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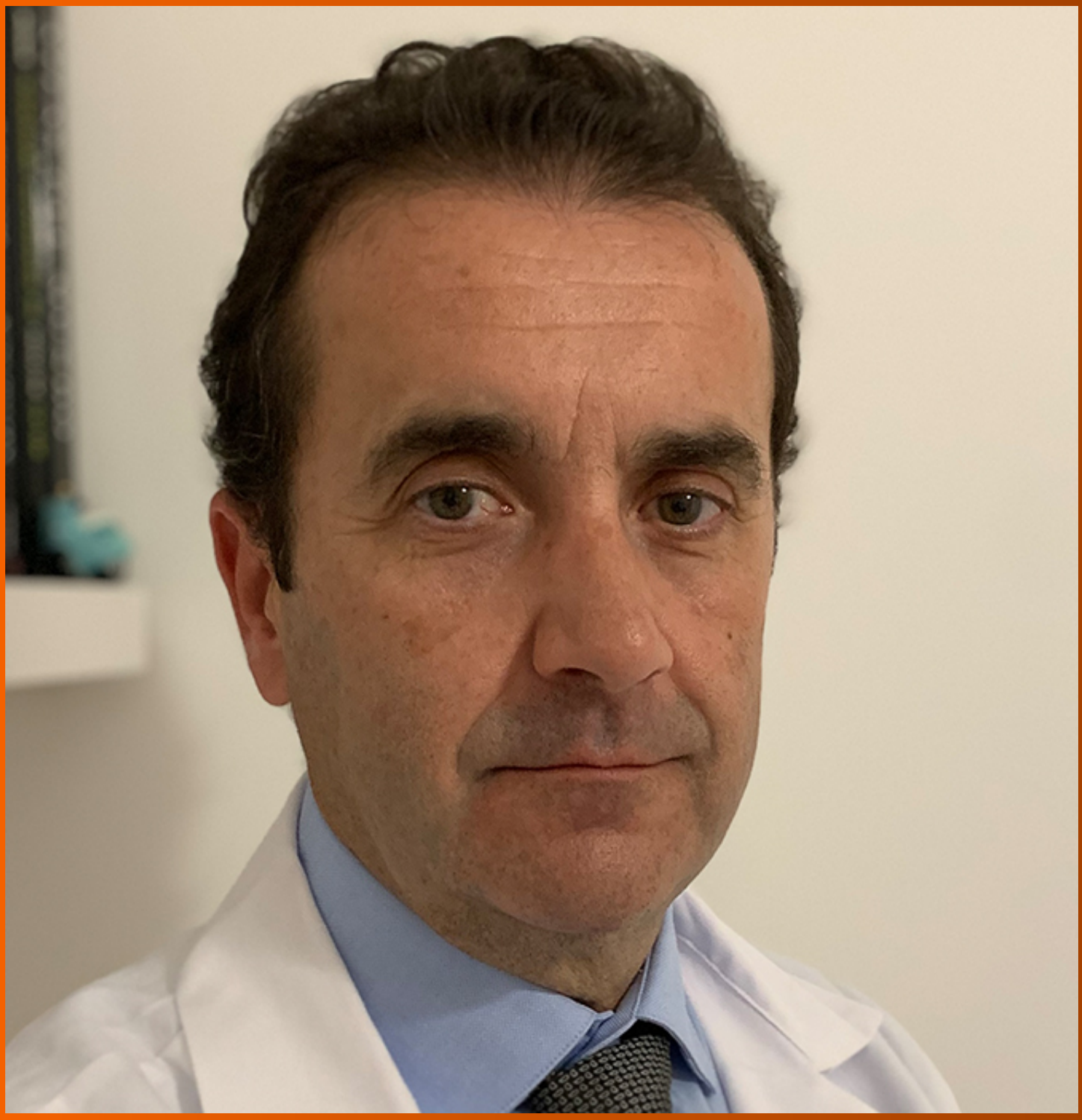

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REVIEW

Microbiota revolution: How gut microbes regulate our lives

Marica Colella, Ioannis Alexandros Charitos, Andrea Ballini, Concetta Cafiero, Skender Topi, Raffaele Palmirotta, Luigi Santacroce

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Abstract

The human intestine is a natural environment ecosystem of a complex of diversified and dynamic microorganisms, determined through a process of competition and natural selection during life. Those intestinal microorganisms called microbiota and are involved in a variety of mechanisms of the organism, they interact with the host and therefore are in contact with the organs of the various systems. However, they play a crucial role in maintaining host homeostasis, also influencing its behaviour. Thus, microorganisms perform a series of biological functions important for human well-being. The host provides the microorganisms with the environment and nutrients, simultaneously drawing many benefits such as their contribution to metabolic, trophic, immunological, and other functions. For these reasons it has been reported that its quantitative and qualitative composition can play a protective or harmful role on the host health. Therefore, a dysbiosis can lead to an association of unfavourable factors which lead to a dysregulation of the physiological processes of homeostasis. Thus, it has previously noted that the gut microbiota can participate in the pathogenesis of autoimmune diseases, chronic intestinal inflammation, diabetes mellitus, obesity

and atherosclerosis, neurological disorders (*e.g.*, neurological diseases, autism, *etc.*) colorectal cancer, and more.

Key Words: Microbiology; Human microbiota; Intestinal microbiota; Immune system; Metabolites; Dysbiosis; Probiotics; Diseases; Cancer

Core Tip: The microbial populations that colonize the human body constitute a complex ecosystem; several cells much higher than the total number of cells in the human body. It is generally accepted that the human gut microbiota is a focus of research interest due to its complexity, involvement with health, and involvement in various pathological conditions. The need to extensively elucidate and document hitherto unknown aspects of the gastrointestinal microbiota, its associations with health fuels the need for further study. Furthermore, on the subject in question it has constituted a trigger for carrying out the present review of the research sources received so far.

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INTRODUCTION

The human organism is colonized by trillions of microbes. The microbiome refers to all the genes of microbes found in various locations in an individual's body. Instead for microbiota we mean the total of microorganisms quantitatively and qualitatively present. The human host and the microbiota have co-evolved for the benefit of both parties especially the intestinal one[1,2]. In fact, on the one hand, the host provides space, suitable conditions, and food for the growth of the intestinal microbiota and this in turn generally participates in obtaining useful substances and induces resistance to various infections. The relationship between the human gut microbiota and the host is symbiotic, in which both the host and the microorganisms are mutually beneficial. For its part, the host offers a place of growth and nourishment to the symbiotic intestinal bacteria, which in turn favors the function of the host on the one hand by inducing resistance to infections and on the other hand by facilitating the absorption of digested food[3]. It appears, therefore, that eukaryotic hosts and symbiotic bacteria have "co-evolved" with mutual interactions based on nutritional benefits enjoyed by both parties. When this balance is disturbed (dysbiosis) for various reasons, such as repeated and inappropriate use of antibiotics or alcohol abuse, pathological conditions may arise, such as mild chronic intestinal inflammation or metabolic disorders[4]. The interactions of (symbiotic) microbes with each other is of particular interest. A negative correlation has been observed between members of the phylum *Bacteroidota*, (such as that of the *Prevotellaceae* spp.) in the intestine, which may reflect alternative "metabolic specializations"[2,5]. Thus, the gut microbiota is of particular importance for the maintenance of human health. In fact, various biological mechanisms are microbiota-dependent because they cannot be performed autonomously and are useful for health homeostasis. In general, the concept of a "superorganism" refers to the bidirectional and therefore beneficial activity between the host organism and the gut microbiota. The microbiota, in addition, has both a protective and trophic role influencing, as we have mentioned, several homeostatic processes of the host organism, *e.g.*, tissue trophism, immune balance, metabolic activity, neuro-endocrine function, *etc.*[6,7]. Intestinal microbiota represents the most populous community of the entire organism. In fact, the stomach has about 10^3-10^4 bacteria, the duodenum 10^5-10^6 and the terminal ileum 10^8-10^9 bacteria (per gram of tissue). However, the most populated is the large intestinal one representing around 10^{12-14} bacteria per gram of tissue[2,6]. The colon is the part of the gastro-intestinal tract where a complex set of microorganisms develops from the moment of birth. The microbiota of the large intestine is denser and more diverse than the human microbiota of the small intestine. Intestine are enriched in the phyla of *Bacillota* and *Actinomycetota*, while *Bacteroidota* and *Lachnospiracae* are more abundant in colonic samples[8]. It is estimated that about 400-500 different genera of microorganisms constitute the intestinal microbiota, while the 90% of them species are predominantly anaerobic. Most of them belong to two genera, *Bacteroidota* and *Bacillota*. The remaining bacterial populations belong to *Pseudomonadota*, *Actinomycetota*, *Fusobacteria* and *Verrucomicrobia*[9]. An estimated 70% of these microbial communities are bacteria that cannot be cultured with conventional microbiological techniques. It has been observed that the microbial populations of the intestinal mucosa differ from those found in the intestinal lumen. The large intestine hosts the most numerous microbial cells which vary in species from individual to individual and are organized in localized microbial communities along its path. These microorganisms appear not to follow stable topological patterns along the intestine as they are influenced by the local oxygen concentration. Thus, facultative aerobic communities reside near the intestinal walls, which are sites of high oxygen concentration, and anaerobes prefer the intestinal lumen, where oxygen concentration is lower[10]. Although there is great diversity in the microbes that colonize the gut, it has been found that the gut microbiota of most individuals can be classified into three main microbial groups or "enterotypes" depending on the predominance of genera: *Bacteroides* (enterotype 1); *Prevotella* (enterotype 2) or *Ruminococcus* (enterotype 3). More recent data, however, show that the division into three enterotypes is a simplification

and that there are many intermediate states in the gut. The prevalence of each enterotype is mainly determined by dietary factors[2]. However, it appears that everyone has a fixed bacterial strain, even though the composition may vary. One of the main functions of the intestinal microbiota is to protect the intestine from pathogenic microorganisms, it also contributes to the development of a healthy immune system, regulates intestinal motility and participates in metabolism. More specifically, the gut microbiota promotes the regulation of the immune system through: (1) Stimulation of the immune response against potential pathogens; and (2) Suppression of the immune response against food and symbiotic antigens[11]. It is estimated that 80% of antibody production occurs locally in the gut, mainly supplying immunoglobulin A. In addition, they participate in host metabolism, drugs metabolism, maintenance of mucosal structural integrity, bile salts metabolism, plant fibers, mucus, and fatty acid catabolism[12]. In addition to bacteria that have already been studied quite a lot, recently it has been hypothesized that yeasts are involved in starch metabolism. Understanding the relationship between yeasts and the immune system is characterized as difficult and there are few data on their action. In some special cases, they can also act therapeutically[13]. Thus, there is an amphidromic relationship between microbes and hosts (Figure 1).

Finally, the probiotics bacteria are the "good" bacteria that are found in gut microbiota but also are added in specific dietary or ferment foods. Probiotics aid the organism by strengthening our immunity and help with gastrointestinal health, especially in conditions such as the irritable bowel syndrome (IBS)[14]. In the 1989, Fuller listed some beneficial effects and therapeutic applications of probiotic bacteria. We find probiotics in dairy products, such as yogurt and aged cheeses, but also in other foods such as pickled vegetables, *etc.* The consumption of probiotics promotes the growth of desirable microorganisms, overcoming potentially harmful bacteria and strengthening the body's defences. Several scientific articles commenting on these results, refer to studies using cultures such as of *Lactobacillus acidophilus* and *Bifidobacterium*, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*[14,15]. Consuming probiotic products can cause a plethora of positives effects on the human body. Probiotics, thanks to their antimicrobial properties action can fight intestinal and other diseases, aid in various infections (such as those from coronavirus disease 2019 and other), reduce levels serum cholesterol, stimulate the immune system, reduce allergy symptoms, aid with lactose intolerance, can prevent hypercholesterolemia and osteoporosis and other[15].

MAIN FACTORS AFFECTING THE INTESTINAL MICROBIOTA

Inheritance

The mechanisms of host-bacteria interactions have not been fully described. Little is known about the relationship between the host genotype and its gut bacteria. However, there is strong evidence that it affects intestinal populations. Simple genetic mutations can lead to changes in the composition of microorganisms in the gut[16]. Further investigations are needed to delineate the mechanisms by which this occurs. There are few studies comparing the gut microbiota between family members. It has been observed that monozygotic and dizygotic twins had a greater similarity in gut microbiota between monozygotic than dizygotic siblings, indicating the importance of genetic background. Furthermore, it was observed in an animal model that the similarities in the gut microbiota of the same mouse strain were greater than in mice of a different species even with a common environmental effect. The role of parental genetic influence on the colonization of microorganisms in the intestine has not been elucidated.

