Influence of non-steroidal anti-inflammatory drugs on intestinal permeability and non alcoholic fatty liver disease

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Summary

The incidence of non-alcoholic fatty liver disease (NAFLD) and its related conditions including obesity and metabolic syndrome has dramatically increased in Western countries. Gut microbiota contributes to body weight regulation and related disorders like NAFLD by influencing metabolic and immune host functions. High fat diet may induce small intestinal bacterial overgrowth (SIBO) and dysbiosis which determine a malfunction of tight junctions (TJ) playing a critical role in the increase of intestinal permeability and the translocation of bacteria and their products. The endotoxaemia induces an inflammatory response that determines the development of insulin resistance, body weight gain, lipogenesis, fibrogenesis and hepatic oxidative stress which contributes to the second hit mechanisms in the pathophysiology of non-alcoholic steatohepatitis. In these categories of predisposed patients these mechanisms may be exacerbated by the administration of non-steroidal anti-inflammatory drugs (NSAIDs) that causes an initial alteration of intestinal permeability up to more serious complications. NSAIDs enteropathy is due to enterohepatic recycling of drug resulting in a prolonged and repeated exposure of the intestinal mucosa to the compound and its metabolites leading to so called topical effects. The pathogenesis of NSAID enteropathy is less linked to their ability to suppress cyclooxygenase activity and to the presence of gastric acid, even if more and more emphasis is placed on co-administration of antisecretory agents which determine hypocloridria, associated with small intestinal bacterial overgrowth and bile acid dysmetabolism, responsible of exacerbation of NSAID-induced intestinal damage. Prevention and treatment of NSAID enteropathy and his systemic complication like NAFLD have become major priorities, consequently novel approaches directed toward other pathomechanisms and research of new therapeutic strategies are needed.

KEY WORDS: non-steroidal anti-inflammatory drugs, non-alcoholic fatty liver disease, obesity, gut microbiota, intestinal permeability.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most consumed drugs in the world thanks to their benefits as analgesic, anti-inflammatory and antipyretics agents (1). However, these benefits are in part overshadowed by the recurrence of digestive system complications which can arise during the course of therapy and which are sometimes severe. The most impacted and damaged sites are the oesophagus, the stomach and the duodenum, due to an observed increased mortality and morbidity. Nevertheless, NSAIDs can cause a variety of functional and structural abnormalities, even in the small intestine for patients who make long-term use. About 60-70% of patient taking NSAIDs develops subclinical mucosal damage (2, 3), including the increase of intestinal permeability, intestinal inflammation, erosions, protein loss, but also more serious complications such as anemia, bleeding, ulcers, perforations, obstruction, diverticulitis, ileal dysfunction and diaphragm-like strictures (4-6) (Table 1). It is estimated that about
1/3 of all complications associated with the use of NSAI
D is represented by severe injuries of the small bowel (7).

At the base of this broad spectrum of lesions there are structural and func-
tional alterations of different components, which make up the intestinal barrier, and also the appearance of factors that interfere with the maintenance and homeostasis of normal bowel functionality. These aspects are better explained in the next paragraphs.

Due to intestinal barrier and its constituent elements alteration, in particular intestinal permeability, luminal substances including toxins, microorganisms and their components can access the portal circulation causing toxemia with pathological effects also in the long term. In this regard, there are many evidences that confirm the liver as one of the main targets of toxemia resulting from the alteration of intestinal permeability. As a consequence, process of inflammation, as well as alteration of metabolic processes can occur in the liver, contributing to the pathogenesis of Non Alcoholic Fatty Liver Disease and to its various manifestations (8), which, however, is beyond the description of this discussion.

NAFLD and microbiota
Nonalcoholic fatty liver disease (NAFLD) is a very common disease. This pathology ranges from simple steatosis disease, characterized by excessive deposition of fat in liver cells without evidence of inflammation or necrosis, to non-alcoholic steatohepatitis (NASH), characterized by hepatic steatosis and inflammation (9) until the fibrotic tissue formation and frank cirrhosis.

NAFLD is currently the leading cause of liver disease worldwide, with a prevalence of 3-10% dependent on age, gender, ethnicity and other risk factors. This illness is the hepatic expression of metabolic syndrome, which is frequently associated with obesity, insulin resistance, dyslipidemia and hypertension, and so with a greater cardiovascular risk (10). Therefore, its prevalence increases along with global epidemic of obesity and metabolic diseases.

It is supposed that NAFLD is characterized by an accumulation of triglycerides in the liver. This event can result in an increase of liver susceptibility to injury mediated by factors such as inflammatory cytokines, adipokines, mitochondrial dysfunction and oxidative stress. These factors, in turn, would be able to lead to steatohepatitis and liver fibrosis. Although NAFLD is a multifactorial disease, whose pathogenesis is not fully understood, in recent years considerable progress has been made to elucidate the mechanisms responsible for liver damage. In particular, more and more importance is given to the role of the microbiota, which seems to be crucial in determining its development, starting from the accumulation of fat in the liver until triggering liver inflammation.

In fact, the gastrointestinal tract is the habitat for a large number of bacteria, whose genome is 150 times larger than the nuclear genome of the eukaryotic cell and whose density reaches about 1012 bacteria per gram of luminal contents in the distal colon. There are hundreds of bacteria species, and the two most representative are the phylum Firmicutes and the Bacteroidetes (11, 12). This “microbial organ” is responsible for a variety of physiological protective functions and metabolic regulation. In fact, the genes of the human intestinal microbiota encode for proteins, enzymes and other products involved in the various processes related to the immune system (immuno-
competence and immunetolerance), glucose metabolism, lipid and protein metabolism, the maturation of the mucosal surface of the gastrointestinal tract and the catabolism of xenobiotics and drugs.

Recent studies suggest a role of intestinal flora in insulin resistance and obesity (13). The specific individual bacterial composition is intricately influenced by multiple factors including the epithelial and mucin turnover, peristalsis, food molecules, pancreatic and bile secretions, drugs, pH changes, the host’s immune system, sex, age,
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genetic host and finally environmental factors such as infections or chronic diseases of the gastrointestinal tract and diet (14). In this regard, several studies shown that in obese patients there is a reversal of the Bacteroides and Firmicutes ratio, with Bifidobacteria reduction, resulting in an increase of bacteria able to metabolize carbohydrates, to take energy and thus increasing adiposity (15). These studies also indicate that obese patients do not have a predetermined microbial composition, but it is rather the specific Western diet, which is high in fat, to influence this composition by increasing the Firmicutes. It is also possible to observe how the composition of the intestinal microbiota in patients with NAFLD differs from that of healthy subjects. Particularly, there is a different microbiota composition in obese and lean individuals under introduction of the same quantity of food, with a prevalence of Firmicutes compared to Bacteroidetes, unlike patients with NAFLD characterized by a small representation of Bacteroidetes and an increased one of Prevotella and Porphyromonas (16) (Figure 1).

In particular, the intestinal microbiota can stimulate liver fat deposition and promote NASH through different mechanisms (Figure 2):

1) obesity promotion by increasing the energy extraction from food;

Figure 1 - Comparison of the relative abundance at different taxonomic levels between the 53 NAFLD patients and the 32 healthy subjects. (A) Average phylum distribution; (B) Primary genus composition in the two groups. *P, 0.05 and **P, 0.0. From Jiang et al. (16).

Figure 2 - Possible pathways involved in NAFLD pathogenesis, by gut microbiota. From (17).
2) increased expression of genes involved in lipogenesis (de novo lipid synthesis); 
3) modulation of the choline metabolism; 
4) regulation of bile acids metabolism; 
5) increasing of the production of endogenous ethanol of bacterial origin; 
6) regulation of the intestinal permeability; 
7) regulation of low - grade inflammation and the immune system.

Obesity promotion

Intestinal microbiota plays an important role in obesity and metabolic syndrome determinism thanks to its ability to increase the energy extraction from food (18). This can happen through the following mechanisms:

- **Interaction between short-chain fatty acids (SCFAs) and receptors coupled to G-protein** (19): this mechanism is able to ferment the non-digestible carbohydrates (derived from vegetable), producing monosaccharides and SCFA, which, besides providing energy to the enteroocytes, are also precursors of fatty acids, cholesterol and substrates for the hepatic gluconeogenesis. The SCFA also binds specific receptors on the intestinal neuroendocrine cells by inducing the release of peptide YY and leptin: the first reduces the intestinal transit and stimulates by promoting the absorption of nutrients, the second is a hormone that stimulates appetite.

- **Suppression of fasting induced adipose factor (FIAF):** the lipoprotein lipase (LPL) plays a fundamental role in the hydrolysis of triglycerides and release of fatty acids, as well as in their transport within the adipocytes. Fatty acids, once entered in adipocytes, are esterified to triglycerides and stored as fat. Secreted by adipose tissue, intestines and liver, the angiotriproteina-like 4/(FIAF) antagonizes the activity of LPL, thereby preventing the storage of triglycerides as fat. The group of Bakhed et al. showed that the intestinal microbiota suppresses the expression of the FIAF in response to a great quantity of food, thus increasing the LPL activity and finally the deposition of fat in adipocytes (16-18).

- **Suppression of adenosine monophosphate-activated protein kinase (AMPK):** AMPK is a heterotrimeric enzyme that plays an active role in energy homeostasis. It has the task to compensate an energy deficient state, by stimulating the oxidation of fatty acids, ketogenesis, the absorption of glucose and insulin secretion, while, as a result, it inhibits the synthesis of cholesterol, lithogenesis and triglyceride synthesis. The microbiota reduces the AMP-kinase phosphorylation, resulting in increased oxidation of fatty acids (18).

