation [24]. Some studies have shown that the liver is one of the main targets of LPS, which binds to LPS-binding protein (LBP) and CD14, activates hepatic TLR4, TLR9-associated inflammatory cascade and promotes the progression of NAFLD [26, 32]. LPS also stimulates hepatocyte apoptosis, which is important in the NASH development and liver fibrosis promotion [26, 32].

It is important to note that dietary factors may contribute to increased endotoxemia in healthy people [14]. Some studies have reported that consuming a Western diet during one month resulted in increased endotoxemia in healthy people compared to a healthy diet (low fat, high fiber) [13, 14]. The cumulative effects of such food can be manifested in oxidative stress, chronic low-grade inflammation and insulin resistance [14, 27].
Methods for intestinal permeability examination

The association of NAFLD with impaired intestinal permeability has increased attention to its investigation as a key factor in the NAFLD progression. Intestinal permeability can be assessed via measurements of the transepithelial resistance, but also by measuring passage of solutes over the epithelium via different passage routes.

The intestinal barrier regulates the translocation of substances from the intestinal lumen, such as antigens and bacteria, that pass through a layer of epithelial cells between epithelial cells (paracellular pathway) or through cells (transcellular pathway) into the submucosa [37, 59]. Paracellular passage of substances can occur by diffusion or activation of junctional proteins, depending on the solute properties.

For the first time intestinal permeability studies in vivo were performed using inert probes of various sizes, which were absorbed in different parts of the digestive tract and excreted in the urine. The most common markers used previously were small pore markers — polyethylene glycol (PEG) 400 Da, monosaccharides (mannitol and rhamnose) and large pore markers — chromium-ethylenediaminetetraacetic acid (51 Cr-EDTA), disaccharides (lactulose) and PEG with molecular weight approximately 1000—4000 Da [37, 59]. Although the in vivo permeability test does not distinguish between paracellular and transcellular permeability, most large molecules pass through the mucosa by the paracellular route [37]. Increased lactulose flux increases the ratio of lactulose to mannitol or L-rhamnose and marks the loss of intestinal barrier integrity. Over the years, this technology has become more sophisticated, and researchers now use a multi-sugar test that includes five different sugar probes: sucrose, lactulose, L-rhamnose, erythrol, and sucralse. The analysis of the urine concentration of probes at different times evaluates the permeability of different parts of the intestine. The permeability test assessing the ratio of lactulose/mannitol or lactulose/L-rhamnose is often used to assess the permeability of the small intestine, sucralse (erythrol) — the large intestine, and sucrose — the stomach [37].

Other opportunities for the intestinal permeability examination in vivo are serum biomarkers investigation, which include zonulin, proteins that bind fatty acids, citrulline, glucagon-like peptide, lipopolysaccharide, lipopolysaccharide-binding protein, D-lactate, alpha-glute, alphas-glut-3, ovalbumin, as well as antibodies to zonulin, occludin, lipopolysaccharide, actomyosin, vinculin. Fecal biomarkers of intestinal permeability include alpha-1-antitrypsin, lipocalin-2 [37, 59].

In vitro techniques offer possibilities to study mechanical processes of the intestinal barrier and individual cells in humans. The most common cell lines used for studying intestinal barrier function are Caco-2 cell line, T84 cell line, SK-CO15, HT29 cell line, co-culture of cell lines, intestinal epithelial cells [37, 59].

Conclusions

Increased intestinal permeability is a key factor in NAFLD development and progression. Further study of intestinal permeability at different degrees of hepatic steatosis in children with NAFLD and obesity may help to identify predictors of NAFLD progression. Timely determination of increased intestinal permeability in the initial stages of NAFLD is needed to identify risk groups and prevent disease progression. Further in-depth study of the influence of intestinal permeability on the progression of NAFLD will be the basis for the development of new directions for the prevention and treatment of NAFLD in children.

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Роль кишкової проникності в прогресуванні неалкогольної жирової хвороби печінки у дітей з ожирінням

Резюме. Численні дослідження останніх років підтвердили підвищення кишкової проникності при усырені неалкогольної жирової хвороби печінки (НАЖХП) і прогресуванні до неалкогольного стеатогепатиту (НАСГ) і фіброзу печінки. У статті розглянуто поширеність, перебіг та діагностичні методи кишкової проникності при НАЖХП у дітей з ожирінням.

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