Geographical location

The diversity of the gut microbiota in children living in rural areas is greater than that of children in developed countries. It was noted in a study among Caucasians and Asians (Chinese) in the United States and Hong Kong that there were qualitative differences and quantitative in the intestinal microbiota[17-19]. It was found that children from a country in West Africa (Burkina Faso) that had a high presence of *Bacteroidota* with a greater presence of the genus *Xylanibacter* and *Prevotella* (which allow the hydrolysis of xylan and cellulose) with a reduced presence of *Bacillota*. Indeed, the higher presence of short-chain fatty acids (SCFAs) was noted. Therefore, the intestinal microbiota can help the host to optimize its energy intake from dietary fibers according to nutrition and needs, thus also protecting against infective and inflammatory processes[20].

Intrauterine period and delivery

There is a growing body of data suggesting that the human gut microbiota begins before infant birth. Meconium (the baby's first stool) contains bacteria, where *Bacillota* phyla predominate, the bacteria through the placenta and circulatory system enter the intestinal lumen of the foetal intestine. After human birth, the gut is colonized by many microbial strains, and everyone has their own gut microbiota which changes further throughout their life[2,6]. How the baby is born is very important as it affects the variety of microorganisms which will be installed. During normal delivery the new-born receives microorganisms from the mother's vagina or intestinal tract, whereas in caesarean section the new-born is exposed to those from the hospital environment. The gut microbiota in these newborns can be disturbed for months to years. Newborns born with normal delivery develop a microbiota that reflects the vaginal microbiota, while those born with caesarean section develop a skin microbiota resulting in delayed microbial colonization by *Bacteroides, and Bifidobacterium* spp.[16,21].

Diet

Many are observed in the first months of a person's life changes in the gut microbiota, while the stability and diversity of

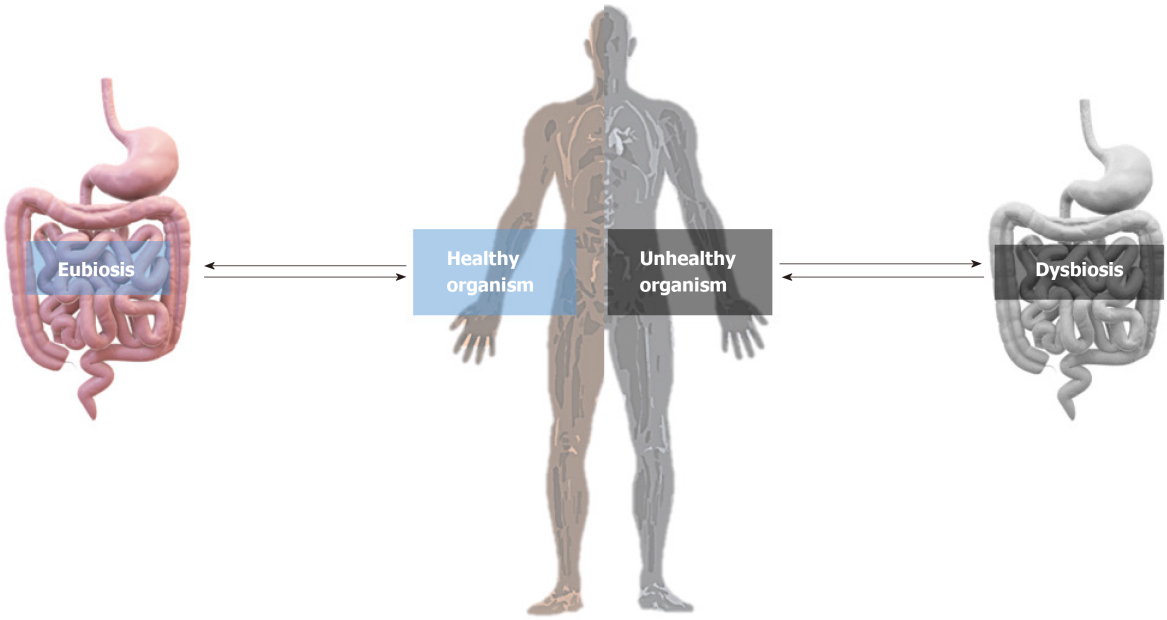

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Figure 1 The cross-talking axis host/gut microbiota: The gastrointestinal microbiota plays an important role in host physiology, metabolism, and nutrition. An organism with a regular maintenance of the physiological homeostasis leads to eubiosis of the gut microbiota and vice versa. Conversely, an altered physiological homeostasis leads to gut microbiota dysbiosis and vice versa. An alteration in the gut microbial community is linked to several disturbances gut conditions, including cancer, obesity, and a variety of gut disorders. The contribution of beneficial components of the gut microbiota to host physiology, metabolism, and immune function has become the focus of scientific research and will undoubtedly lead to new therapeutic approaches.

microbiota genes increase after the first three years of life[21]. Breastfeeding or consuming breastmilk substitutes in infancy has significant effects on intestinal microbiota of the new-born and in the development of the immune system [22]. In animal models, the two different ways of feeding babies develop different populations of microorganisms in the gut which lead to differences in the immune system. Immunity is affected up to the first five years of life[23]. In humans there are few studies on the different administration of milk to newborns. Undoubtedly, however, the intestinal microbiota of the child influences the development of the immune system and its metabolism, just as in adult life[24]. Breastfed infants are exposed to the gut microbiota offered by milk, which contains more than 700 species of bacteria. Breast milk contains many protective factors that breast milk does not contain. In addition, changes in the infant's gut microbiota appear to predispose to diseases and his later life[2,16]. Subsequently adult dietary habits are one of these most important environmental factors shaping the growth of microorganisms in the human gut. Short or long-term consumption of animal or vegetable products changes the structure of the intestinal microbiota. The short-term diet based on animal foods increases the microorganisms of genera such as *Bacteroides*, *Alistipes*, *Bilophila* and decreases the levels of *Roseburia*, *Eubacterium rectale*, *Ruminococusbromii* belonging to the *Bacillota* phyla (populations that mainly metabolize polysaccharides of plant origin)[25]. Instead, it has been noted that long-term consumption of fruit and vegetables by the elderly is associated with an increase in populations of the genus *Prevotella*. It was noticed that a change of nutrition style (*i.e.*, sugar-rich foods, a shift from a low-fat vegetable polysaccharide to a high-fat vegetable polysaccharide, and so) modifies the microbiota, qualitatively and quantitatively, through a change of the microhabitat in 24 h and so on changes in metabolic pathways also occur. Although the gut microbiota responds to short-term changes in diet, long-term nutrient changes appear to determine the type of qualitative and quantitative microorganism population also for other such as the oral ones[26,27]. There are differences in the presence of gut bacteria in the two diets, reflecting differences in metabolism. of carbohydrates and proteins. In both cases, the colonization of fungi, viruses and yeasts is observed[25]. It has been observed that the consumption of foods of plant origin is also associated with a beneficial set in the intestinal microbiota. A high correlation has been noted between a plant-based diet and increased levels of SCFAs and some dietary fibres that degrade *Bacillota* phyla. Indeed, the characteristics of the Mediterranean-type diet, *i.e.*, increased intake of cereals, fruit, vegetables, and legumes offer many benefits for the human intestine[28]. An observational study found that dietary fiber from beans, fruits, and vegetables was associated with an abundance of and *Actinomycetota* phyla and *Clostridium* spp. But also, the consumption of sour milk, many dairy products, or the consumption of fermented foods (such as Greek yogurt, kefir, and others) affect the intestinal microbiota and change its structure. The use of substances of abuse (such as alcohol, cocaine and other) or chemical xenobiotics can cause in human health is associated with quantitative and qualitative changes in the intestinal microbiota (dysbiosis)[29,30].

Lifestyle

Modern lifestyles can influence the intestinal microbiota, sedentary life contributes to obesity and combined with a positive energy balance and the consumption of foods of animal origin rich in saturated fat leads to the rearrangement of populations at the intestinal level. Although studies show conflicting results, an increase in *Bacillota* and a decrease

in *Bacteroidota* have been observed in individuals consuming foods of animal origin[6]. Frequent and moderate exercise influences the intestinal defence as well as on the genes of intestinal microorganisms. In a study on the effect of exercise on the intestinal microbiota of 22 athletes, the beneficial influence of exercise on the diversity of intestinal microorganisms is underlined and the combination with diet is claimed to affect the gut microbiota structure[31,32]. Smoking also affects the composition of the gut microbiota, increasing populations of the *Bacteroides-Prevotella* genera. Stress affects gut motility, which can change the structure of microbial populations, which has even been blamed for the development of inflammatory bowel disease (IBD)[33].

Use of antibiotics

Antibiotics target pathogenic microorganisms, but they also have a negative effect on intestinal symbionts. Unfortunately, they affect the intestinal microbiota especially if they are broad-spectrum antibiotics that are used for all diseases[34]. Gut microbiota diversity is reduced, many strains are lost, and their re-emergence is gradual and long-term, which is a major concern of experts in the field of intestinal health. It was observed that most of the bacteria affecting antibiotics response mainly belong to the *Bacillota* and *Pseudomonadota* phyla[35,36].

THE ROLE OF THE HOST/INTESTINAL MICROBIOTA CROSS-TALKING AXIS

Nutritional and gut-influenced energy conservation by metabolic processes (metabolome)

Several studies have shown that the intestinal mucosa needs to be colonized by microorganisms to take on its integral structure. For example, mice raised in a sterile environment developed fewer blood vessels in their intestinal villi. Sterile growth also showed that there is defective growth in gut-associated lymphoid tissue and antibody production. Also, in the context of the sterile environment, fewer Peyer's patches develop, there are fewer cells in the dermis, and fewer plasma cells in the germinal centres of the mesenteric lymph nodes than in the development data in a nonsterile environment[37]. The intestinal microbiota could be considered a "metabolic organ" performing multiple functions needed for maintaining the health status of the host organism orchestrating an amphidromous communication with it. The catabolism of a number of both endogenous and exogenous indigestible molecules (*i.e.*, cholesterol, fibers, bile acids, excreted mucus, *etc.*), is one of the most important activities of the intestinal microbiota accounting for 10% of the host's energy requirement every day[38,39]. So, the gut microorganisms (especially bacteria and commensal fungi) produce SCFAs to gain energy but also give trophic substances and energy to the host organism. Finally, certain bacterial species can synthesize vitamins such as B12, folic acid, thiamin, biotin, vitamin K, amino acids and more[40]. As proof, *Bacteroides thetaiotaomicron* can metabolize polysaccharides that reach the large intestine as is in Figure 2. Indeed, it has many enzymes such as glycoside hydrolases and polysaccharide lyases that break down pectins, arabinose, *etc.*

Archaea, such as *Methanobrevibacter smithii*, establish beneficial relationships with other bacteria to eliminate the H_2 by-products, thus facilitating the yield of ATP[41,42]. The gut microbiota benefits the host in many ways contributing to the conservation of energy from several common non-digestible polysaccharides through enzymes such as glycoside hydrolases and other non-encoded enzymes in the human genome[43]. Studies of mice with a germ-free gut microbiota revealed that the gut microbiota improves the regulation of fat accumulation and obesity mainly due to an increase in energy production from food. These (germ-free) mice are protected against obesity and metabolic syndrome[44]. It was noted that restoring an eubiotic intestinal microbiota in these animals leads to an increase in insulin resistance and fasting blood glucose, but also of the level of liver triglycerides and body fat amount.

The intestinal microbiota improves the absorption of monosaccharides, which in turn leads to an increased lipogenesis with consequent accumulation of triglycerides both in the liver and in the fat tissue. With food utilization, obese mice have been reported to conserve energy from food more efficiently than lean-wild-type mice[45]. Distal gut microbiota's composition of obese mice revealed that the relative increase of *Bacteroidota* and *Bacillota* resulted in a modified metabolic potential of the gut microbiota, giving it a greater ability to use energy from the diet. Interestingly, this obesity trait of his was transmissible through faecal transplants from obese mice as opposed to lean germ-free mice. Obese mice also possess multiple methanogenic archaea which can increase the efficiency of bacterial fermentation through H_2 removal[46]. It was observed that, after gut co-colonization, both *Bacteroides thetaiotaomicron* and *Methanobrevibacter smithii* boost up the efficiency and specificity of bacterial fermentation by removing bacterial polysaccharides, increasing adiposity compared to mice colonized with only one of the two organisms[47,48]. Further studies have highlighted further correlations regarding the possibility of increased energy saving through induced microbiota nutrition or genetically induced obesity microbiota[49]. For that, age and diet are important factors not only for the composition of the gut microbiota, but also for energy saving possibilities. In addition, the link between gut microbiota and liver diseases has been studied mainly in obesity and non-alcoholic steatohepatitis/nonalcoholic fatty liver disease patients, and in liver failure conditions (*e.g.*, cirrhosis, hepatic encephalopathy, infections, hepatocellular carcinoma), total parenteral nutrition-associated liver disease, cholangitis primary sclerosing, primary biliary cirrhosis[50]. It has been noted that dysbiosis of the intestinal microbiota leads to intestinal motility disorders (stasis, with bacterial proliferation). These conditions lead to increased intestinal permeability, impaired immune response with an excessive increase in tolerance in bacterial translocation by the intestinal microbiota with subsequent liver injury[50,51].