Lipogenetic expression of genes

The microbiota regulates lipogenesis through monosaccharides and SCFAs, which, once absorbed, reach the portal circulation and so the liver. The rise in insulin and blood glucose seems to have the ability to induce the expression of lipogenic enzymes in the liver: acetyl - CoA carboxylase (ACC1) and fatty acid synthase (Fas). These two enzymes are controlled by two nuclear factors: the carbohydrate response element-binding protein (ChREBP) and the sterol regulatory element-binding proteins (SREBP - 1) (17) (Figure 3).

Modulation of choline metabolism

Choline is an important component of membrane’s phospholipids and it is central in the hepatic metabolism of lipids and VLDL assembly. In the gut, it is cleaved by bacterial enzymes into toxic...
metabolites (dimethylamine and trimethylamine) which are subsequently converted into trimethylamine-N-oxide, responsible for liver inflammation. If an excessive amount of choline is converted, there is less bioavailability for the production of VLDL in the liver. Therefore, the damage can occur by means of two mechanisms: one implicates a greater production of this toxic metabolite, while the other one causes a greater accumulation of triglycerides in the hepatocytes. In fact, VLDL lipoproteins have very low density and contain large amounts of triglycerides and few cholesterol, phospholipids and fat-soluble vitamins, which are able to transfer triglycerides from the liver to the tissues. In particular, after being synthesized in the liver, they are released into the bloodstream and absorbed mainly by the muscle tissue and adipose tissue (20).

Regulation of bile acid metabolism

The microbiota changes bile acids secretion and their interaction with the signaling pathways of FXR and TGR5. Bile acids, included into the bile, are secreted in duodenum which emulsify fats and fat-soluble nutrients facilitating their digestion and absorption. They also exert an antimicrobial function damaging the bacterial membrane by interacting with phospholipids. Recent studies have shown that a diet rich in fatty changes the composition of bile acids influencing the microbiota and giving dysbiosis. Conversely, however, the microbiota is able to modulate the metabolism of bile acids through the stimulation of the farnesoid X receptor. Bile acids are ligands for G-protein coupled receptors such as TGR5 / Gpbar-I, and activate their nuclear receptors such as FXR. The latter is expressed in the liver and in the gut and controls lipogenesis, the export of triglycerides by VLDL and their plasma turnover. Bile acids also bind the TGR5, which is implicated in the control of glucose metabolism by stimulating the secretion of glucagon-like peptide-1. It was observed that TGR5 reduces serum and hepatic triglyceride levels, and consequently the degree of steatosis (21), following the administration of specific agonists.

Increased endogenous ethanol production

Dysbiosis and SIBO have been associated with endogenous ethanol production of bacterial origin due to a high proportion of ethanol producing bacteria (E. Coli) that contributes to the morphological and functional damage in the small intestine by increasing the permeability to endotoxins coming from the lumen with subsequent hepatic damage (22-24). The microbiota produces a large quantity of hepatotoxic compounds, including ethanol, phenols, ammonium, which are transported to the liver by the portal circulation. These compounds activate kupffer cells and stimulate the production of NO, cytokines and TNF-alpha. Therefore, ethanol can contribute to NASH in two ways: by inhibition of the Krebs cycle, which stimulates the production of ROS and determines direct liver injury, and by contributing to the increase of intestinal permeability destroying the integrity of the barrier and causing a passage of endotoxins into the circulation via NOS. Zhu et al. studied the intestinal microbiota of patients with and without NASH in obese individuals and children in health. They noticed an increase in alcohol-producing bacteria in children with NASH, associated with an increase in blood alcohol without an alcohol intake (21).

Altered intestinal permeability

The microbiota sustains the intestinal barrier through different mechanisms: firstly, it provides nourishment to epithelial cells, to the proliferation and tissue repair via the SCFA, which is derived from the fermentation of carbohydrates; it also regulates the biosynthesis and catabolism of mucin, increases the expression of tight junctions and finally plays a protective effect against pathogenic microorganisms (14). These mechanisms may fail in the process of homeostasis alteration between microbiota and host, which means a possible damage of the barrier and of the intestinal permeability, which will be increased, resulting in the phenomenon called “leaky gut syndrome” (25). Through the alteration of the intestinal barrier, a translocation of bacteria can happen together with toxic substances produced by them, which, entering the portal circulation, can reach the liver and determine a large number of pathological changes (24). Also food antigens pass through the damaged intestinal mucosa causing an immune response and inflammatory reactions that may be the cause of inflammatory syndromes, autoimmune diseases and allergic phenomena. In fact, the pathogenetic theory of NAFLD has been enriched by another aspect, known with the
name of “gut -liver axis”. There is a close anatomical and functional relationship between the intestine and liver, because the liver receives about 70% of its blood supply from the intestines through the portal vein. Thus, it represents the first line of defense against intestinal antigens and it is one of the organs more exposed to toxic factors coming from the intestines, such as bacteria and their products (18). The gut -liver axis plays a central role in the pathogenesis of NAFLD, especially through interactions between microbiota and the host’s immune system modulating inflammation, insulin resistance and intestinal permeability. Therefore, the intestinal microbiota may play an important role in maintaining gut -liver axis health and in the pathogenesis of NAFLD.

There are many scientific evidences concerning the possible role of the gut-liver axis malfunction as a determinant of NAFLD development (26). Especially the conditions involved in the physiopathogenetic process are SIBO (which stands for “Small Intestinal Bacterial Overgrowth”) (27) and the intestinal dysbiosis, or intestinal dismicrobism. Dysbiosis is a condition characterized by an imbalance of the enteric bacterial flora and increased intestinal permeability that may be due to physiological phenomena such as fasting, intense physical effort, intestinal infection or a chronic and non-physiological process. In both cases, the intestine allows the passage of massive toxins, foods only partially digested, antigens, pathogenic organisms including bacterial, fungal and parasites that are associated to a systemic inflammatory condition causing an excessive load for the liver and the nervous system.

In patients with NASH, cirrhosis, obesity and glucose intolerance there is a predisposition to develop autonomic neuropathy. This pathology leads to a reduced intestinal motility and, therefore, in a delayed gastric emptying and retention of undigested material in the lumen. All these events promote a retrograde colonization of the small intestine resulting in Small Intestinal Bacterial Overgrowth (SIBO) (25). The study conducted by Miele et al., which compared the biopsy of 35 patients with NAFLD with healthy controls, showed an increase in intestinal permeability (leaky gut) with alteration of tight junctions in patients with NAFLD. This proves how the increased intestinal permeability and SIBO are correlated with the degree of severity of steatosis (28). The study of Abele et al. conducted on rats after administration of a high fat diet and an agent that causes epithelial damage, showed liver fibrosis due to bacterial translocation. Jiang et al. looked at duodenal tight junctions by TEM in patients with NAFLD. They observed a larger width of the tight junctions, irregular microvilli with loss of barrier integrity and increased intestinal permeability compared to healthy subjects. Specifically, they observed through immunohistochemical studies an increased expression of occludin protein in healthy subjects compared to patients with NAFLD (16).