Immunological actions and resistance to colonization by pathogens

The host's organism meets both the pathogenic microbes of the environment and the microbes of the intestinal microbiota. Previous studies of the immune system have focused on the mechanisms by which this system can defend itself against pathogenic microbes. In addition, the microorganisms of the intestinal microbiota produce antimicrobial

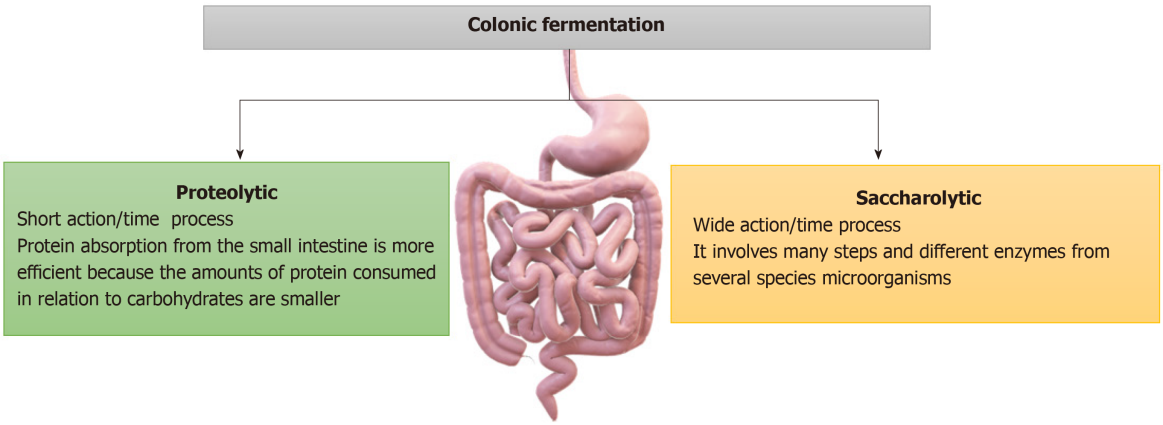

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Figure 2 The metabolic activity by the colonic microbiota. The intestinal microbiota finds an environment rich in polysaccharides which are not digested by stomach enzymes. Fermentation of polysaccharides by intestinal bacteria leads to the production of acetate, butyrate, and propionate, which are used as a carbon source by intestinal mucosal cells. The initial fermentation of the carbohydrate that escaped digestion in the small intestine is followed by the utilization and cross-distribution of metabolites by various members of the microbiota, and then the synthesis of short-chain fatty acids (butyrate, propionate, acetate). Proteolytic fermentation differs from saccharolytic fermentation because it releases many potentially toxic nitrogen and sulfur metabolites, such as ammonia, amines, nitrates, nitrites, and hydrogen sulfide[32,40].

substances such as bacteriocins and hydrogen peroxide which inhibit the growth of others with pathogenic behaviour [51]. The immune system has evolved in such a way that it can accommodate symbiotic bacterial communities of increasing complexity while retaining the ability to fight pathogenic bacteria. The microbiota regulates the development and function of the innate and acquired immune systems[11]. Even in healthy conditions of the host, there is a continuous stimulation of the immune system by the intestinal microbiota. This condition leads to a basal state of "low physiological inflammation" representing an effective first line of defence against pathogenic microbes. Furthermore, both resident and pathogenic microbes compete for available sites and nutrients, so the microbiota exerts a protective role metabolizing those nutrients that are necessary for the pathogens survival and producing molecules that inhibit their growth[2,52]. In fact, it has been shown that the introduction of certain molecules produced by *Bacteroides thetaiotamicron* and *Eubacterium rectale* can induce the production of specific mucosal glycans. These can be metabolized by these 2 bacterial species only but not by pathogens, thus preventing their proliferation. Hence, diet appears to have a pivotal role in microbial composition modifications[53]. The immune system works by learning, *i.e.*, at the beginning of life it has the necessary components (cells, internal cellular mediators, *etc.*) but does not have data available from the environment, which it acquires in the first years of life through contact with other people and the natural environment. If these early childhood data are inappropriate, the regulatory mechanisms of the immune system could fail. As a result, the immune system attacks not only pathogenic microorganisms but also harmless targets such as pollen, house dust and food antigens, leading to the onset of allergic diseases[54]. The microorganisms together with digestive enzymes, the mucus layer, intestinal peristalsis, and the epithelial barrier with "tight" connections (tight junctions) constitutes the non-immune component of the body's immune response. The functions of the intestinal microbiota in terms of defence of the organism are on the one hand to influence in a decisive way the arise of the gut immunity (for which reference was made to the trophic role) and on the other to prevent possible invasion of pathogens through a direct effect on them and/or through the "activation" of the host's immune mechanism[55,56]. As far as natural immunity is concerned, it has the ability, by recognizing characteristic pattern molecules [pathogen associated molecular patterns (PAMPs)] on microorganisms, to separate potentially pathogenic microbes from "unwanted" antigens. More specifically, the cells of the natural immunity using proline rich proteins (PRPs) receptors (pattern recognition receptors) detect PAMPs[57]. PRPs also participate in the release of cytokines and the activation of acquired immunity. However, there are many types of PRP, and of the most important are the Toll-like receptors (TLRs) found in dendritic cells, neutrophils, macrophages, and intestinal mucosal epithelial cells. The most known PAMPs recognized by PRP receptors are bacterial carbohydrates (*e.g.*, mannose, glucides of lipopolysaccharide), bacterial peptides (*e.g.*, flagellin), peptidoglycans and lipoteichoic acid of Gram+, fungal lipoproteins, glycans, viral genomes. Since these molecules are also found in symbiotic microorganisms, they are called microbe-associated molecular patterns (MAMPs)[58]. Thus, MAMPs seem to be able to modify the expression of TLRs in natural immunity cells. Thus, the recognition of MAMPs triggers the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, which results in the production of cytokines, the activation of other auxiliary and necessary molecules on the antigen-presenting cells, which ultimately results in the activation of T-cells, *i.e.*, acquired immunity[59]. However, microorganisms can modulate the natural immunity changing the quality and the amount of mucus from the intestinal mucoid cells. Mucus forms a natural barrier to stop an infection directly adhering to them, collects the bacterial by-products, and protect epithelial cells from secretions thanks to its low pH rich and richness in lytic enzymes. These events activate body's defences[60]. As far as the acquired immunity in the gastrointestinal system is concerned, it is "based" in the gut associated lymphoid tissue which is made up of the Peyer's Patches and the mesenteric lymph nodes. The spleens of germ-free mice were found to contain increasingly smaller germinal centres in the lymph nodes and reduced numbers of memory CD T4 cells in the intestinal epithelium, that the production of

cytokines belongs to a Th2-type of immune response, and that these animals have a reduced ability to secrete germicidal agents[61]. Recolonization of these muscles with muscle-specific bacteria can reverse some of these perturbations, as experimentally demonstrated by the restoration of systemic T-cell deficiency and Th1/Th2 imbalance of sterile muscle microbes after single colonization of the muscle gut with the bacterium *Bacteroides fragilis*. Such studies collectively highlight the importance of the gut microbiota for the normal development of the peripheral immune system in immunocompetent hosts[62].

Influence of the host's health condition

According to clinical studies that have been done in recent years, they show a correlation of the change in the composition of the gut microbiota with several serious pathological conditions such as: autoimmune diseases, type 2 diabetes (T2D), weight gain and obesity, IBD, asthma and chronic sinusitis, mental health disorders, dermatological problems, Alzheimer's disease, poor immune system health, gastroesophageal reflux disease, constipation or diarrhea, cancerous conditions and other[63].

INTESTINAL MICROBIOTA ROLE IN DISEASES

IBS and IBD

Functional bowel disorders such as IBS are defined solely by symptom-based diagnostic criteria. IBS is characterized by abdominal pain or discomfort and changes in bowel habits. Although the aetiology is multifactorial, recent studies to understand the pathophysiology of IBS have revealed that changes in the normal gut microbiota may play a role in IBS-associated low-grade intestinal inflammation[2,40]. Indeed, intestinal microbial dysbiosis is involved in the pathogenesis of IBS by facilitating the adhesion of the pathogenic microorganism to the intestinal wall and several studies revealed that. Indeed, there is a clear separation between the gastrointestinal microbiota of patients with IBS and that of healthy controls[64]. That is, IBS is characterized by an increase in the genera *Dorea*, *Clostridium* (as *Bacillota* phylum) and *Ruminococcus* with an important loss in the population of the genera *Bifidobacterium* and *Faecalibacterium*. According to a trial on IBS in young patients who were divided into subgroups with different bacterial fingerprints, an increase of *Bacillota* phylum compared to *Bacteroidota* phylum exists, differing from healthy patients[65,66]. Finally, another study on a paediatric population with IBS showed a variation of *Bacillota* and *Pseudomonadota* phyla amount, with many of the genera *Dorea* and *Ruminococcus*, and *Haemophilus parainfluenzae* species. The *Bacteroides* genus was found to be smaller in paediatric patients with IBS than in healthy controls. Typically, these studies could ultimately lead to the design of targeted therapies[67].

Idiopathic IBDs are immune-driven chronic diseases and represented by ulcerative colitis and Crohn's disease. Today they are an adverse immune response to endogenous symbiotic gut microorganisms with or without the involvement of the autoimmunity process. Studies have shown a change in the composition of the gut microbiota in IBD[68]. Furthermore, there is a qualitative reduction in it, *i.e.*, on the bio-diversity of the population with a typical reduction of *Bacillota* phylum strains (such as *Bifidobacteria*, spp., *Faecalibacterium prausnitzii*, and *Lactobacillaceae* families) while an increase in the microorganisms that are attached to the mucus is observed. *Bacillota* strains are the main producers of SCFAs, such as butyric acid, which has immunomodulatory properties[69]. However, it has not been clarified whether the disturbance in the microbiota is the cause of the disease or is the result of it. The normal, non-inflamed gut contains many immune cells which are in such a state of activation that there is no complete immune response to the microbes of the normal microbiota and food antigens. This is due to the activation of potent mechanisms of immune regulation, such as the stimulation of regulatory T cells expressing the transcription factor FoxP3 to suppress inflammation[66]. But, under environmental stimuli (*e.g.*, certain infections) the activation of the intestinal immune system starts on a large scale but is subsequently suppressed. In IBD patients this suppression of this immune response may not be adequately regulated. The eubiosis of the intestinal microbiota is under the control of the host through immune and epithelial responses, diet, drugs use (especially antibiotics), genetics, and more[2]. In turn, the microbiota, has significant effects on host epithelial and immune function thanks to its structural components and metabolism, that can become permanent by epigenetic effects. From childbirth, when the human microbiota is established, these host effects may influence the risk of developing IBD later in life[63]. It is worth noting here that in most studies the incidence of IBD increases particularly in the second to fourth decades of life, while some studies even report a second peak in the sixth and seventh decades. Specifically, therefore, components of the microbes can promote or protect against disease. The community microbes in patients with ulcerative colitis and Crohn's disease have been shown to be different from unaffected individuals, a state of dysbiosis: The presence of disease-causing microorganisms (such as those form *Pseudomonadota* phyla and adherent *Escherichia coli*) and to which directed immune response and/or loss of microorganisms that inhibit inflammation (*e.g.*, such as *Faecalibacterium prausnitzii*). But many changes and inflammation results in changes in the microbial community. Also, antibiotics (such as nitroimidazoles, quinolones) and certain diets change the gut microbiota and may improve Crohn's disease symptoms. In fact, the use of antibiotics really has a place in the treatment of Crohn's disease[2,16,70].