Regulation of low-grade inflammation and activation of the immune system

Toll-like receptors (TLRs) and NOD-like receptors recognize bacterial products PAMPs (pathogen associated molecular patterns) and DAMPs (damage associated molecular patterns) (29), which are highly conserved microbial molecules (Figure 4). NAFLD and other states of insulin resistance are associated with the activation of the innate immune system. The intestinal flora is involved in the development and homeostasis of host immunity. In fact, the cross-talk between host and bacteria in the mucosal interface layer is responsible for innate and adaptive immune responses that protect the guest and help to maintain intestinal homeostasis. This communication depends on specific receptors, including TLRs and NOD-like, which are called pattern recognition receptors. They recognize bacterial and viral components (30, 31) and are present on many liver cells including hepatocytes, Kupffer cells, stellate cells, endothelial cells of sinusoidal, biliary epithelial cells and immune cells (32, 33). TLRs signaling is normally suppressed in healthy liver, but it acts as a sensor of the immune system of PAMPs and DAMPs: they start an adaptive immune response, which is the first line of defense against invading pathogens through the production of anti-viral cytokines and anti-bacterial (29). In fact, the following signal cascade leads to the activation of pro-inflammatory genes, such as tumor necrosis factor- α (TNF-α), IL-6, IL-8, IL-12 and interferon (34). In mammals 13 different kinds of TLR have been identified, including TLR2, which is associated with the pathogenesis of NAFLD. TLR4, TLR5, and TLR9 recognize lipopolysaccharide, peptidoglycan, flagellin and bacterial DNA (35-37). The most studied PAMP is the active portion of endotoxin, lipopolysaccharide (LPS), a component of the cell membrane of gram...
negative bacteria, which reaches the liver via the portal vein (38). The LPS binds lipopolysaccharide-binding protein (LBP), which in turn binds the CD14. The complex LPS-LBP-CD14 activates TLR4, present on Kupffer cells, triggering an essential inflammatory cascade which includes the protein kinase and stress-activated mitogen-activated, the Jun N-terminal kinase (JNK), p38, interferon regulatory factor 3 and the way of nuclear factor NF-kB (39). The translocation of NF-kB to the nucleus induces the transcription of several pro-inflammatory genes in the Kupffer cells, such as TNF-α and IL-6, IL-8, IL-1 β and generates reactive oxygen species (ROS). The synthesis of proinflammatory cytokines causes inflammation, liver and prolonged damage (35). This inflammatory response induces the production of profibrotic factors from stellate cells through the signaling of transforming growth factor-β (TGF-β); insulin signaling impairs the consequent increase of the inflow of FFAs, alters the mitochondrial beta-oxidation resulting in hepatic steatosis and inflammation (40). There are also the inflammosomes, large intracellular multiprotein complexes that play a central role in innate immunity. This responds to a large range of PAMPs, including bacterial flagellin, and DAMPs. Among the inflammosomes there is a member of the family of the NOD-like receptors that recruits the inflammosome-ASC adapter protein. This factor interacts with caspase-1 activating the inflammosome and, as a result, the proinflammatory cytokines IL-1β and IL-18. This mechanism is thought to be involved not only in maintaining the state of chronic inflammation in obesity, but also in the progression of NAFLD / NASH, as well as in multiple aspects of the metabolic syndrome through modulation of...
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the microbiota (25-41). In particular, the NOD-like receptor protein 3 (NLRP3) is implicated in the activation of caspase-1. The resulting IL-1β activation promotes insulin resistance, beta-cell death and the formation of atherosclerotic plaques (40). However, other bacterial endotoxins, such as peptidoglycan, help to stimulate the pro-inflammatory cascade, leading to the activation of the receptor NOD1 (42). TLR2 binds the bacterial cell wall components of gram-positive, like peptidoglycane and lipoteicoic acid. Many studies, mostly conducted with animal models, allowed us to correlate the LPS-TLR4 activation signal to insulin resistance and NASH. Cani et al. have described as a diet conducted for 4 weeks with high fat content leads to a moderate increase in the plasma concentration of LPS in mice. They have defined this effect as metabolic endotoxemia, because the LPS increase was still 10-50 times lower than the values that could be achieved during septicemia. Four weeks of continuous infusion of LPS in mice mimicked the phenotype of the high-fat diet, that is to say, an increase of insulin resistance, the content of triglycerides in the liver and inflammation of the adipose tissue (43). The role of the intestinal flora has been further demonstrated by means of antibiotic treatment which reduced the intensity of the disease in high-fat diet in ob/ob mice (44, 45). Since then, other studies in TLR-4 null mice (-/-) have confirmed that the TLR-4 is essential for the deposition of fat liver and the development of NASH (46, 47). All these mechanisms lead to the development of NAFLD.

The Primum movens is thought to be made up of increased influx of fatty acids to the liver (closely related to the accumulation of visceral fat) because of the alteration of lipid metabolism. This last phenomenon is due to the imbalance between lipogenesis and lipolysis (increased synthesis of fatty acids, beta oxidation deficits, deficits synthesis phospholipids, cholesterol esters and lipoproteins). The insulin resistance plays a key role in determining the initial accumulation of lipids in the hepatocytes, as it determines on the one hand the increased hepatic blood flow of fatty acids and, on the other hand, hyperinsulinemia. Both conditions cause the inhibition of mitochondrial β oxidation in the liver cells and an increase in β peroxidation in peroxisomes. The requirements which predispose the development of NASH are the exposure to a second or subsequent pathogenic noxae (intestinal flora changes, drugs, xenobiotics, etc.) and genetic factors. The following events are lipid peroxidation, production of reactive oxygen species and resulting inflammation. The reactive oxygen species produced in this way determine a mitochondrial injury, revealed by the presence in the hepatic cells of paracrystalline mitochondrial inclusions and by the loss of mitochondrial crests, typically found in biopsies of patients with NASH (48, 49). The lipid peroxidation and the increase in production of oxygen radicals result in decreased ATP synthesis and the production of cytokines, which induces the hepatic cells to go towards necrosis and apoptosis via the mitochondrial damage: this stimulates the induction of pro-apoptotic interleukins. In fact, such processes are able to stimulate the production of cytokines like TNF alpha, TGF beta, IL8, which, in turn, stimulate neutrophilic chemotaxis, necrosis, and the synthesis of collagen. The death of liver cells and the inflammation caused by this process induce liver fibrosis that can progress to cirrhosis (50). The main cells involved in fibrogenesis and in the abundant extracellular matrix deposition are myofibroblasts, monocytes and macrophages, lymphocytes and cholangiocytes (51). Some subpopulations of macrophages seem rather involved in the resolution of fibrosis, using the collagen lysis carried out by proteolytic enzymes such as matrix metalloproteases (MMPs). The liver myofibroblasts produce fibrogenetic factors (TGF-β1, VEGF, PDGF and IL-13), promote angiogenesis and the production of pro-inflammatory cytokines (52). Fibroblasts are present in inactive form in the portal connective and, after the chronic liver injury, differentiate into myofibroblasts thanks to the activation induced by reactive cholangiocytes (53).

The cholangiocytes and hepatocytes exposed to chronic damage gain the ability to migrate and produce extracellular matrix (54). In fact, cholangiocytes undergo processes of dedifferentiation and transdifferentiation called “ductal reaction”, characterized by profound changes both on the phenotypic profile and on their secretory capacity (55). The Kupffer cells and circulating monocytes produce TNF-α, TGF-β and PDGF-BB. These factors stimulate perisinusoidal quiescent stellate cells, which have important contractile activity by promoting angiogenesis and increased vascular resistance in the portal circulation (56). An important role is played by adipokines whose secretion would seem increased in patients with NASH. Especially in obese patients there is an increase in leptin that mediates the fibrogenesis by activating specific receptors on stellate cells and
promotes angiogenesis linked to fibrosis through the production of free oxygen radicals. Leptin also stimulates the expression of TGF-beta by interacting with the Kupffer cells and endothelial cells. Instead, adiponectin increases insulin sensitivity and has antiinflammatory effects thanks to the induction of IL-10 secretion by blocking the activation of NFkB, reducing the activation of TLR 4 in macrophages, inhibiting the release of TNF-alpha, IL1, and chemokine. Finally, it reduces the levels of ROS and therefore the inflammation and liver damage. There can be many causes that may contribute to increased intestinal permeability and among these, food intolerances (eg. Celiac disease), intestinal ischemia, excess pathogenic gut flora, poor production of immunoglobulin A, intestinal dysbiosis, the prolonged use of cortisone, laxatives, excessive intake of castor oil and NSAIDs. In particular, the effect of NSAIDs on intestinal permeability will be discussed. Indeed, through the alteration of the intestinal barrier, as happens in the NSAID enteropathy, a translocation of bacteria and toxic substances of intestinal origin can occur: entering the portal circulation, they can reach the liver and can lead to a large number of pathological alterations. It can be assumed that this condition promotes the development of NASH in patients with predisposition factors towards the development of NAFLD, such as obesity and metabolic syndrome. Not least to be considered, the direct effects of NSAIDs in determining liver damage, which is a predisposing factor for the development of nonalcoholic steatohepatitis, as will be described hereinafter. Specifically, NSAIDs would act at colic level exposing the patients with NASH to a susceptibility for gut leakiness. In fact, one of the mechanisms proposed for the second hit in the pathophysiology of non-alcoholic steatohepatitis (NASH) is the hepatic oxidative stress triggered by high levels of endotoxins, for example due to the destruction of the integrity of the intestinal barrier. Then, the endotoxemia represents the stimulus required to trigger the necro-inflammatory cascade in hepatocytes, already affected by alteration in lipid homeostasis induced by obesity. According to a study by Farhadi et al. (2008), the intestinal permeability was measured in patients with steatosis or with NASH and in healthy patients, before and after the administration of aspirin, through the urinary excretion: the lactulose/mannitol (L/M) ratio was evaluated after 5 hours, while the sucralose after 24 hours. It was observed that the aspirin increases the urinary excretion L/M in the majority of patients, but, especially, it significantly increases the intestinal permeability in patients with NASH. According to this model, patients with NASH would not have a constantly altered intestinal permeability, but this situation can happen because of the stress factors such as aspirin, NSAIDs, psychological and physical stress or other (57). For this reason it could be reasonable that patients with particular susceptibility to oxidative stress, such as those with metabolic syndrome (obesity, diabetes, NAFLD and insulin resistance) and altered metabolism of fatty acids, avoided agents such as alcohol and NSAIDs that increase intestinal permeability.

Intestinal permeability

Therefore, it is important to specifically analyze the intestinal barrier, with its constituents and intestinal permeability, and the various factors that interact and affect them (Figure 5). The intestinal barrier covers an area of approximately 400 m² and requires about 40% of body energy use. Its function is to prevent the dispersion of water and electrolytes, the inlet of antigens and microorganisms. It allows the exchange of molecules between the host and the environment and the absorption of nutrients (58). Water-soluble compounds can pass through the intestinal barrier. They easily penetrate the intercellular junctions when their molecular weight does not exceed 250-300 Dalton. Also fat-soluble compounds can cross the intestinal barrier. Anyway, they do not pass through the tight junctions, but according to their size, to the charge and their lipophilicity, can penetrate the brush border due to bile acids and the formation of micelles. The mechanisms of adaptation and specialization of the intestinal mucosa of mammals throughout evolution allowed it to reach a peaceful coexistence with the intestinal symbionts without triggering a chronic inflammation and to maintain an inflammatory and measured defensive response against pathogens (59, 60). It is a complex system which consists of an external and an internal “functional” immunological “physical” barrier (61). Some experiments showed that a disruption of the peaceful coexistence with intestinal symbionts in the early stages of life causes a severe immune deficiency and risk of disease (62, 63).
The intestinal barrier is a functional entity that separates the intestinal lumen by guests, protecting us from bacterial invasion or other microorganisms and toxins. This barrier consists of mechanical elements (mucus and epithelial layer), humoral factors (defensins and IgA), immunological factors (lymphocytes and cells of the innate immune system), mucosal and neurological elements (64). It is considered a set of different constituents, which include the intestinal permeability, the intestinal microbiota and mucosal immunology, each of which with a precise definition. Specifically, the physical barrier includes the cellular components constituted by the vascular endothelium, by the layer of epithelial cells and by that of mucus. Next to this physical barrier, there are chemicals that contribute to the barrier function, such as digestive, immune molecules substances, cytokines, inflammatory mediators and antimicrobial peptides, produced mainly by Paneth cells in the crypts of the small intestine (64) (Figure 6). Instead, the intestinal wall comprises four layers: the mucosa, submucosa, muscle and serosa (64).