Immune dysregulation (allergies and autoimmunity)

Effectively stimulated immunity of both local and systemic mucosa is required for a mature gut microbiota. If this does not happen, a dysregulation can occur which can lead to allergic manifestations or an asthmatic phenotype from early life. It has been noted that developing countries have a lower incidence of allergic diseases such as asthma than countries developed. Thus the "hygiene hypothesis" was developed, which according to this lack of exposure to pathogenic bacteria or products of non-pathogenic bacteria can cause this condition by a negative effect on the development of the

immune system[71]. Subsequently, the "microbiota hypothesis" was formulated, in which it is proposed that changes in diet, as well as increased use of antibiotics in developed societies, lead to a less diverse gut microbiota in its microbial components. This "immature" gut microbiota, as it has been defined, alters the development of the immune system interrupting the proper evolution of events that allow the development of immune tolerance and thus increasing the risk of developing allergic hypersensitivity[72]. More specifically, levels of *Bifidobacterium* and *Enterococci* appeared to be related to allergic symptoms in the first months of life. An increased *Bacteroidota*/*Bifidobacterium* ratio was reported during the second year of life in children who developed symptoms of atopy children who eventually developed allergies were less frequently colonized with *Lactobacillaceae* phyla, *Bifidobacterium* and *Clostridiodies difficile* strains during the 2nd mo of life[73]. Autoimmune diseases (such as rheumatoid arthritis and other) can be sharing a common pathogenesis, an immune-mediated attack on the body's own organs. Autoimmune diseases usually show a variety of characteristics and symptoms. Concerning these characteristics, the most common may include, for example, an increase in epithelial (such as intestinal) and vessel permeability, mitochondrial dysfunction, progressive inflammation and chronic infections, imbalance of the hypothalamic-pituitary-adrenal (HPA) axis, and microbiota dysbiosis. Symptoms, on the other hand, can manifest as chronic fatigue, allergic phenomena, poor cognitive function, mood and mental disorders and pain, skin rashes, gastrointestinal disorders, and so[74,75]. An example of the genetically predisposed autoimmune diseases of the small intestine is celiac disease, an age-independent condition. The symptoms occur after the ingestion of the toxic epitopes of gluten (*i.e.*, the proteins present in wheat, rye, barley and, less so, in oats). In celiac disease there is an increase in intraepithelial lymphocytes and an atrophy of the intestinal villi. The autoimmune mechanism is due to the presence of various autoantigens, where the most important is tissue transglutaminase that is also an important diagnostic marker. Furthermore, it is often associated with other autoimmune diseases, such as insulin dependent diabetes mellitus (IDDM), or type 1 diabetes (T1D), thyroiditis, *etc.* This suggests that these diseases share common pathogenetic pathways[76-79]. Finally, the intestinal microbiota seems to play a fundamental role in the development and course of celiac disease. There is an unfavourable qualitative and quantitative composition of the intestinal microbiota characterized by a greater presence of the genus *Bacteroides* and *Escherichia coli* and a minor presence of *Bifidobacterium* spp. (*e.g.*, *B. longum* compared to healthy controls). Furthermore, this condition does not seem to change even after a gluten-free diet[80,81]. Finally, it has been noted that children born by caesarean section have a higher risk to develop the disease[82,83].

Diabetes mellitus

The exact role of the gut microbiota in the pathogenesis of T1D remains unknown. Data from experimental models support the idea that some bacterial families may act protectively against divalent metal transporter 1 (DMT1). Studies have shown that the use of antibiotics in experimental animals can prevent the occurrence of type 1[84]. But also, the administration of probiotics with bacterial strains in experimental animals prevented the onset or delayed the progression of the disease[85]. In another study, it was found that accidentally infecting mice with a spore forming bacterium resulted in a reduction in the incidence of DMT1[86]. Similarly, incubation of mycobacterium and streptococci in laboratory animals protected them from developing diabetes. Studies of the gut microbiota in mice that developed DMT1 and mice that did not develop diabetes reported that, at the onset of the disease, the two groups differed in the concentrations and type of microbes[87]. Stool samples from animals (mice) that developed diabetes contained higher concentrations of so-called probiotic bacteria, such as from *Lactobacillaceae* phyla and *Bifidobacterium* spp., in contrast to mice that did not develop diabetes which showed higher concentrations of *Bacteroides*, *Eubacterium* and *Ruminococcus*. It is worth mentioning that the *Lactobacillus johnsonii* strain prevents the development of diabetes when administered to mice[88,89]. Because of the possible relationship between the microbiota and IBD, most of the research around the gut microbiota has been done in patients with IBD, whereas studies in patients with type 1 diabetes mellitus (T1DM) are limited. In addition to increased intestinal permeability, patients with T1DM show an increase in inflammatory cells in the gut and decreased numbers of CD4, CD25, and T-cells, which are the master regulator of the immune system. Another study showed that individuals who developed T1DM had higher concentrations of *Bacteroidota* and lower concentrations of *Bacillota* compared to healthy controls. In addition, individuals who developed DMT1 were colonized with a lower number of bacteria compared to healthy controls[90,91].

The manifestation of T2DM is due to the combination of reduced insulin secretion from the β-cells of the pancreas and increased insulin resistance, as well as the disruption of incretin secretion from the gastrointestinal system. Several studies associated the microbiota with the development of T2DM, which is particularly characterized by a decrease in the concentrations of the phylum *Bacillota* (such as the genus *Roseburia* from the *Lachnospiraceae* family and the *Enterococcus faecalis* from genus *Enterococcus*, and other). Changes in the number of *Bifidobacteria*, *Lactobacillaceae* phylum, *Clostridioides* genus, as well as the *Bacillota*/*Bacteroidota* ratio have also been observed in the intestinal microbiota of children with T1D [91-93]. Obesity and T2D share the presence of low-grade inflammation that occurs in tissues involved in the regulation of metabolism, such as the liver, adipose tissue, and muscle. Inflammation is characterized by an increase in cytokines, interleukin (IL)-6, IL-1 and tumor necrosis factor-alpha with the result being insulin resistance. There are several studies in which the gut microbiota has been associated with the presence of obesity, insulin resistance and T2D, such as, and that probiotic treatment affects the metabolic control of patients with T2D. The gut microbiota contributes to the development of T2D[94]. Both studies showed that people with T2D had reduced concentrations of *Clostridiales* bacteria (*Roseburia* spp. and *Enterococcus faecalis*)[95]. T2D, independent of obesity, may also affect the structural composition of the microbiota. T2D may have a high presence of Gram-negative intestinal bacteria, such as *Bacteroidota*. In fact, in diabetic mice a reduction in *Bacteroides/Prevotella* spp. has been linked to an improvement in metabolic endotoxemia and lowering of laboratory tests of inflammation[96]. Changes in the number of *Bifidobacterium*, *Lactobacillaceae* phylum, *Clostridium* as well as the ratio *Bacillota*/*Bacteroidota* have also been observed in the intestinal microbiota of children with T1D[97]. Similar changes in the composition of the intestinal microbiota have been reported in patients with T2D. A lot of different theories have been proposed about the biomechanisms to explain the effect of the intestinal microbiota on insulin

resistance and T2D, the main ones being metabolic inflammation, modification of incretin secretion and the production of hydroxybutyric acid. Lipopolysaccharides are endotoxins that are usually found in the outer membrane of Gram-negative bacteria and cause so-called metabolic inflammation, which is characterized by the release of pro-inflammatory factors [98]. The role of lipopolysaccharides in the pathogenesis of metabolic diseases was demonstrated by a study in mice fed a normal diet, in which the infusion of lipopolysaccharides caused insulin resistance in the liver, glucose intolerance, as well as an increase in adipose tissue. In addition, lipopolysaccharides may can lead to the expression of NF-κB and stimulate the activity of mitogen-activated protein kinase metabolic pathways in adipocytes. A study in fed leptin-deficient mice physiologically showed that increased intestinal permeability to lipopolysaccharide resulted in a change in the proportion of Gram-negative bacteria in the intestinal lumen, which was associated with the presence of insulin resistance. The modification of the intestinal microbiota by administering probiotic treatment to obese mice acted favourably on the intestinal barrier, reducing lipopolysaccharide-induced metabolic inflammation[99]. It has been found that increasing concentrations of *Bifidobacterium* modifies the inflammatory response in obese mice by increasing the production of glucagon like peptides (GLPs), while reducing intestinal permeability. The increase in *Bifidobacterium* concentrations induced by probiotic treatment is probably associated with an increase in the levels of gut-secreted peptides GLP-1 and YY, which exert a favourable effect, reducing insulin resistance and improving β-cell function. In addition, probiotic treatment caused an increase in GLP-2 levels in the colon, improved intestinal barrier function, and ultimately reduced plasma lipopolysaccharide levels[100]. It was found that people with T2D had an increase in the number of various opportunistic gut pathogens and a decrease in the concentrations of hydroxybutyric acid-producing bacteria. Hydroxybutyric acid is the main source of energy for maintaining the function of the cells of the digestive system. In the large intestine, hydroxybutyric acid is mainly produced by the bacteria *Clostridium coccoides* and *Eubacterium rectale*. However, changes in gut bacteria were found in colon cancer patients and in elderly subjects, suggesting that hydroxybutyric acid-producing bacteria could potentially have a protective role in the functioning of the gut microbiota[101,102].

Obesity and atherosclerosis

In obese individuals the accumulation and thus the increase of energy is related to the transfer of hydrogen between certain bacterial taxa. In fact, this effect has been noted both from the hydrogen-producing *Prevotellaceae* and from the methanogenic archaea that use hydrogen[103]. In obese individuals this increase and accumulation of energy due to the relative abundance of *Gammaproteobacteria* and the less presence of *Clostridium* genus. Additionally, obese individuals harbour clusters of H_2-producing bacteria, primarily members of the family *Prevotellaceae* and some groups of *Bacillota*. These H_2-producing bacteria coexist in the gastrointestinal tracts of obese individuals with relatively large numbers of H_2-oxidizing methanogens belonging to archaea. Methanogens account up to 10% of all anaerobes in the colon[104,105]. Plant polysaccharides and dietary fibers are fermented by intestinal bacteria to produce SCFAs. An increase in methanogenic oxidation of H_2 facilitates fermentation, which produces more SCFAs[106]. It can also be utilized directly by hydrotrophic methanogenic agents, while propionate, butyrate and lactate can be fermented with acetate and H_2, where the latter is utilized by hydrotrophic methanogenic bacteria. As a consequence, the increase in methane oxidation should increase the conversion of plant polysaccharides into SCFAs, especially the acetate. SCFAs produced by fermentative bacteria are absorbed through the human intestinal epithelium, whereas H_2 is an energy exchange factor within bacterial communities[107].

Atherosclerotic vascular disease is caused by environmental and genetic factors such as food and associated microorganisms. We currently know three circulating phospholipid-related molecules considered promoters of atherosclerosis: Trimethylamine N-oxide (TMAO), choline and betaine that could be used as biomarkers to predict cardiovascular disease risk[108]. These three phospholipid-related molecules were identified by analysis of plasma metabolites from 50 patients with atherosclerotic disease using liquid chromatography/mass spectrometry compared to 50 healthy samples. It has been noted that used apoE-deficient mice as a model of atherosclerosis and showed that plasma TMAO levels in apoE-deficient mice correlated positively with the area of aortic damage. The activity level of hepatic flavin monooxygenases, which convert trimethylamine (TMA) to TMAO, correlated positively with plasma TMAO levels in both mice and humans[109,110]. In the apoE-deficient mice an antibiotic treatment modify plasma TMAO level and atherosclerosis size, suggesting that gut microbiota significantly influence the development of atherosclerosis in apoE-deficient mice reducing it. This was demonstrated by adding 1% choline to the diets of apoE-deficient mice, as it increased the formation of foam cells as well as the expression of the scavenger receptors CD36 and SRA1 on macrophages, which is normally prevented by the administration of broad-spectrum antibiotics. A new pathway linking dietary lipid intake, gut microbiota and atherosclerosis has been found. Dietary L-carnitine is metabolized to TMA by gut microbiota and further converted to TMAO in the liver, which accelerates atherosclerosis in mice[111]. 16S rRNA sequencing of cecum bacteria in mice fed a diet supplemented with L-carnitine showed that the family *Prevotellaceae* were increased and positively correlated with plasma TMA level[112]. Furthermore, circulating TMAO in individuals with non-selective diet seems to be much higher than in those with a vegetables-based diet. Faecal microbiota analysis and plasma TMAO levels have been correlated in individuals with *Prevotella* enterotype in respect of those with *Bacteroides* enterotype, showing that these are higher in the first. L-carnitine use in individuals with different diet habits showed that a varied diet led to the production of more TMAO than in vegans or vegetarians. This confirm that both diet and lifestyle can change both the gut microbiota composition and its ability to metabolize TMA and TMAO from dietary L-carnitine[113]. Based on metagenomics, the genus *Collinsella* in faecal microbiota seems to be increased in patients with symptomatic atherosclerosis, while the genera *Eubacterium* and *Roseburia* is abundant in healthy subjects. Furthermore, in patients with atherosclerosis, metabolomics of faecal microbiota suggests an increase of the expression of genes coding for peptidoglycan synthesis, and a fall of hydrogenase levels. In addition, serum carotenoids, especially β-carotene, were decreased suggesting that symptomatic patients with atherosclerosis could have changes in the gut microbiota and a basic inflammatory state[113,114].