**Intestinal barrier**

The physical barrier between the mucosa and intestinal lumen is constituted by a single layer of epithelial cells, constituted for 80% by absorptive cells and 20% by Paneth cells, enteroendocrine cells and goblet cells. Intestinal cells are bound together by tight junctions (TJ), the zonula adherens (also called apical junctional complex), by gap junctions and desmosomes (24). The TJ form a multifunctional complex, which seals the intercellular space, but can also act as pores that regulate the flow of water, ions and small molecules through the combination of claudin and other proteins of the junctional complex (56-65). The TJ complex consists of intramembrane protein and can be single or tetra-transmembrane domain: the former consists mainly of junction adhesion molecules, the latter, also composed of occludin, claudin and tricellulina (specifically the isoforms 2, 7, 12 and 15 of claudin), determines a selective permeability barrier (24). Occludine, claudine and tricellulina join the adjacent cells’ actin cytoskeleton through scaffolding proteins such as zonula occludens cytoskeletal proteins (ZO-1, ZO-2 and ZO-3), which are localized in the intracellular part of plasmamembrane. The claudins are the seal of molecules and pores that facilitate the loss of electrolytes and water. Tricullina and occludin, as well as a new protein called marve ID3, can be substituted with each other, but if all three are missing or deficient arises, a severe impairment of the permeability with leakage can happen (66). Under the tight junctions there are the adherence junctions (AJ). They are important in signaling between cell and cell and in the epithelial repair, as...
well as desmosomes that support the epithelial stability. 
Among the other constituents of the intestinal barrier there are:
• The mucous layer is composed of glycosylated and polymerized mucins produced by goblet cells and constitute an enormous structure similar to a network. Among these, Mucina 2 is the major component produced in the small and large intestine and it is critical in keeping the microbes faraway from the epithelial surface (67). The mucous layer is colonized by microbiota only in the outer layer where it interacts with various oligosaccharides and glycoproteins (68).
• The glycocalyx protects the apical part of the epithelium and is made up of mucins that are glycosylated after being bound with the microorganisms. Then, they are sent as a defense and prevention mechanism of colonization (69, 70).
• The Paneth cells produce a set of antimicrobial factors that protect cells of the crypt by microorganisms such as alpha-defensins (whose production would seem reduced in Crohn’s disease), and lysozyme REG3-proteins; among these last proteins, there is the RegIIIg (71, 72), antimicrobial peptide that keeps the bacteria at a distance of about ~50 micron.
• Other structures such as blood vessels, smooth muscle cells and the enteric nervous system, which help to regulate the mucous membrane and its ability to start specific defensive processes (62, 65, 73).
• The B lymphocytes, after their activation and proliferation, produce antigen-specific IgA which activates a response of T-dependent mucosa-associated lymphoid tissue (GALT) (74).

The intestinal barrier may be altered and damaged by several possible causes as shown by the Table 2 (64), some of which will be detailed hereinafter.

**Microbiota**

The intestinal microbiota contributes to “gut health”, by being involved in metabolic processes, and modulates the intestinal barrier. Its specific composition would seem to be less influenced by age, sex, body mass or geographical provenance of the host, but more by diet and by the genetic background (75). The microbiota has a symbiotic relationship with the human body and it is involved in the digestion and absorption of nutrients, vitamins, in the production of hormones and in the prevention of colonization by pathogens. It also has a central role in regulating
epithelial barrier. Moreover, the intestinal barrier plays a key role in limiting the inflammatory response against the microbiota and is regulated by a fine network of immunological mechanisms for the recognition and tolerance of the microbiota (76, 77). Many conditions such as atopic diseases, IBD, diabetes, obesity, cancer, neuropathy, and some medications can alter the microbial population or reduce its diversity. In addition, pathogenic bacteria can destroy the tight junctions, especially the occludine or perijunctional acto-myosinic ring through effects mediated by kinases (78, 79). Pathogens and antibiotics can also increase the mucinic degradation or inhibit the normal production of mucus (80).

Dietary factors

Some dietary factors may increase the permeability and consequently bacterial translocation. This event leads to liver inflammatory response, in white adipose tissue, in the brain and in other organs, triggering metabolic disorders such as insulin resistance, type 2 diabetes, cardiovascular disease, and non-alcoholic fat liver disease and non-alcoholic steatohepatitis (81, 82).

• An example is the energy-rich high-fat diet also called Western diet which alters the microbiota and increases intestinal permeability, resulting in endotoxemia. The diet rich in fructose would also be able to determine steatosis and dyslipidemia without altering glucose homeostasis, liver function or intestinal permeability, also reducing the Bifidobacterium and Lactobacillus with endotoxemia increase (86).
• Vitamin A and its derivatives regulate the differentiation and growth of intestinal cells (58).
• The short-chain fatty acid (SCFA), which includes acetate, propionate, butyrate and valerate, is produced by bacterial fermentation of indigestible carbohydrates in the colon.
• Prebiotics and probiotics may stabilize the intestinal barrier and prevent some of the consequences of its damage through the modulation of the intestinal microbiota and the intestinal barrier.

The measurement of the permeability can be studied by means of in vivo or in vitro tests, which study the ability of certain electrolytes or sugars of different molecular weight to cross the epithelium or the mucous layer, finally entering respectively into the submucosa and in Ussing chamber (which uses animal or human tissues for research purposes) (87, 88), or in the blood (tests with sugars) (89).

NSAID-mediated gastrointestinal lesions

More than 30 million people make daily use of anti-inflammatory drugs, which therefore constitute 60% of analgesic drugs in the US market. Like many
Influence of non-steroidal anti-inflammatory drugs on intestinal permeability and non alcoholic fatty liver disease

Table 3 - Risk factors for nonsteroidal anti-inflammatory drug-related gastrointestinal complications.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>GI Complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age ≥ 65 years (especially &gt; 70 years)</td>
<td>2: acclofenac, ibuprofen and celecoxib</td>
</tr>
<tr>
<td>History of peptic ulcer</td>
<td>RR of GI comp. 2 to 4: rofecoxib, meloxicam, nimesulide, sulindac, diclofenac and ketoprofen</td>
</tr>
<tr>
<td>Use of two or more NSAIDs at the same time</td>
<td>RR of GI comp. 4 to 5: tenoxicam, naproxen, diflunisal and indomethacin</td>
</tr>
<tr>
<td>Concomitant therapy with antiplatelet agents, anticoagulants, corticosteroids and selective serotonin reuptake inhibitors. Severe illness Helicobacter pylori infection</td>
<td>RR of GI comp. &gt; 5: piroxicam, azapropazone and ketorolaco</td>
</tr>
<tr>
<td>Use of more gastrolesive NSAIDs</td>
<td></td>
</tr>
<tr>
<td>RR of GI complications &lt; 2: acclofenac, ibuprofen and celecoxib</td>
<td></td>
</tr>
<tr>
<td>RR of GI complications 2 to 4: rofecoxib, meloxicam, nimesulide, sulindac, diclofenac and ketoprofen</td>
<td></td>
</tr>
<tr>
<td>RR of GI complications 4 to 5: tenoxicam, naproxen, diflunisal and indomethacin</td>
<td></td>
</tr>
<tr>
<td>RR of GI complications &gt; 5: piroxicam, azapropazone and ketorolaco</td>
<td></td>
</tr>
</tbody>
</table>

GI, gastrointestinal; NSAID, nonsteroidal anti-inflammatory drug; RR, relative risk. From (90).

other drugs, they are associated with a wide spectrum of side effects that include gastrointestinal and cardiovascular events, renal toxicity, hypertension or other. The 30-50% of NSAID consumers have endoscopic gastric lesions mainly located in the gastric antrum, often in the absence of clinical manifestations. During the treatment about 1-2% of patients experienced a serious complication of a GI lesion especially in the presence of concomitant risk factors (Table 3) (90). Several videoenterocapsule studies have shown that use of NSAIDs (both nonselective and selective Cox-2 inhibitors) may be associated with a high incidence of small-bowel erosion and ulceration (of the order of 55 to 75%) (91-94); chronic use of low dose aspirin has also been shown to be associated with the presence of similar small-bowel lesions (95, 96).

Recent publications have shown that the incidence of complications of the lower GI tract, many of these due to the use of NSAIDs and ASA, is on the rise while the incidence of upper GI lesions is declining (Figure 7) (97). The ratio high/low complications was 4.1 in 1996, but dropped to only 1.4 in 2005 (4, 97). In fact, it would appear that NSAIDs increase the risk of bleeding and perforation of the lower GI tract in a way similar to what was seen for the GI superior tract (98). These adverse effects associated with NSAIDs involving the duodenum, the jejunum and ileum are grouped under the name of enteropathy, while in the case of more distal involvement they are called colonopathy. A study performed with videoenterocapsula showed that about 2/3 of consumers of long-term NSAID (> 3 months) and short-term (<1 week), had from mild to severe

Figure 7 - Time trends of gastrointestinal events. Estimated number of events per 100,000 person-years on the basis of the adjudication of events in the validation process. Figure constructed using data from (97). GI, gastrointestinal.
drug lesions in the small intestine (Fortun and Hawkey 2007; Bjornsson et al. 2008)(99, 100).

Effect of NSAIDs on the intestinal barrier

Mechanisms of jejunal and ileal mucosal damage: Multi-hit concept

The mechanisms underlying NSAID-induced damage in the distal intestine are distinct from those of NSAID-induced gastroduodenal damage (101). The damaging effects of NSAIDs in the stomach and proximal duodenum are closely related to their ability to suppress cyclooxygenase (COX) activity and dependent on the presence of gastric acid. In contrast, the pathogenesis of NSAID enteropathy is less linked to COX suppression and gastric acid and more related to bile, enteric bacteria, and enterohepatic circulation of the NSAID (102-105).

The mechanisms underlying NSAID enteropathy are primarily represented by the so-called “topical effects”, i.e. those adverse effects coming from the high local concentration of NSAID in the intestinal lumen (106). The topical effects are by definition independent from the impact of NSAIDs on cyclooxygenase (COX) (107).

From a pharmacokinetic point of view NSAIDs are weak acids (pKa 3-6) which are protonated and then absorbed in the stomach according to their lipophilicity (108, 109). Subsequently, the NSAID-containing carboxylic acids, after the introduction by oral or intraperitoneal way, reach the liver via the portal system where the drug is glucuronidated: it is conjugated to glucuronic acid (110, 111) or taurine (112) or sulfate, and excreted into the bile in large quantities. Specifically, it is exported inside the bile canaliculi against a concentration gradient through the ATP-dependent transporters present on the apical membrane hepatocyte, the MRP2 (ABCC2) (113) or Bcrp1 (ABCG2) (114); the specific carrier of tauro-conjugates is less defined.

At this point the small intestine is exposed to the drug and to its oxidative conjugated metabolites that reach the most distal part where the glucuronide is cleaved by bacterial beta-glucuronidase. Thus, the enterocytes are exposed to aglycones, which are free derivatives of NSAIDs or oxidative metabolites (115). There are also the acyl-glucuronides which are generated in the liver from the NSAID-containing carboxylic acid and the iso-glucuronides, which are electrophilic reactive intermediates, secondary metabolites formed after the acyl migration of the aglcone along the sugar ring (Dickinson, 2011) (116) and are associated with the topical effects of NSAID enteropathy (Boelsterli and-Alcantara Ramirez, 2011) (117). Another release mechanism of the aglycones is the spontaneous hydrolysis of the glucuronide, which is pH-dependent (117). Then, the drug is transferred again into the enterohpatic circulation (Figure 8). Several studies have demonstrated that NSAIDs that do not undergo enterohpatic circulation do not cause small intestinal damage (118-121). In fact the increase in gut permeability is not observed with all NSAIDs, because those that do not undergo enterohpatic recirculation may not have this effect. It is now clear that long-term ingestion of most conventional NSAIDs (indomethacin, piroxicam, naproxen, ibuprofen, sulindac) (3, 99, 122, 123) is the most responsible for enteropathy.

An initial increase in small intestine permeability is a prerequisite of the subsequent development of small intestine inflammation, which is associated with blood and protein loss, but it is often silent (124).

It would appear that the enterohepatic recycling results in a prolonged and repeated exposure of the intestinal mucosa to the compound (10, 121).

Topical effects are the result of the oral administration of drugs, but also of the parenteral and intraperitoneal one due to hepatobiliary excretion of metabolites and enterohpatic circulation. They

Figure 8 - Enterohepatic circulation of NSAIDs. From (107).
are called luminal and include the uptake of the drug and its metabolites in the enterocytes where they are metabolized by cytochrome P450 (CYP450) in order to potentially reactivate intermediates with possible bioactivation and induction of mitochondrial (125-127) and endoplasmic reticulum stress (128, 129) (Figure 9). Therefore, the production of reactive metabolites occurs through CYPs of enterocytes, ER stress, oxidative stress and mitochondrial damage (107). The electrophilic reactive metabolites of NSAIDs are produced in the liver and transported in the small intestine through the hepatobiliary transport or they are generated directly in the intestinal epithelium (107). In the small intestine a large amount of forms of CYP (130, 131) is produced, expressed in enterocytes in a different manner depending on the different sections of the small intestine. Their distribution along the small intestine may correlate with the severity of ulceration (132). In humans it is mainly CYP2C8 / 9/19 to be involved in the oxidative biotransformation of many FANS (133). This step is called the “first hit”. After this initial insult of enterocytes, the mucosal epithelium becomes more permeable and the LPS present in the lumen can penetrate deeply into the mucosa and activate toll-like receptor 4 (TLR4) of macrophages in the lamina propria. This can cause cell damage mediated by tumor necrosis factor, and subsequently the activation of the innate immune system with recruitment of inflammatory cells into the injury site. The inflammatory response that follows is the “second hit” (134).

Therefore, the role of enteric bacteria in determining the NSAID enteropathy is twofold:

- Bacteria can invade the deeper layers of the mucous membrane when the tight junctions become more permeable after toxic insult and the subsequent activation of TLR.
- They can metabolically convert NSAIDs glucuronide in aglycones by activation of beta-glucuronidase, bacterial enzyme used to obtain sugars. Such enzymatic activity would seem greater in the distal part of the small intestine than in the other parts (135). However, the gus gene, which codes for this enzyme, is not present in all bacterial strains, but only in 50% of the human gut symbiotic bacteria (136).

**First hit**

1) **Mitochondrial damage**: most of NSAIDs causes a decoupling of oxidative phosphorylation in the mitochondria (OXPHOS) both *in vivo* and *in vitro*, dissociating the breathing from the production of energy and dissipating the inner transmembrane potential of mitochondria (137). During the absorption of the NSAIDs there is an intracellular accumulation of drug proportional to its acidity and even at micromolar concentrations: this is able to uncouple oxidative phosphorylation at the mitochondrial level. This event can have two effects on enterocytes. Firstly, an attenuation of ATP production with gradual depletion of cellular

![First hit and second hit in NSAIDs enteropathy. From (107).](image-url)
ATP; secondary, a collapse of the gradient can determine the opening of the mitochondrial permeability transition pore (mPT) leading to cell death (106). This ability is due to the structure of NSAIDs. In fact, NSAIDs are weak and lipophilic acids which induce enteropathy through mitochondrial energetic depletion (106). Some NSAIDs inhibit several complexes of the electron transport chain. Other NSAIDs, such as indomethacin and diclofenac, inhibit the activity of rotenone-sensitive complex I in mitochondria and therefore increasing the production of superoxide. This inhibition is reversible with the administration of quercetin, an ubiquinone-mimetic (coenzyme Q) (138).

2) Interaction with biomembranes: it’s due to the direct effect of NSAIDs on cell membranes by altering the biophysical properties. One example is the electrostatic interaction between the NSAID and hydrophobic anions and the positive charged nitrogen of phosphatidylcholine, which alters the biophysical properties of the membrane, its fluidity and finally increases the permeability to protons and to bacterial toxins (139).

3) Detergents properties: NSAIDs are invariably lipophilic weak acids and this makes them the detergents for phospholipid components of the brush border. This causes direct damage to the epithelial surface.

4) Mitochondrial permeability: NSAIDs can induce mitochondrial permeabilization followed by the release of apoptotic factors from the intermembrane space inside the cytosol. This mechanism is mediated by the opening of MPT pore, involving both the internal and external membrane, and can be triggered by an increase of calcium (mechanism introduced by many NSAIDs), oxidative stress or by the collapse of mitochondrial membrane potential (140, 141).

5) Intestinal permeability: this effect also affects TJ, which are under the control of the actin-myosin ATP-dependent complex. The consequence is an increase in the intestinal permeability (142). The reduction of mitochondrial ATP production causes loss of the intestinal barrier function and this can be tested quantitatively by oral administration of dextran (143).

6) Oxidative stress: there is only indirect evidence of its involvement in NSAID enteropathy. For example, indomethacin raises the expression of heme oxygenase (HO-1), an antioxidant enzyme induced by oxidative stress (144). Another pathway activated by oxidative stress is that of the MAPK (via phosphorylation of JNK). These effects can be induced by mitochondrial dysfunction that increase oxidative stress. This last event may be a side effect triggered by the inflammatory response of the innate immune system cells (145).

7) ER stress: according to some studies performed on patients taking diclofenac, there is an increase of markers of endoplasmic reticulum stress proteins, like GRP78 and CHOP. CHOP is a transcription factor that induces cell death mediated by mitochondria (146).

Second hit

It consists of the innate immune system and the inflammatory response. The innate response is triggered by bacteria and proinflammatory mediators coming from bacteria that invade the mucous layer over the epithelium. As a result, the signaling pathway TLR-mediated is activated and the neutrophils infiltrate the damaged areas. Instead, the adaptive immune system does not seem to play a critical role in NSAID enteropathy (107).