Neurological and psychiatric disturbance

As for the brain, everyone knows that it sends messages throughout the body. The intestine seems to respond. One of the most interesting effects of probiotics on the body focuses on the presence of the gut-brain axis (GBA). The GBA is the connection and the two-way communication between the gut [enteric nervous system (ENS)] and the central nervous system (CNS). This axis is a complex two-way pathway essential for metabolism homeostasis, the influence it has on emotions, mood and in general higher cognitive functions. It is a complex system in which it participates the CNS, the autonomic nervous system, the brain, the spinal cord, and the HPA axis[2,115]. The GBA axis indicates the bidirectional relationship and interdependence between CNS and ENS. It is important to understand this interaction and how a healthy intestinal microbiota can affect the body's nervous system and vice versa. Through a multitude of mechanisms, hundreds of substances and neurotransmitters, the relationship between the two complex systems is in constant change which can have both positive and negative effects. Stress is a quite common factor that can affect mood, psychology and, apparently, the body's intestinal microbiota[116,117]. When the organism exposed to a stressful social situation even for a period of only two hours, the microbiota undergoes significant changes, a significant change in its profile and change in the ratio of the main races of bacteria. Additionally, the brain, under the right conditions, can influence composition and functionality of the intestinal microbiota. It may alter intestinal permeability thereby allowing bacterial antigens to enter the epithelium and stimulate a mucosal immune response. Acute stress can increase colonic permeability, leading to overproduction of interferon-γ[116]. Finally, dysbiosis, *i.e.*, alterations caused in the gut due to stress, facilitate the expression of infectious bacteria. An example is the secretion of norepinephrine during surgery which causes expression of *Pseudomonas aeruginosa*, which can lead to intestinal sepsis. In addition, norepinephrine can stimulate the proliferation of enteric pathogens and increase the infectious properties of *Campylobacter jejuni*[117-119]. Finally, it can favour the overgrowth of non-pathogenic *Escherichia coli* isolates, as well as pathogenic *Escherichia coli* type 0157:H7[3,116]. In continuation of the above, it seems that probiotics have the mechanisms to deal with the complications of stress. Cortisol is a substance produced when the organism is in a state of stress and experiences situations related to anxiety and depression. It was found that probiotics led to a decrease in its release of this substance. In addition, the metabolic products of probiotics, SCFAs, offer beneficial actions. The ENS becomes a recipient of bacterial metabolites. SCFAs such as butyric, acetic, and propionic acids are the main metabolic products of bacterial metabolism[120]. In addition to their essential presence for the multitude of beneficial actions they offer, they can stimulate the sympathetic nervous system, release serotonin in the mucosa and positively affect memory and the learning process[116]. The above-mentioned negative effects of unpleasant psychological states indicate this strong dependence and relationship between CNS and ENS. It is worth focusing on the reverse course, that is, how the health of the body's microbiota can, through probiotic bacteria, give the appropriate signals to the brain to influence possible diseases. Studies show that balance in the microbiota is related to our emotions, as well as how our brains process information from our senses, such as sights, sounds, tastes. Scientists suspect that gut microbiota disorders may play a role in autism spectrum disorders, depression, anxiety, and chronic pain[121]. The GBA is a communication system between the gut and the brain *via* neural, hormonal, and immune circuits, offering the gut microbiota and its metabolites a potential pathway to access the brain. This communication system is bidirectional and allows the brain to regulate gastrointestinal functions such as peristaltic movements and mucus production as well as immune functions[122]. Considerable progress has been made in the past decade in understanding the ways in which the gut microbiota is linked to the brain. Stress conditions affects the gut microbiota composition and that two-way communication between gut microbiota and the CNS influences the host's response to stress. Stress has been shown to affect the integrity of the intestinal epithelium and alter peristalsis, secretions, and mucus production, thereby altering the gut microbiota environment and causing changes in microbial composition and/or metabolism[2,123]. Finally, regarding autism, it has been found that children with autism typically have a higher abundance of *Pseudomonadota*, *Bacteroidota* and a lower abundance of *Bacillota* and *Bifidobacteria* than healthy children. In fact, many classes of bacteria that make up *Bacillota* and *Clostridia* spp., have been found to appear in higher percentages in autistic children with a history of gastrointestinal problems, while at the same time and despite the overall higher abundance of *Bacteroidota*, and lower *Prevotella* were observed. Therefore, in addition to quantifying the relative increase or decrease of the populations of some phyla, it has been deemed necessary to determine the populations of specific intestinal symbiotic organisms to understand the significance of some physiological changes in the gut and/or brain[124].

Colorectal cancer

Colonic metabolism may be protective against carcinogenesis under eubiotic conditions[125] (Figure 3). Several environmental and individual factors associated with the development of colorectal cancer, and the interactions between them. It is estimated that 20% of human cancers are related to conditions of chronic inflammation and/or persistent infections. As for example *Helicobacter pylori* infection is often associated with ulcer and gastric cancer, hepatitis B virus and hepatitis C virus can facilitate hepatocellular carcinoma, IBDs can develop colorectal cancer[126]. The composition of the gut microbiota according to studies may contribute to the mechanism of carcinogenesis either through diet or through its anti-inflammatory effect on the gut mucosa. A number of research has showed a link between *Fusobacterium* genus and the development of colorectal cancer. Most recently an association between *Fusobacterium nucleatum* and colorectal cancer was established by a molecular cytogenetic technique[127,128].

The gut microbiota in patients with colorectal cancer is characterized by increased diversity in *Clostridiaceae* family (such as those from *Clostridium* genus), and an increase in *Bacteroides* and *Bifidobacterium* spp. In contrast, the gut microbiota of individuals with a reduced risk of developing cancer has a high population of lactate-producing bacteria, such as *Eubacterium aerofaciens* and *Lactobacillaceae* phyla[129]. Studies on animals led to propose a model of carcinogenesis based on the consideration that certain mutations in the intestinal epithelial cells lead to a loosening of the intercellular junctions and a reduced production of mucus, compromising the integrity of the intestinal mucosa. So, there

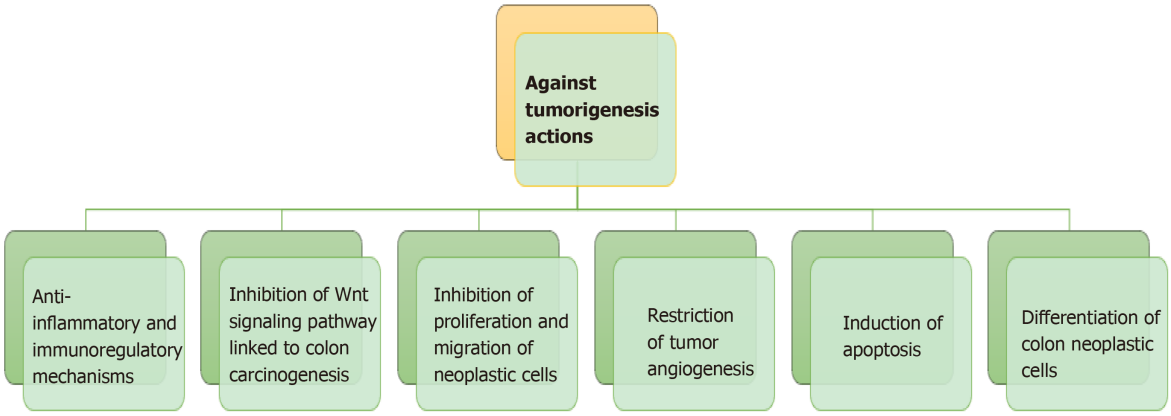

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Figure 3 The inhibitory properties of butyrate on tumorigenesis through various mechanisms by the colonic microbiota. Many of the metabolites of protein fermentation can be taken up by other microorganisms and synthesized into active carcinogens. For example, amines and nitrates can be used by facultatively anaerobic and anaerobic colonic bacteria and catalysed the formation of N-nitrosamines, which are among the strongest procarcinogens. Reduced levels of butyrate in the body are not only an indication of the possibility of cancer, but also indicate the severity of the cancer and its course in the body.

is a translocation of bacteria from the lumen to the epithelium where microbial products bind to tumor-associated macrophage receptors inducing the release of inflammatory factors (such as IL-1, IL-6, IL-23) which in turn leads to the production of IL-17 stimulating T-helper lymphocytes. IL-17 activates the transcription factor signal transducer and activator of transcription 3 in epithelial cells which will lead to increased survival and proliferation of epithelial cells resulting in further mutations facilitating carcinogenesis[130,131]. These changes in the epithelium burden the already compromised epithelial integrity, exacerbating bacterial allostasis and contributing to the vicious cycle: Bacterial allostasis - inflammation - cancer. The IL-23/IL-17 axis in tumour-induced inflammation is caused by barrier loss. Thus, otherwise "good" bacteria in the gut microbiota can be transformed into a carcinogen due to the altered host defence mechanism, indicating the uniqueness of the mucosal environment in preventing the development of cancer and may can lead to a cancer progression in other sites such as make the gut/breast microbiota's axis[132-135].

CONCLUSION

A large population of microorganisms of various composition gradually colonizes the human organism, externally and internally, and forms the so-called microbiota. The most important and then populous is the intestinal one. The organism represents a stable and nutrient-rich habitat for the gut microbiota to thrive, and therefore the health of the host is of primary importance to them. In turn, the host benefits because they not only prevent colonization by pathogens, but also provide a continuous and dynamic effect on their host's homeostasis. Thus, this symbiosis creates the host/gut microbiota axis that influences its health throughout an individual's life. In fact, a gut microbiota disorder has been related to many diseases (such as asthma, diabetes, obesity, neurological disorders, *etc.*) but also psychic and mental disorders. However, not all correlation mechanisms are yet understood, which is why more research is needed into the composition of the microbiota that is the cause or result of a disease. The aim of this study is on the human microbiota and its composition for the correlation of conditions during the life course that can be physiological or pathological. In fact, the scientific community is still trying to understand and relate its development and change to various pathological conditions that occur in humans.

FOOTNOTES

Author contributions: Colella M contributed to the conceptualization and data collection; Charitos IA involved in the original manuscript writing and drafting, and resources; Ballini A take part in the manuscript revision and formal analysis; Cafiero C and Palmirotta R contributed to the investigation; Palmirotta R and Santacroce L involved in the validation of this manuscript; Santacroce L contributed to the conceptualization, project administration, and funding of this manuscript; and all the authors have read, discussed, and approved the original manuscript and the final version of it.

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REVIEW

Advances in application of novel magnetic resonance imaging technologies in liver disease diagnosis

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Abstract

Liver disease is a major health concern globally, with high morbidity and mortality rates. Precise diagnosis and assessment are vital for guiding treatment approaches, predicting outcomes, and improving patient prognosis. Magnetic resonance imaging (MRI) is a non-invasive diagnostic technique that has been widely used for detecting liver disease. Recent advancements in MRI technology, such as diffusion weighted imaging, intravoxel incoherent motion, magnetic resonance elastography, chemical exchange saturation transfer, magnetic resonance spectroscopy, hyperpolarized MR, contrast-enhanced MRI, and radiomics, have significantly improved the accuracy and effectiveness of liver disease diagnosis. This review aims to discuss the progress in new MRI technologies for liver diagnosis. By summarizing current research findings, we aim to provide a comprehensive reference for researchers and clinicians to optimize the use of MRI in liver disease diagnosis and improve patient prognosis.

Key Words: Diagnostic imaging; Liver diseases; Fatty liver; Liver fibrosis; Hepatocellular carcinoma; Magnetic resonance imaging

Core Tip: Accurate evaluation of liver disease is essential for effective treatment strategies and better patient prognosis. Magnetic resonance imaging (MRI), a non-invasive diagnostic tool, has become a necessary technique for detecting liver diseases. The advancements in various magnetic resonance techniques have significantly enriched the diagnostic methods for liver diseases, each with a different focus and expertise in examining the liver. This article reviews the principles, advantages, limitations, and clinical applications of these new technologies in the diagnosis of liver disease, providing necessary references for researchers and clinical physicians to enhance the application of MRI in liver disease diagnosis and improve patient prognosis.

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INTRODUCTION

Liver disease is a major global health concern with high rates of morbidity and mortality[1]. Chronic liver diseases often lead to liver fibrosis and cirrhosis, which are the primary risk factors for hepatocellular carcinoma (HCC)[2]. The accumulation of large molecules such as collagen protein and glycosaminoglycans in the extracellular matrix is the basic pathological feature of liver fibrosis[3]. Reports suggest that 80%-90% of new HCC cases occur in individuals with cirrhosis[4]. Liver cancer is the sixth most common malignancy in humans and the fourth leading cause of cancer-related deaths worldwide. HCC accounts for 90% of all liver cancer cases[5]. Surgery is the preferred method for treating early-stage HCC[6]. However, due to the absence of apparent symptoms in the early stages, most patients are diagnosed late or with distant metastasis[7]. In major Western centers, the incidence of HCC recurrence within 5 years after curative liver resection is estimated to be 60%-70%[8]. Therefore, early diagnosis and recurrence monitoring are crucial for these patients.

Imaging examinations are vital in diagnosing chronic liver disease and liver cancer. HCC can be diagnosed non-invasively without requiring pathological confirmation, unlike most solid tumors[9]. MRI is a commonly used imaging technique that provides detailed information on the pathological and physiological aspects of liver cancer. It reflects changes in the tissue structure, metabolic status, tumor microenvironment, and other relevant factors.

Recent advancements in magnetic resonance imaging (MRI) technology, such as diffusion weighted imaging (DWI), intravoxel incoherent motion (IVIM), magnetic resonance elastography (MRE), chemical exchange saturation transfer (CEST), magnetic resonance spectroscopy (MRS), hyperpolarized MR (HP MR), contrast-enhanced MRI (CE-MRI), and radiomics, have significantly improved the accuracy and effectiveness of liver disease diagnosis. These new technologies are expected to provide further important information on tumor biological behavior.

This review aims to discuss the progress in new MRI technologies for liver disease diagnosis. By summarizing current research findings, we aim to provide a comprehensive reference for researchers and clinicians to optimize the use of MRI in liver disease diagnosis and improve patient prognosis.

NEW MRI TECHNOLOGIES FOR LIVER DISEASE DIAGNOSIS

DWI and IVIM

DWI is a non-invasive imaging technique that provides information on liver tumors, diffuse liver lesions, and liver function status without the need for contrast agents[10]. DWI has become a popular tool in tumor chemotherapy response assessment and follow-up after treatment due to its ability to detect recurrent lesions earlier than traditional imaging techniques[11].

IVIM, an extension of DWI, has shown great potential in liver function assessment[12], diagnosis of diffuse liver lesions [13] and liver tumors, and liver lesion characterization[14]. It is commonly used to estimate blood flow and microvascular perfusion[15]. The IVIM analysis includes the *D* value (representing pure diffusion factors), the *D** value (representing transport and perfusion factors), and the *f* value (representing microvascular perfusion factors). Research has demonstrated that IVIM has a higher diagnostic efficacy for displaying different liver lesions than DWI. Additionally, the diagnostic efficacy of the *D* value derived from IVIM is significantly higher than that of the apparent diffusion coefficient (ADC) value[16]. IVIM can also be used to predict histological grade, as the *D* value exhibits good diagnostic performance in distinguishing high-grade from low-grade liver cancer[17].

The perfusion-diffusion ratio (PDR) is a recent concept proposed to enhance the effectiveness of DWI. PDR is the ratio of the decreased signal rate caused by IVIM to the decreased signal rate caused by diffusion. A study was conducted to compare the effectiveness of IVIM parameters, ADC value, and PDR in distinguishing solid benign and malignant liver diseases in patient imaging data. The results indicate that PDR had better accuracy compared to IVIM parameters and the ADC value, with an accuracy rate of 79%, while also exhibiting high sensitivity and specificity[18].

The technologies of DWI and IVIM show great promise in aiding the diagnosis and treatment of liver cancer. Furthermore, the introduction of PDR has provided new insights for DWI, allowing for a more precise evaluation of liver cancer. However, DWI does have some limitations, such as the low repeatability of the ADC value, which prevents it from being a dependable imaging biomarker[19]. In addition, DWI is prone to several artifacts such as blurring, ghosting, and distortion which pose a challenge in achieving the repeatability of IVIM parameters and the ADC value[20]. To address this issue, Simchick *et al*[21] proposed a two-dimensional (2D) *b*-M1-optimized data acquisition technique that offers better stability and repeatability in measuring IVIM parameters. This technique can be instrumental in establishing IVIM quantitative biomarkers for liver disease.

MRE

Liver biopsy is widely accepted as the most reliable method for diagnosing and staging liver fibrosis[22]. However, its invasiveness, cost, and potential for complications limit its use. Furthermore, the diagnostic reliability of liver biopsy is questionable due to its sampling variability and subjectivity[23]. Ultrasound elastography is a low-cost and easy-to-use alternative, but it is highly influenced by obesity[24]. For example, vibration-controlled transient elastography (VCTE) is one of the most widely used ultrasound-based methods for diagnosing liver fibrosis. Its rapid, safe, and reproducible nature has made it widely used for bedside diagnosis. However, its reliability is lower than that of MRE as 15% of its conclusions are inaccurate, which is mainly due to the impact of obesity and insufficient experience of doctors[25,26]. According to a large sample meta-analysis, the summary area under the curve (AUC) values of VCTE for diagnosing significant fibrosis, advanced fibrosis, and cirrhosis were lower than those of MRE, with values of only 0.83, 0.85, and 0.89, respectively, while MRE had AUC values as high as 0.91, 0.92, and 0.90, respectively[27]. Serum biomarkers have also been explored for liver fibrosis evaluation, but their lack of specificity poses a challenge as they may also be released during inflammation in other tissues[28].

MRE proves to be a better method for diagnosing and staging liver fibrosis as it is not influenced by factors such as obesity, ascites, inflammation, or etiology[29]. The accuracy and reliability of MRE in diagnosing all stages of liver fibrosis, especially late-stage fibrosis and cirrhosis, have been confirmed by multiple meta-analyses[27,30]. However, the clinical application of MRE is still restricted due to its long examination time and high cost.

Encouragingly, recent studies suggest that combining serum biomarkers and MRE could have unexpected benefits. For instance, fibrosis-4 (FIB-4) index, an indicator used to assess liver injury and fibrosis, includes aspartate aminotransferase, alanine aminotransferase, age, and platelet count[31]. Tamaki *et al*[32] conducted a study where they used a two-step strategy combining FIB-4 index and MRE to detect late-stage fibrosis. They found that the accuracy of the two-step strategy was equivalent to that of using MRE alone, which could lead to cost reduction by reducing excess MRE. Therefore, the two-step strategy can serve as a screening method for large populations. Another study also demonstrated that the combined use of MRE and FIB-4 index has excellent negative predictive value in liver decompensation, which has significant clinical implications[33]. In addition, MRE has acceptable specificity and sensitivity in evaluating splenic stiffness and portal hypertension[34], which is crucial for assessing the overall health status of patients with cirrhosis.

Currently, liver fibrosis assessment can be carried out using 2D MRE, but a more advanced option now available is three-dimensional (3D) MRE. Unlike 2D MRE, 3D MRE images allow analysis of the entire volume of the liver in a 3D manner, providing better accuracy by collecting and processing information in all directions. In a study by Li *et al*[35], the diagnostic performance of 2D MRE and 3D MRE was compared, and both were found to have strong performance in detecting and staging liver fibrosis. However, 3D MRE provided significantly better image quality than the 2D MRE method and had higher inter-observer consistency in measuring liver stiffness (LS). Furthermore, in their study, Catania *et al*[36] found that 3D MRE is more repeatable due to its lower sensitivity to artifacts and provides a more comprehensive liver evaluation by including a larger area of liver parenchyma.

MRE has been found to be useful in predicting the occurrence and recurrence of HCC. Reports suggest that the risk of developing HCC increases with LS as measured by MRE[37]. Late-stage HCC recurrence is predicted by LS ($P < 0.001$), which has a high specificity (90.0%)[38]. MRE has also shown potential in diagnosing microvascular invasion (MVI) in HCC. A study by Zhang *et al*[39] found that tumor stiffness (TS) increased with MVI severity, with TS/LS > 1.47 ($P = 0.001$), TS > 4.33 kPa ($P < 0.001$), and irregular tumor margins ($P = 0.006$) being important independent predictors of MVI positivity.

In summary, MRE is a valuable tool for the thorough evaluation of liver fibrosis, cirrhosis, HCC, and portal hypertension in individuals with liver disease. Although MRE has demonstrated high accuracy in identifying advanced fibrosis or cirrhosis, there are discrepancies in the diagnostic threshold for fibrosis staging based on retrospective data meta-analyses[40], which require further validation.

CEST MRI

The CEST technique, first introduced by Ward *et al*[41] in 2000, utilizes off-resonance saturation pulses to saturate a specific substance, which in turn affects the signal intensity of free water through chemical exchange, providing relevant information about the substance. There are several types of CEST, including Glu CEST, Cr CEST, LATEST, and Gluco CEST, which can, respectively, map glutamate, creatine, lactate, and glucose, all of which are important substances involved in tumor metabolism. CEST can be divided into two categories: Exogenous CEST and endogenous CEST, depending on whether exogenous CEST agents are used. In liver imaging, CEST MRI can be used to evaluate tumor metabolism and tumor microenvironment, and monitor tumor treatment response.

Tumors are known to exhibit increased glucose uptake and glycolysis. To monitor tumor glucose metabolism and treatment response, ^{18}F-fluorodeoxyglucose positron emission tomography (^{18}F-FDG PET) is commonly used. However, the high cost and radioactivity of this method limit its application. CEST MRI has emerged as a potential alternative. A study by Chan *et al*[42] showed that using D-glucose, a simple carbohydrate, to detect tumor metabolism was possible.

The Gluco CEST image showed a significant enhancement in the tumor region, consistent with PET results. However, the CEST signal decreases rapidly due to the rapid metabolism of D-glucose after entering the cells[43]. In recent years, 3-O-methyl-D-glucose (3OMG) has emerged as a promising option for monitoring tumor progression and treatment efficacy. Unlike D-glucose, 3OMG is not metabolized after being taken up by cells, and accumulates in tumor cells, giving it a different kinetic profile[44]. Its similar CEST contrast efficiency to D-glucose makes it a suitable reporter for tumor cell glucose transporters. The Gluco CEST technique, which utilizes 3OMG, offers a low-risk option for patients with renal diseases to display tumor metabolism without using radioactive tracers.

In addition, CEST MRI provides a non-invasive method for evaluating tumor pH, which is important due to the Warburg effect. This effect leads to increased lactate production within cancer cells and results in an acidic extracellular pH in the tumor microenvironment. This acidic environment can enhance tumor invasiveness, metastasis, angiogenesis, and resistance to radiotherapy and chemotherapy[45]. The CEST measurement of pH is not limited by observation depth and can be used for whole-body imaging. While amide proton transfer (APT) CEST MRI is capable of measuring pH[46], it is limited in its ability to calculate the absolute value of pH. To address this limitation, exogenous agents have been utilized to measure extracellular pH in the tumor microenvironment. The use of iodinated agents in CEST MRI has emerged as a promising non-invasive technique for pH assessment[47], as these clinically approved agents are not affected by concentration and offer advantages over APT MRI.

CEST has found another novel application in image-guided nanoparticle (NP) drug delivery. This technique can monitor the transformation and distribution of NP drugs in the body, thereby assisting in adjusting treatment plans. CEST-guided NP drug delivery systems can be divided into labeled and unlabeled systems, depending on the labeling strategy used to achieve the CEST signal[48]. For example, Law *et al*[49] successfully used CEST MRI to detect the specific distribution of iohexol-labeled liposome drugs in the brain of an animal model. In a separate study, Liu *et al*[50] introduced a new labeling-free strategy that uses CEST MRI imaging technology to detect the delivery efficiency of anti-cancer drug lentinan-functionalized SeNPs and achieve image-guided drug delivery. The ability of CEST MRI to visualize NP drug delivery provides a unique opportunity for the integration of tumor diagnosis and treatment. However, this technology is still in its infancy, and nearly all related research is currently being conducted in preclinical animal models. Therefore, extensive validation is necessary to ensure its accuracy and acceptability for clinical translation.

CE-MRI

CE-MRI is a technique used to enhance liver lesion imaging by injecting a contrast agent intravenously. The contrast agent distribution patterns in the liver can be classified into four categories[51]: (1) Extracellular fluid agents, such as Gd-DTPA and Gd-DOTA, rapidly enter the hepatic capillary network and diffuse into the tissue space after injection, eventually being excreted by the kidneys; (2) Hepatobiliary agents, such as Gd-EOB-DTPA and Gd-BOTPA, can be absorbed and metabolized by normal liver cells. During the hepatobiliary phase, regions with premalignant liver lesions or malignant tumors display low signal due to decreased contrast agent uptake[52]; (3) Superparamagnetic iron oxide (SPIO) particles can be taken up by Kupffer cells in normal liver tissue, resulting in a 'black liver' effect. Tumor tissue displays high signal because it almost does not take up SPIO particles; and (4) Molecular targeted MRI contrast agents can be modified with specific antibodies or ligands, allowing them to bind specifically to tumor tissue and highlight the tumor.

The development of MRI contrast agents with tumor targeting, safety, and efficacy has been extensively researched. The use of molecular targeted MRI contrast agents with specific antibodies or ligands has shown promising results in actively targeting tumor tissue, enabling early detection of tumors. Various specific targets can be utilized to produce specific contrast agents. This review will introduce glypican-3 (GPC3) and alpha-fetoprotein (AFP) as examples.

GPC3 is a cancer embryonic polysaccharide present on the cell membrane. Studies have shown that GPC3 is highly expressed in over 70% of HCCs, but not in hepatitis, cirrhosis, benign liver lesions, or healthy adult tissue. GPC3 promotes the progression of liver cancer by binding to molecules such as Wnt signaling proteins and growth factors[53]. In terms of diagnosis, GPC3 has a diagnostic specificity similar to AFP but with higher sensitivity[54]. Additionally, GPC3 can be used to distinguish AFP-negative HCC[55], making it a more reliable diagnostic marker for HCC. Overall, GPC3 shows promise as a potential diagnostic tool for HCC. Zhao *et al*[56] have developed a novel liver cancer-targeting probe by combining USPIO with GPC3-specific ligands. The probe demonstrated preferential binding to GPC3 in both *in vitro* and *in vivo* experiments. Furthermore, it exhibited good stability and biocompatibility, making it a promising candidate for a targeted MRI contrast agent.

AFP is not typically expressed in adults, but in liver cancer patients. AFP levels may be very high, making it a potential target for detecting liver cancer[57]. AFP antibodies can be used to create targeted probes, but as previously mentioned, AFP is not highly expressed in all liver cancer patients. To address this issue, Ma *et al*[58] conjugated AFP antibodies and GPC3 antibodies to ultra-small SPIO NPs (USPIO), resulting in the development of a dual-antibody-conjugated MRI probe that can detect heterogeneity and small HCC with higher sensitivity than single-target probes. The experiment demonstrated that using dual-antibody-conjugated USPIO probes for targeting cancer cells was more efficient and sensitive compared to using single-labeled probes that targeted AFP-USPIO and GPC3-USPIO, as well as non-targeted USPIO. This finding highlights the potential of using multi-target probes to overcome tumor heterogeneity and improve sensitivity to HCC.