1) TLR and LPS: TLRs recognize specific molecular patterns associated with pathogens, and trigger the inflammatory response. In particular, TLR4 is the LPS receptor and it is expressed in monocytes and macrophages of the lamina propria as an extracellular domain rich in leucinic repetitions and a IL-1R signal intracellular domain (147). So, the TLR4 activates the NF-κB with consequent production of pro-inflammatory cytokines including TNF and IL-1 beta (148).

2) HMGB1: acronym for high mobility group box 1, it belongs to a family of proteins that are released from necrotic cells and macrophages during inflammation. In this case, HMGB1 has the role of proinflammatory cytokine. In fact, it is an endogenous ligand for TLR4 and other TLR contributing to their activation (149).

3) TNF: prostaglandins, and in particular PGE2, inhibit TNF synthesis, while the reduced levels of prostaglandins induced by NSAIDs lead to an increase of its synthesis (150). TNF is implicated in apoptosis of enterocytes and in the inflammatory response in the intestine. However, according to some studies, TNF appears to have cytoprotective effects on the intestinal mucosa by inducing the expression of COX2, mediated by EGFR transactivation (151). Also IL-17A should be mentioned. It is produced by T cells of
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4) **Neutrophils:** the NSAID enteropathy is characterized by a massive infiltration of neutrophils in the ulcerated areas, which aggravate the damage through the production of ROS or protease. The biomarkers used for their study is the time-dependent increase in the activity of myeloperoxidase (134).

5) **Bile acid metabolism:** the critical role of bile in the pathogenesis of NSAID enteropathy is evident from studies showing that bile duct ligation prevented NSAID-induced intestinal damage in rats (103, 121, 153, 154). According to some animal models, the NSAIDs, especially indomethacin, are rapidly excreted via the bile and then enter the enterohepatic circulation through the small intestine, resulting in a high concentration of drug in the liver and bile. Bile acids are cytotoxic because of their cleansing effect, by binding to phospholipids and directly altering the integrity of the membrane. The phospholipids and cholesterol are considered luminaries factors with direct effects that appear to be involved in protective mechanisms on gastrointestinal and liver cells by cleansing effect of bile acids. NSAIDs are highly amphiphilic molecules and create stronger links with the phospholipids. Animal and laboratory studies show that NSAIDs reduce the hydrophobic properties of the upper GI barrier, partly determined by the active surface phospholipids. Therefore, NSAIDs secreted in bile interact with its amphipathic components, such as phosphatidylcholine and bile acids; this leads to an alteration of the structure and the stability of these components, and consequently the toxicity of bile in the small intestine is modified (155). Especially Dial et al. examined the biliary phosphatidylcholine (PC), which appears to have protective effects on enterocytes, cholangiocytes and erythrocytes against damage induced by bile salts. NSAIDs appear to determine intestinal damage in proportion to their ability to be secreted via the bile because of their capacity to chemically bind with the micelles and with the PC, lowering their effects. As mentioned before, NSAIDs bind the PC and this process takes place in the gastrointestinal tract, where the drug-induced loss of PC, that protects the mucosa, causes mucosal damage. The PC would protect it from both damage induced by bile salts and by NSAIDs (156, 157).

**Adverse effects mediated by inhibition of COX**

The pharmacological target of NSAIDs is cyclooxygenase (COX or prostaglandin endopeptidase synthetase) with consequent reduction in the production of prostaglandins (PGE)(158). The PGE are implicated in a significant number of critical functions in the bowel, such as the maintenance of blood flow, the turnover of epithelial cells and the resolution of inflammatory processes. In this last case, their suppression is related to enteric toxicity (106). Some experiments were performed on the small intestine of rodents in which the concentration of PGE2 (the predominant factor in the intestinal mucosa) after the administration of NSAID was measured. This study showed not only the reduction of PGE2 after 3 hours of a single dose of indomethacin, but also how this reduction clearly precedes the development of an ulcer (159). In fact, the PGE2 promotes healing of small intestinal lesions by stimulating angiogenesis, which is mediated by the prostaglandin EP4 receptor subtype, and reversible after administration of EP4 agonists (160).

The cyclooxygenase consists of two isoforms with distinct functions:

- the COX-1, encoded by the gene PTGSI1, which represents the shape constitutively expressed in many tissues. COX-1 catalyzes the formation of many cytoprotective PGE and is related to inflammation development;
- COX-2, encoded by the PTGSI2 gene and inducible form implicated in the resolution of inflammation processes. It is responsible for the production of a variety of PGE that may cause or protect against inflammatory processes. It is produced at low levels in normal hepatocytes, but its production is markedly increased in chronic hepatitis and liver cirrhosis (161).

The concept that inhibition of both isoforms determines enteropathy is strengthened by a study, conducted by Adler et al. in 2009. In this investigation, patients with functional deficiency of the gene encoding the cytosolic phospholipase A2alfa, rapidly developed ulcers in different sites of jejunum and ileum because of the loss of the protective functions of the PGE. In fact, A2alfa is an enzyme which releases arachidonic acid from the membrane phospholipids: this acid is the substrate for COX in the production of PGE (or for LOX in producing leukotrienes). In addition, the inhibition of the COX can divert arachidonic acid metabolism in other directions, as the way of lipogenase (5-...
LOX), and this would lead to oxidative stress due to the production of superoxide, thus contributing to the development of NSAID enteropathy. According to some evidence, also the inhibition of COX-1 would result in an inability to increase the flow of blood in the microcirculation of the lesion, thus suggesting an initial damage of ischemic origin. While it seems to be clear the role of COX-2 inhibition on the damage mechanism at the gastric level, on the other hand, there is less knowledge on the effect at enteric level. One hypothesis is represented by the probable immunomodulator role in the small intestine performed by COX-2; while another one affirms that its inhibition is proinflammatory. A study performed by administering R-2-phenylpropionic acid (which has the same effects of topical NSAIDs in absence of interaction with the enzymes COX) and celecoxib in mice deficient in COX-1 and COX-2, suggests the great importance of COX-2 in maintain the integrity of the small intestine. In fact, the combination of the absence or inhibition of COX-2 and the topical effects of NSAIDs results in a change of the characteristics of NSAIDs enteropathy without concomitant inhibition of COX-1 and/or decrease of mucosal prostaglandins (162).

**NSAID associated enteropathy and PPIs**

The ulceration and bleeding produced by NSAIDs in the stomach and duodenum can be diminished by co-administration of inhibitors of gastric acid secretion, such as proton pump inhibitors and histamine H2 receptor antagonists (163). However, it’s clear that these drugs offer no protection to the small intestine NSAID-induced damage. In fact more and more emphasis is placed on strategies to prevent bleeding in the upper GI tract induced by NSAIDs, and especially by aspirin at low doses (low-dose aspirin, LDA), through the identification and modification of the associated risk factors and co-administration of antisecretory agents. This is necessary for the increasing prescription of aspirin in low doses, thanks to its use for primary and secondary prevention of cardiovascular events, as well as for its protective effects against colon cancer and other gastrointestinal sites (113). Especially, the use of PPIs in patients taking LDA seems to be the best strategy in reducing the peptic ulcer and the complications of the upper gastrointestinal tract (164). This strategy doesn’t have a direct protective effect on the NSAID-mediated damage in the most distal part of the small intestine, where the incidence is estimated to be about 1 - 6 cases per 1000 patients per year. Furthermore emerging evidence indicates that inhibitors of gastric acid secretion significantly worsen the small intestinal damage caused by NSAIDs (165-167), as confirmed in a recent study analyzing the effects of co-administration of pantoprazole sodium and diclofenac sodium (168). The PPI would be able to alter the defense mechanisms of the gastrointestinal tract in different ways: they determine hypocloridia causing abnormal growth of bacteria that can colonize the small intestine causing SIBO (the risk of which increases after one year of therapy), with increase bacterial translocation (169-170); they seem to facilitate the transport of various molecules enlarging the intraepithelial spaces by the increase of gastric mucosal permeability and reduce the viscosity of the intestinal mucus (171, 172); finally they reduce intestinal motility (inhibiting the contractile activity and relaxing the smooth muscle) and retard the speed of gastric emptying (173, 174).

A multicenter study, carried out in Japan by Endo et al. investigated through the use of a videointerceptive capsule the presence of factors associated with increased risk of small bowel bleeding in patients taking LDA for more than three months. It was concluded that the association with the PPI increased this risk (175). This increase could be due to the alteration of the microflora determined by the PPI and a consequent increase in the susceptibility of the small intestine to the damage mediated by NSAIDs. According to the findings from the study by Wallace et al. the contribution of bacteria to NSAID enteropathy was highlighted demonstrating that PPIs exacerbated NSAID enteropathy in rats and that this effect was a consequence of specific alterations in the intestinal microbiota by the PPI. In fact, in this study, the rats were treated with antisecretive doses of omeprazole and lansoprazole for 9 days and with the combined administration of NSAIDs, such as celecoxib and naproxen, in the final days. They analyzed hematocrit variations, the damage of the small intestine and alterations of intestinal microflora. From these analyses, it was verified that both PPI significantly exacerbate the intestinal ulcers and bleeding determined by the two considered NSAIDs. Moreover, they also determined an alteration of the number and types of enteric bacteria, with a reduction of about 80% of
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actinobacteria and bifidobacteria spp. A reduction in the incidence and severity of adverse events mediated by these NSAIDs were obtained from the prevention or reversal of dysbiosis through the reintroduction of these two bacterial species. A report of 2012 showed that when the PPI are administered in high-risk patients, who receive dual antiplatelet therapy with aspirin and an inhibitor of the platelet receptor, the incidence of bleeding in the upper GI tract is very low (176), while that of the upper portion increases. Finally, there are two aspects to consider: the first is that gastrointestinal bleeding events may arise from pre-existing lesions, such as ulcers, H. pylori, or de novo lesions induced by LDA; secondly, it have to be considered that the size of the coated tablet seems to have no influence on the risk compared to non-coated formulation. Similar effects have now been shown for another class of inhibitors of gastric acid secretion, the histamine H2 receptor antagonists (165, 177).