Gadolinium-based contrast agents (GBCAs) are commonly used in MRI, but they carry the risk of allergic reactions and nephrogenic systemic fibrosis[59]. Furthermore, even patients with normal kidney function can experience gadolinium accumulation in all tissues with GBCA exposure[60]. Therefore, the development of effective Gd-free MRI contrast agents is becoming increasingly important. Manganese (Mn) has emerged as a promising alternative to GBCAs[61], with researchers discovering that Mn oxide nanoparticles have negligible toxicity and produce good T1-weighted contrast effects[62]. Mn^{2+} has also demonstrated potential as an anti-cancer drug or adjuvant, making it a fascinating area of

research[63]. In a recent study, researchers found that administering Mn^{2+} to a mouse model induced a strong systemic anti-cancer response. This was achieved through promoting natural killer cell function, macrophage and dendritic cell maturation and activation, CD8+ T cell differentiation and activation, and memory T cell survival in tumors. As a result, tumor growth and metastasis were greatly inhibited. Another group of researchers developed an intelligent therapeutic probe called MnTBs, which decomposes quickly in acidic and reducing cellular environments, releasing Mn^{2+} and triggering chemodynamic therapy. This probe was found to be effective in inhibiting tumor growth and metastasis, as well as detecting millimeter-sized liver metastases with a high contrast of 316%[64]. These findings suggest that Mn-based contrast agents have potential in targeted tumor therapy.

MRS

MRS is a non-invasive method that monitors organ metabolites, making it particularly suitable for the liver due to its high concentration of multiple metabolites such as ATP, glutamine, and glycogen. The liver MRS analysis primarily uses ^{1}H, ^{31}P, and ^{13}C. Its major applications include the diagnosis and grading of fatty liver and liver fibrosis, as well as the diagnosis of liver tumors.

The incidence of non-alcoholic fatty liver disease (NAFLD) is increasing, and some patients with NAFLD may develop non-alcoholic steatohepatitis, which can progress to liver cirrhosis and HCC[65]. Therefore, early diagnosis is crucial for preventing and treating NAFLD. ^{1}H-MRS is a non-invasive method that can accurately measure the content of triglycerides in the liver[66]. However, it has some limitations, such as long processing time, motion artifacts, and sampling errors.

The use of MRI proton density fat fraction (MRI-PDFF) has become a popular method for measuring liver fat content in recent years[67]. MRI-PDFF has a strong correlation with MRS ($r = 0.983$, $P < 0.001$) and can quantitatively measure the lipid content of the entire liver[68], overcoming the limitations of MRS and liver tissue biopsy. This makes MRI-PDFF a widely used reference standard for image-based fat quantification with broader application prospects than MRS[69]. In addition, the recent use of the multi-echo Dixon sequence has shown promising results in detecting NAFLD[70].

In the diagnosis and staging of liver fibrosis, ^{1}H-MRS and ^{31}P-MRS have distinct values. Ding *et al*[71] discovered that choline (Cho) levels measured by ^{1}H-MRS increased in proportion to the severity of liver fibrosis. They also found that the Cho/lipid ratio was the most significant diagnostic indicator of liver fibrosis based on the receiver operating characteristic curve. The diagnostic thresholds for liver fibrosis and early cirrhosis were ≥ 0.028 and ≥ 0.131, respectively. In comparison to ^{1}H-MRS, ^{31}P-MRS is advantageous in diagnosing cirrhosis as it can distinguish between different causes of liver disease. Through quantitative analysis of metabolite concentrations such as phosphate monoester (PME), phosphate diester (PDE), Pi, and ATP, ^{31}P-MRS has the potential to differentiate between alcoholic liver disease, viral liver disease, NAFLD, and cirrhosis[72]. However, larger-scale multi-center studies are needed to confirm the clinical relevance and usefulness of this finding.

Liver cancer patients can benefit from the use of ^{1}H-MRS and ^{31}P-MRS as well. In ^{1}H-MRS, the Cho peak is a significant indicator of focal liver lesions, and its elevation could suggest the proliferation of tumor tissue due to increased synthesis of cell membrane phospholipids. Furthermore, the information provided by the Cho peak can be further developed. Liao *et al*[73] conducted a study to compare the diagnostic performance of Cho peak area, Cho peak amplitude, and combined methods for early detection of rabbit liver cancer. The results showed that the combined method had higher accuracy in the early diagnosis of liver cancer, and the correlation between Cho peak amplitude and tumor volume was the best. In ^{31}P-MRS, the contents of PME and PDE can reflect cell membrane synthesis and breakdown, and their increase means cell proliferation. Liver tumors can be associated with an increase in PME/PDE and PME/Pi, and changes in PME/PDE after treatment are significant. However, further multi-center studies are needed to confirm their accuracy due to high heterogeneity[74]. ^{31}P-MRS is also reliable for measuring ATP consumption and identifying the degree of acute liver ischemia-reperfusion injury (IRI)[75], which is particularly meaningful for patients who require hepatectomy and liver transplantation. Diagnosis and treatment of IRI during surgery have always been a challenging task.

^{13}C MRS is considered the gold standard for imaging human liver glycogen metabolism after oral ingestion of ^{13}C-labeled glucose[76]. However, the low natural abundance and sensitivity of ^{13}C nuclei can limit their application. To overcome this challenge, researchers have developed hyperpolarization techniques, which will be discussed below. For readers' convenience, the targets of MRS and HP MR in liver metabolism evaluation are summarized in Figure 1.

However, motion still has an impact on the accuracy of MRS. Additionally, liver tissue has significant heterogeneity, so excluding extraneous tissue when setting the observation area is crucial. As technology continues to advance, MRS is expected to become an increasingly important tool for diagnosing and grading liver tumors, potentially replacing the need for invasive biopsies.

HP MR

The development of HP MR can be attributed to the dynamic nuclear polarization (DNP) technique, which was initially proposed by Ardenkjaer-Larsen *et al*[77]. This technique can increase MR signal by more than 10000 times. Currently, the leading HP biomarkers primarily consist of ^{13}C, with [1-^{13}C] pyruvate being the most commonly used due to its central role in cellular metabolism. When absorbed by liver cells, [1-^{13}C] pyruvate can produce three observable metabolic intermediates: Lactate, alanine, and CO_2, thus allowing visualization of liver metabolism.

Cancer cells often have higher levels of the enzyme lactate dehydrogenase[78], which convert pyruvate to lactate. Measuring the metabolic transformation from pyruvate to lactate is important for analyzing tumor invasiveness, grade, and prognosis[79]. In a study, HCC was induced in rats, and tumor cells were extracted and re-implanted into another group of nude mice. The study found that tumors re-implanted from cells with higher lactate/pyruvate ratios showed higher lactate signals. Therefore, using HP MR to evaluate glucose metabolism differences in liver cancer tissue could potentially reveal tumor phenotypes[80].

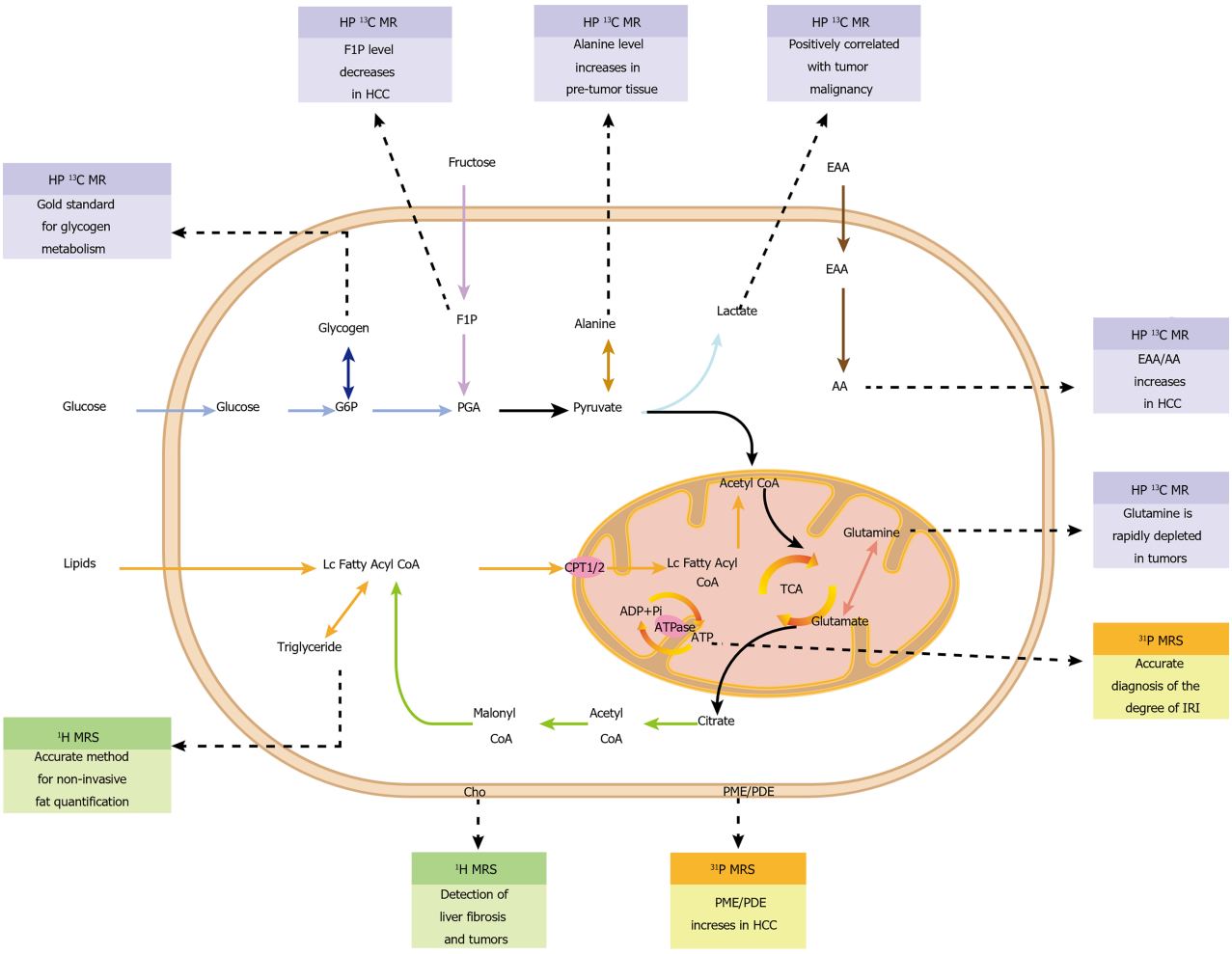

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Figure 1 The targets of magnetic resonance spectroscopy and hyperpolarized magnetic resonance in liver metabolism evaluation. AA: Acetoacetate; CPT: Carnitine palmitoyl transferase; Cho: Choline; EAA: Ethyl acetoacetate; F1P: Fructose-1-phosphate; G6P: Glucose-6-phosphate; IRI: Ischemia-reperfusion injury; HCC: Hepatocellular carcinoma; PDE: Phosphate diester; PGA: Phosphoglyceric acid; PME: Phosphate monoester; TCA: Tricarboxylic acid; MRS: Magnetic resonance spectroscopy.

Alanine is another potential biomarker for liver cancer diagnosis. It was found that an increase in alanine generation was the earliest metabolic change detected in liver cancer models, even before the formation of primary tumors. In pre-tumor tissues, the conversion rate of pyruvate to alanine significantly increased, and the area with the most abundant alanine signal in pre-tumor tissues was often the region where tumor nodules form[81]. Thus, [1-^{13}C] pyruvate HP MR can be used for early diagnosis of liver cancer by monitoring alanine generation.

Recent studies have shown an increased interest in the use of other hyperpolarization probes, including HP [1,3-$^{13}C_2$]-ethyl acetoacetate, HP [2-^{13}C]-fructose, and HP [5-^{13}C, 4,4-$^{2}H_2$,5-^{15}N]-L-glutamine. Ethyl acetoacetate (EAA) is converted to acetoacetate (AA) at a reduced rate in HCC cells due to lower concentrations and activity of carboxylesterases. In a rat liver transplant tumor model, HP [1,3-$^{13}C_2$]-ethyl acetoacetate MR revealed that the EAA/AA ratio in tumor tissue was approximately four times higher than that in healthy tissue (P = 0.009)[82]. Similarly, HP [2-^{13}C]-fructose can be used to explain HCC cell metabolism. HCC cells have reduced generation of fructose-1-phosphate (F1P) due to lower expression of ketohexokinase[83]. HP MR has successfully detected F1P and its loss in an HCC model. Finally, evaluation of glutamine metabolism is also beneficial for liver cancer diagnosis because it is rapidly consumed by proliferating cells in tumor tissue. The newly developed HP [5-^{13}C, 4,4-$^{2}H_2$,5-^{15}N]-L-glutamine proved to be the best choice for determining *in vivo* glutamine metabolism[84].