Some evidence suggests that PPI-induced dysbiosis determines changes in bile that may be responsible for exacerbation of NSAID-induced intestinal damage (177). In several studies chronic use of PPIs is associated with small intestinal bacterial overgrowth and bile acid dysmetabolism (178-180). PPI-induced dysbiosis probably triggers bile acid dysmetabolism, since many intestinal microbes are capable of enzymatic modification of bile acids (181).

Methods of investigation

To estimate the relative importance of all mechanisms involved in the pathogenesis of NSAID enteropathy, it is necessary to use specific investigations aimed at the study of intestinal permeability, the number of intestinal bacteria and enterohepatic recycling. Intestinal permeability studies were conducted in the late ’80s, in which healthy subjects took aspirin, indomethacin and ibuprofen and showed increased intestinal permeability and a destruction of the intestinal barrier, whose morphological alterations resided in the intercellular junctions (122). A study by Brian et al. in 1997 examined these parameters after the administration of the diclofenac and its derivative nitrofenac. These drugs have the same anti-inflammatory and antipyretic activity. The nitrofenac is a derivative belonging to the so-called NO-NSAIDs, that is, NSAIDs with attached nitric oxide-releasing compounds. It has an equal inhibitory effect on COX-1 and COX-2, but it has the typical characteristic of the NO-NSAID to reduce the damaging effects on the stomach and intestine, while inhibiting the prostaglandin production like the traditional NSAIDs. In this study the intestinal permeability was analyzed through the urinary excretion of [51Cr] EDTA administered orally. In particular, the content of enteric bacteria was studied through the analysis of tissue samples of caecum, jejunum and ileum, while the enterohepatic recirculation was studied through the analysis of bile. Specifically, it was obtained that the diclofenac causes a progressive increase in intestinal permeability, the number of gram-negative bacteria and frank intestinal ulcers. The nitrofenac causes similar effects after the first administration and such effects don’t increase with repeated administrations. Moreover, nitrofenac would not lead to the appearance of ulcers or an increase in the bacterial content. This is probably due to the fact that the nitrofenac doesn’t take part to enterohepatic recycling (as well as aspirin and nabumetone) and this hypothesis gets down the importance of reduced synthesis of PGE in NSAID enteropathy (119).

Another study by Sigthorsson et al. in 1998 highlighted as most of NSAIDs are equally associated with inflammation and alteration of intestinal permeability in the small intestine. In this case absorption tests were performed at three different osmolarities, by administering to approximately 68 patients iso-, hypo- and hyperosmolar compounds for six months. Also in this study it was confirmed the minor pathogenicity of drugs such as aspirin and nabumetone (182).

The visible damage on the mucous membrane of the small intestine of chronic users can be checked by endoscopy, using for example the videoenteroscopula, as done in a study undertaken by David et al. in 2005. This investigation focused on ambulatory patients with various types of arthritis and which took daily NSAIDs. In particular, mucosal lesions of the small bowel resulted endoscopically visible in the patients than in the control group. Among these lesions, there were red spots, large and small erosions and ulcers, which can lead to a chronic bleeding and to an anemic state (183).

NSAIDs and NAFLD

It should be emphasized that NSAIDs are able to determine a direct damage and the consequent liver steatosis (micro or macrovesicular type) and
statohepatitis. In fact, these drugs are implicated in the pathogenesis of the so-called drug-induced liver injury (DILI) (184), which is diagnosed when the worsening of liver function is given by prescribed medications or not. According to RUCAM criteria (Roussel-Uclaf Causality Assessment Method) is likely to talk about DILI when it appears within 90 days of starting therapy, with improvement within 15-30 days from its suspension in case of hepatocellular and cholestatic injury (185, 186). Steatohepatitis caused by drugs can occur many months after their use and cannot be resolved within 15 days. However, it can be possible that the drugs exacerbate a pre-existing NAFLD (187, 188).

The drugs that determine steatosis and NASH interfere firstly with the mitochondrial respiration, beta-oxidation, or both, as shown in one of the first studies performed on pirprofen (189). When the hepatic mitochondrial beta oxidation is severely inhibited, the damage of the beta-CoA oxidation increases the levels of non-esterified fatty acids, which are converted into triglycerides determining hepatic steatosis (190). An increased production of ROS is the result of this process, and, in the most severe cases, this increase leads to liver necrosis (191, 192) (Figure 10).

Drugs, that inhibit mitochondrial beta oxidation, make it through different mechanisms:

- inhibit the entry of LCFA into the mitochondrial matrix, which consequently inhibits the mitochondrial acyl-CoA synthase (troglitazone) (193);
- appropriation of CoA thioester in the form of Farmaco.CoA (valproate) (194);
- inhibition of the enzymes catalyzing the beta-oxidation, such as the acyl-CoA dehydrogenase (glucocorticoids) (195);
- increased synthesis or reduced secretion of hepatic triglycerides (dexamethasone) (196, 197);
- increase in the de novo synthesis of fatty acids (antipsychotics) (198).

### Therapeutic approaches

Currently, there are not approved pharmacological strategies that can treat or completely prevent NSAID-mediated enteropathy. Some compounds suitable to reduce the inflammatory response or to stimulate the effects mediated by prostaglandins were used, but with limited effectiveness or adverse effects (102-199). Most of the experiments and of the therapeutic approaches are focused on the inflammatory component that constitutes the second hit, while approaches to protect from the first hit (mitochondrial stress, endoplasmic reticulum stress, electrophilic stress) or acting on the release of glucuronide and aglicoles, have not yet been fully explored. Since there are multiple mechanisms involved in NSAIDs enteropathy and in the subsequent development of NAFLD, also the therapeutic approach has to aim at applying multiple strategies simultaneously.

- **Mucosal protective agents (MPAs):** an animal study has shown that misoprostol, irsogladine, rebamipide and mucin of porcine stomach inhibited the formation of intestinal lesions...
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caused by a high dose (10 mg/kg) of diclofenac alone and prevented the exacerbation of diclofenac-induced lesions by antisecretory drugs (200). The group of Fujimori in 2011 studied the effectiveness in NSAIDs enteropathy of a mucosal protective agent used for the treatment of gastric ulcer, the rebamipide. This agent acts according to a mechanism not completely known, but presumably through the increase of PGE and the mucus secretion (201).

- **Co-treatment with prostaglandins**: a significant reduction in intestinal permeability was observed during co-administration of PGE or analogues PGE; regardless, they were not able to normalize or abolish the changes of the permeability and, therefore, even to prevent the development of enteropathy when taken in the long-term. A study performed with the videointerocapsule showed that co-administration of misoprostol (an analogue of PGE1) reduces by 75% the lesions of the small intestine and inflammation associated with the use of diclofenac (Fujimori et al. 2009). However, this approach is limited by the multidaily administration and the side effects such as diarrhea and abdominal pain. Its use is also forbidden in pregnant women because of its abortive effect (202).

- **Pharmacological inhibition of mitochondrial stress**: the release of signaling mediators in the mitochondrial cell death is regulated by a large number of factors, including the cyclophilin D, which regulates the pore mPT. A study, in which ulcerogenic doses of diclofenac and alisporovir (a selective inhibitor of mitochondrial cyclophilin D) were co-administered, showed their protective effect on the increase of the intestinal permeability and on the enteropathy caused by NSAIDs (Lo Giudice et al. 2010) (121).

- **Pharmacological inhibition of bacterial beta-glucuronidase**: in the pathogenesis of NSAIDs enteropathy the site-specific release of glucuronidase come from bile, and the local release of aglicoles in jejunal and ileal lumen by bacterial enzymes have a key role. According to recent studies, the selective inhibition of mitochondrial beta-glucuronidase with a bacterium-specific chemical inhibitor would seem to relieve ulcers of the small intestine caused by diclofenac (102). These molecules belong to a group of compounds tested for their effectiveness against the toxicity of CPT-11 (irinotecan) with the same mechanism (Ahmad et al. 2011) (203). They are selective and non-toxic molecules that do not act on the beta-glucuronidase of mammals and are not lethal to human cells.

- **Micronutrients**: other experimental studies analysed the possible action on the uncoupling of mitochondrial oxidative phosphorylation. In one study, the administration in man of a compound containing glucose and citrate together with indomethacin abolished the increase in intestinal permeability. The same findings were observed in another study in which ATP was added to therapy with indomethacin. Similar effects would also seem to be obtained with the administration of metronidazole.

- **Reduction of the acid**: the administration of H2-receptor agonists with NSAIDs do not seem to have any effect in reducing the damage despite the pH changes would affect their pharmacokinetics.

- **TNF-α block**: TNF-α together, alone or in combination with other proinflammatory cytokines, induces and modulates the induction iNOS expression (inducible isoform of NO synthase) and regulates the inflammatory response. In a study, daily doses of theophylline or pentoxifylline (phosphodiesterase inhibitors) were administered to rats for 2 days. Then, a jejuno-ileitis was induced via two cutaneous injections of indomethacin (7.5 mg/kg) and subsequent administration of theophylline or pentoxifylline for 12 hours or 4 days. Other rats were treated with a single intraperitoneal injection of monoclonal antibodies antiTNF-α (TNF-Ab) 30 minutes before of the administration of indomethacin. On the fourth day, the therapy with one of these three drugs caused a significantly reduction of the ulcers induced by indomethacin, of myeloperoxidase activity, of the TNF-alpha levels in serum and tissue, of IL-1beta, the nitrite / nitrate ratio and the induction of iNOS expression (204, 205). A study by Barbuio et al. analysed the monoclonal antibody administration antiTNF-α (infliximab) for 10 days in Wistar rats fed with a high-fat diet, obtaining a significant decrease in the liver of proinflammatory markers such as TNF-α, IL-6, IL-1β and SOCS-3. This event was accompanied by the reduction of fat deposition, fibrosis and inflammation, with an improvement in insulin signal transduction through the receptor (IR)/IR substrate / Akt / FOXO1 and via JAK2 /
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STAT3 (206). Experimentally, pentoxifylline would appear to induce an improvement in the levels of transaminases, but the study is not supported by histological data. The adiponectin induces a reduction in body fat and free fatty acids, improves hepatic and peripheral insulin sensitivity and has anti-inflammatory effect; in animal models it was shown that adiponectin causes a reduction in hepatomegaly, steatosis, levels of transaminases and TNF-α (liver and serum).

• A role of 5-ASA in enteropathy therapy with NSAIDs was suggested by Nandi et al. In this study, the enteropathy was induced in the rat after administration of indomethacin at a dose of 7.5 mg/kg/day subcutaneously for two days. The study of the small bowel during the following 14 days showed small ulcers, as well as an increase in the activity of myeloperoxidase, the expression of iNOS and the serum levels of nitrites/nitrates. These parameters, as well as the width of ulcers, underwent a decrease after administration of 5-aminosalicylic acid ASA, at an orally dose of 10 or 50 mg/kg/day. The best effects were obtained after the administration of dexamethasone (transcription inhibitor of iNOS) at a dose of 3 mg/kg/day (207). Sulphasalazine seems to reduce the inflammation of the small intestine and bleeding.

Among the various approaches, there is the use of selective inhibitors of COX-2 and NO or H2S-releasing NSAIDs. Nitric oxide and hydrogen sulfide are powerful vasodilatory molecules that protect the mucous membrane and maintain its integrity. The new class of H2S-releasing NSAID derivatives has demonstrated vastly improved GI safety, with minimal damage in stomach and small intestine. H2S is an endogenous gaseous signaling molecule, that protects against potentially damaging luminal agents such as acid, bile, and various drugs (208), and modulating the microbiota (209). In particular, ATB-346, a H2S-releasing naproxen derivative, has demonstrated superior GI safety compared with its parent NSAID (naproxen) (210, 211). A recent study of Blackler et al., in which rats were treated orally with naproxen or equimolar doses of ATB-346 over a 5-day period, with or without PPI administration, demonstrated that naproxen caused significant intestinal damage and inflammation, whereas ATB-346 did not. Naproxen dose dependently increased the cytotoxicity of bile, and it was further increased by PPI coadministration, it was greatly reduced in rats treated with ATB-346. The enteric microbiota of naproxen-treated rats was distinct from that in vehicle- or ATB-346-treated rats, and PPI administration caused significant intestinal dysbiosis, increasing an abundance of jejunal gram-negative Proteobacteria including pathogens, such as Salmonella and Escherichia coli, and decreased abundance of jejunal gram-positive bacteria such as Actinobacteria and Firmicutes (212). Furthermore bacterial enzymes converts primary to secondary bile acids, that are more toxic to intestinal epithelial cells and this process is increase in case of dysbiosis (150-213). This could lead to think that their association with NSAIDs mitigates its negative effects due to inhibition of prostaglandin. Particularly, nitric oxide-releasing NSAIDs or COX-inhibiting nitric oxide donator were studied as a potential alternative to selective NSAIDs and not; among these, there is the naproxcinod, one of the first, and perhaps the only one, to be investigated through clinical trials, showing a slight improvement of the intestinal tolerability when compared with naproxen.

Because of the lack of studies on the results and the potential adverse effects, this compound has not yet received the approval to be marketed from the Agency of the drug in Europe and in the US. As an alternative, COX-2 selective drugs such as nimesulide, celecoxib, rofecoxib and lumiracoxib can be used. In fact, they don’t increase the intestinal permeability when taken for short periods. It should also be noted that intestinal bacteria play an important pathogenic role in the development of NSAIDs enteropathy in response to increased intestinal permeability and the consequent inflammatory reaction aggravates the increase of the same permeability induced by NSAIDs. Therefore, it could be reasonable to think therapeutic strategies that aim to restore the intestinal microbiota firstly.

• Dietary interventions: the dietary approach designed to reduce the intake of fat, fructose and carbohydrates is essential. In fact, these elements may affect the modulation and the composition of the microbiota, thus impacting on the onset of obesity, metabolic syndrome and NAFLD.

• Antibiotics: antimicrobials, such as tetracycline, kanamycin, metronidazole, or neomycin plus bacitracin, attenuate NSAID enteropathy, thus giving further support to the pathogenic role of enteric bacteria (4). Recent studies of antibiotic treatments with polymyxin B, tetracycline or metronidazole showed beneficial effects on the liver injury related to SIBO, including hepatic steatosis caused by total par-
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In particular, it was seen that the metronidazole would abolish the increase in permeability induced by indomethacin in some volunteers. It also would reduce intestinal inflammation and bleeding in patients receiving long-term NSAID, without reducing the permeability changes.

**Probiotics:** probiotics are defined as “live microorganisms which, when consumed in adequate amounts, confer a health benefit to the host” (FAO/WHO 2001). Nowadays, they have been considered a promising treatment modality of NAFLD as they modulate the intestinal flora, change the intestinal barrier function, prevent bacterial translocation and epithelial invasion, inhibit bacterial adhesion of the mucus, the production of antimicrobial peptides and immunomodulatory, have anti-inflammatory and metabolic effects (215). The probiotics are able to inhibit growth and invasion of pathogenic bacteria through antibacterial products generated by lactobacilli, that lower the pH and consequently reduce the gram-bacteria, lead to changes in the membrane permeability by stimulating the production of proteins of the tight junctions, reduce the endotoxemia and levels of blood lipopolysaccharides and inhibit the synthesis of cholesterol enzymes, by increasing its elimination in feces via recycling of bile acids and through their reduced absorption (201). Prebiotics are indigestible carbohydrates that stimulate the activity and growth of bacteria. The benefits are observed especially after the intake of lactobacilli and bifidobacteria. Indeed, prebiotics can stabilize the intestinal barrier and prevent some of the consequences of its damage observed by some studies. In these investigations, the galactooligosaccharides (GOS) and fructo-oligosaccharides (FOS), which modulate the intestinal microbiota and the intestinal barrier, were considered and it was found that they can attenuate hepatic steatosis. In particular, they reduce the degree of steatosis in animal models through a reduction in levels of IL-6 and TNFa, the de novo synthesis of fatty acids and body weight, increasing glycemic control. Human studies showed that a diet with oligofructose for 8 weeks reduced liver enzymes and insulin (Daubioul et al.). However, the limits in their use are due to the fact that few trials with limited number of participants, with differences in the strains of probiotics, duration of the trial, added prebiotics and by the need to follow up in the long-term, were carried out. Among probiotics, there are the E.Coli Nissle 1917 (ECN) and some metabolites secreted by the *bifidobacterium infantis*, contained in VLS # 3. According to some studies, these probiotics determine an increased expression of some occludin and zonulin with the consequence strengthening of the TJ (216, 217). On the other hand, the *lactobacillus plantarum* MB452 contained in VLS # 3 induces the transcription of genes for occludin and cingulina (218) and prevents the destruction of TJ induced by cytokines, such as TNF alpha and IFN gamma (219).

**Conclusions**

NAFLD is a rising disease in the Western world due to the increased predisposing diet and lifestyle, and its incidence grows along with that of obesity and metabolic syndrome. Moreover, the simultaneous use of NSAIDs such as analgesics, anti-inflammatory and antipyretic, and the growing prospect of new therapeutic uses also as anti-cancer drugs or in the treatment of Alzheimer’s, make them widespread drugs. The magnitude of serious outcomes from the lower GI tract is not well defined, but recent data suggest that they may be as frequent and severe as upper GI complications. Contrary to what happens in the upper GI tract, treatment and prevention of NSAID enteropathy is difficult, since the pathogenic mechanisms are different and not well understood. Among other options, MPAs, antibiotics, and 5-ASA have been proved to be effective in animal models, but they have not been properly tested in humans. Selective COX-2 inhibition and NO or H2S-releasing NSAIDs are emerging as a potential alternative to NSAIDs in the prevention of damage in the lower GI tract. Therefore attention should be paid to the administration of NSAIDs or aspirin in patients with particular susceptibility to oxidative stress such as those with metabolic syndrome (obesity, diabetes and insulin resistance) and NAFLD and to the co-administration of antisecretory agents which may exacerbate NSAID-induced intestinal damage. Nevertheless, it is necessary to consider the incessant increase of the NSAID enteropathy, and, therefore, research of new therapeutic strategies is needed to prevent or reduce the incidence of this complication and the consequent systemic diseases, like NAFLD.
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