Importantly, HP MR provides a unique advantage in obtaining information on tumor metabolism and perfusion, which is not easily obtainable through other methods. The latest technology utilizes the dual-probe imaging method, which involves the use of [1-^{13}C]-pyruvate and [^{13}C, $^{15}N_2$]-urea for simultaneous tumor perfusion and metabolic imaging [85]. The metabolic conversion rate of pyruvate indicates enzyme activity and transporter expression, whereas HP ^{13}C urea is a non-metabolically active extracellular probe that reflects tissue perfusion and distribution. The combination of these two probes can effectively explain changes in metabolism and perfusion during disease progression and treatment response.

PET is currently the preferred imaging method in clinical practice as it provides metabolic information through non-invasive analysis of cancer metabolism *in vivo*. This is achieved through the injection of ^{18}F-FDG[86]. Although PET is

useful for assessing glucose metabolism, it has limited ability to evaluate downstream metabolism, which can be important in many cases. However, HP ^{13}C-pyruvate MR can provide information about downstream metabolism. To obtain a complete picture of glucose metabolism, a combination of ^{18}F-FDG and [1-^{13}C] pyruvate methods can be used. Hansen *et al*[87] have demonstrated the feasibility of this approach through a technique called hyperPET, which shows the consistency between tumor ^{18}F-FDG uptake and [1-^{13}C] lactate production. Clemmensen *et al*[88] have also shown that combining [^{68}Ga]Ga-NODAGA-E[(cRGDyK)]$_2$ PET and HP [1-^{13}C]-pyruvate MR can enhance the ability of hyperPET to detect tumor angiogenesis. HyperPET is a promising tool for evaluating glucose metabolism direction in order to predict tumor occurrence and evaluate the malignancy degree of the tumor. It achieves this by describing the transformation of pyruvate into alanine, lactate, or entering oxidative phosphorylation in the body. While the complexity and high cost of hyperPET may seem impractical for routine use, advancements in MRI and PET-related technologies may make it a valuable tool for diagnosing liver cancer.

Recent advancements in HP MR have led to improvements in both efficiency and accuracy. The slow production of hyperpolarized pyruvate using DNP has been addressed with the MINERVA protocol, which has greatly facilitated the clinical translation of HP MR[89]. Additionally, quantification bias resulting from the use of surface transmit/receive coils has been addressed by Lee *et al*[90] through the development of a dedicated HP ^{13}C EPSI post-processing pipeline. This method has significantly improved the accuracy of measuring the pyruvate to lactate conversion rate in tumors and adjacent regions, with the average signal-to-noise ratio of pyruvate, lactate, and alanine increased by 37.4, 34.0, and 20.1 times, respectively.

MRI radiomics

The concept of radiomics was introduced by Lambin *et al*[91] in 2012. This method involves extracting features from conventional imaging at the tumor's overall level, providing a non-invasive, comprehensive, and quantitative observation of the tumor's temporal and spatial heterogeneity. Presently, MRI-based radiomics research in the liver primarily focuses on classification of liver fibrosis and hepatitis, liver cancer diagnosis, differentiation degree and immunohistochemistry prediction, and MVI evaluation.

MRI-based radiomics has shown promise in diagnosing and predicting hepatitis and liver fibrosis. While conventional MRI images can detect severe cases by observing changes in water content and distribution caused by inflammation, subtle tissue changes can prove challenging to diagnose. Radiomics technology can capture these tiny changes, allowing for more accurate diagnosis. Wei *et al*[92] proposed a grading system that simultaneously stages fibrosis and inflammation activity, with an AUC of 0.932 and 0.910 for diagnosing early-stage hepatitis and fibrosis, respectively. This study demonstrates the potential for radiomics to improve diagnosis and prediction of liver diseases. A novel technique, known as the dynamic image radiomics model, has been developed using deep learning technology to evaluate liver fibrosis. This method combines imaging features from multi-phase dynamic contrast-enhanced images with temporal features. It utilizes time-varying curves of contrast enhancement and imaging features during enhancement and eliminates manual selection bias by using an automated region of interest extraction method. Overall, compared with traditional radiomics methods and clinical serum parameters, the dynamic radiomics model has stronger predictive performance for various stages of liver fibrosis. The proposed liver fibrosis classification model is highly automated, saving time and effort. This model is significant in predicting new cases and training additional datasets[93].

Research has shown that MRI-based radiomics models are highly effective in distinguishing between HCC and non-HCC, with better discrimination efficiency than visual assessment by novice radiologists ($P < 0.05$)[94]. These models also have significant value in identifying high- and low-grade HCC. In a study by Ameli *et al*[95], radiomics features demonstrated strong differentiation ability in a multi-classification model, with an AUC of 0.83. This outperformed classification based solely on ADC or arterial-phase enhancement value, which had an AUC of only 0.75.

Moreover, research has shown that MRI radiomics features have good predictive efficacy for immunohistochemistry and molecular expression in HCC. For example, CK19+ HCC has higher invasiveness, higher lymph node metastasis rates, higher resistance to radiotherapy and chemotherapy, and poorer prognosis. Researchers have successfully constructed and validated a multi-sequence radiomics model for accurately identifying the CK19 status of HCC patients using multi-center MRI imaging data[96]. GPC3 is also associated with poor prognosis in HCC patients. Gu *et al*[97] developed a useful method for predicting GPC3 positive HCC patients without invasive procedures by combining AFP and radiomics features in a column chart. The tool showed significant predictive performance in both training and validation cohorts, with AUC values of 0.926 and 0.914, respectively. Additionally, radiomics features were found to be correlated with the protein level of the immunotherapy target programmed cell death ligand 1 (r = 0.41-0.47, $P < 0.029$) and the mRNA expression levels of programmed cell death 1 and cytotoxic T-lymphocyte-associated protein 4 (r = -0.48 to 0.47, $P < 0.037$)[98]. The findings of these studies indicate that utilizing a combination of multi-sequence MRI radiomics features can lead to precise classification of immunohistochemistry and molecular expression in liver cancer. This non-invasive approach can facilitate personalized management strategies for patients.

The use of MRI radiomics analysis can also provide a distinct advantage in preoperative diagnosis of MVI in liver cancer, as current diagnosis of MVI can only be made through postoperative histological examination. Feng *et al*[99] developed an MRI radiomics model for predicting preoperative MVI by extracting radiomics features from Gd-EOB-DTPA-enhanced MRI. The model's AUC, sensitivity, and specificity were 0.83, 90%, and 75%, respectively, which outperformed radiologists in predicting MVI. According to Liang *et al*[100]'s meta-analysis of 15 studies and 981 patients, MRI radiomics has a high accuracy in diagnosing MVI with an AUC of 0.87, sensitivity of 79%, and specificity of 81%. Based on our analysis, it can be concluded that the use of MRI radiomics for predicting MVI has a high level of accuracy. This non-invasive method can be considered as an alternative approach for evaluating MVI.

Table 1 Advanced magnetic resonance imaging techniques for liver diagnosis: Comparison of clinical applications, advantages, limitations, and developments

Technique(s)	Applications	Advantages	Limitations	Developments
DWI and IVIM	Assessment of liver tumors, diffuse liver lesions, and liver function. Assessment of liver or tumor blood perfusion	Evaluate liver or tumor blood perfusion without the use of contrast agents	Poor reproducibility of IVIM and DWI	2D *b*-M1 acquisition improves reproducibility. The new parameter PDR improves DWI performance
MRE	Diagnosis and staging of liver fibrosis and cirrhosis. Prediction of the recurrence of HCC	Diagnosis of liver fibrosis is not affected by obesity, ascites, inflammation, and etiology	The diagnostic threshold for fibrosis is variable and conflicting	The two-step strategy can screen for liver fibrosis. LS and TS can predict HCC recurrence
CEST	Assessment of tumor metabolism and microenvironment. Monitoring of tumor treatment response	An FDG substitute that does not involve ionizing radiation	Easily affected by other factors. Long scan time	The new reagent 3OMG has unique advantages. Realization of image-guided drug delivery and integration of tumor diagnosis and treatment
CE-MRI	Diagnosis and staging of liver tumors. Detection of liver metastases and diffuse liver lesions	Provides more information about lesions compared to plain MRI. Specific probes enable visualization at the molecular level	Gd chelates can cause allergic reactions and nephrotoxicity	Multi-target probes have the potential to overcome tumor heterogeneity. Potential applications of Mn-based contrast agents in targeted tumor therapy
MRS	Diagnosis and grading of fatty liver and liver fibrosis. Metabolic evaluation of liver and intrahepatic tumors	Provides quantitative data on liver metabolism non-invasively without the use of contrast agents	Low sensitivity of ^{13}C. Accuracy is affected by liver tissue heterogeneity and motion	Cho peak can provide more information. ^{31}P-MRS can differentiate liver cirrhosis etiology and evaluate IRI
HP MR	Providing metabolic, perfusion, and enzymatic information on HCC	DNP improves MR signal by 10000 times. [1-^{13}C] pyruvate has particular value in evaluating tumor metabolism	Current measurement methods still produce inevitable quantitative deviations	HyperPET is expected to elucidate complete glucose metabolism. More hyperpolarized probes are being used. Double-probe HP MR can simultaneously obtain metabolic and perfusion information
MRI radiomics	Diagnosis and prediction of immunohistochemistry features, MVI, liver fibrosis, and hepatitis.	Comprehensive, non-invasive, and quantitative observation of the spatiotemporal heterogeneity of tumors	Poor reproducibility of MRI features. MVI features are variable and conflicting in different studies	Good application value in the differential diagnosis, immunohistochemical feature prediction, and MVI prediction of HCC

CEST: Chemical exchange saturation transfer; CE-MRI: Contrast-enhanced magnetic resonance imaging; Cho: Choline; DNP: Dynamic nuclear polarization; DWI: Diffusion weighted imaging; HCC: Hepatocellular carcinoma; HP: Hyperpolarized; IRI: Ischemia-reperfusion injury; IVIM: Intravoxel incoherent motion; LS: Liver stiffness; MRE: Magnetic resonance elastography; MRI: Magnetic resonance imaging; MRS: Magnetic resonance spectroscopy; MVI: Microvascular invasion; PDR: Perfusion-diffusion ratio; TS: Tumor stiffness; PET: Positron emission tomography; FDG: Fluorodeoxyglucose; 2D: Two-dimensional.

The current limitation of MRI radiomics is the lack of reproducibility in extracting radiomics features. While previous studies have shown good interobserver reproducibility of HCC radiomics features from specific MRI systems, caution is needed when interpreting data in multi-platform radiomics studies as reproducibility can vary greatly between different platforms[101].

Additionally, there is variability and inconsistency in using MRI features to predict MVI in different studies. To combat this, Hong *et al*[102] conducted a meta-analysis of data from 36 studies involving 4410 participants. They identified seven MRI features, namely, large tumor volume, arterial edge enhancement, arterial tumor surrounding enhancement, hypointensity around the tumor in the hepatobiliary phase, irregular margins, multifocality, and low T1 signal, as important predictors of MVI in HCC. These MRI radiomics features are valuable references for future research and of great significance for developing more reliable MVI prediction strategies. Table 1 summarizes various novel MRI technologies' clinical applications, advantages, limitations, and latest developments discussed in this review for the diagnosis of liver diseases. Table 2 lists the diagnostic performance of some MR techniques for the diagnosis of liver diseases.

CONCLUSION

MRI is a promising technology for diagnosing liver diseases due to its non-invasive and radiation-free nature. The development of new technologies such as DWI, IVIM, MRE, CEST, MRS, HP MR, CE-MRI, and radiomics has expanded the capabilities of MRI, allowing for more comprehensive and accurate diagnostic results. Although these technologies have limitations, their role in liver disease diagnosis will continue to improve as they are updated and enhanced. It is important for researchers and clinical doctors to thoroughly study the application value of these new technologies in clinical practice to better guide the diagnosis, treatment, and rehabilitation of liver diseases and ultimately improve patient prognosis.

Table 2 Comparison of performance indicators of new magnetic resonance techniques in diagnosis of liver disease

Technique	Disease	Subjects	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)	Ref.
DWI	HCC	34	54.8	90.9	34.5	95.8	[103]
DWI	LF	40	85	82	85	83	[104]
PDR	MT	83	81	77	NA	NA	[18]
MRE	LF	59	69.0	88.2	53.6	93.5	[105]
MRS	Liver steatosis	4715	72.7-88.5	92.0-95.7	NA	NA	[106]
Gd-EOB-DTPA MRI	HCC	77	88.2	96.7	90.6	95.7	[107]
SPIO MRI	HCC	30	66.0	98.0	91.4	90.0	[108]
MRI-PDFF	NAFLD	60	96	100	92.6	89.5	[70]
Radiomics	MVI	50	90	75	NA	NA	[99]
Radiomics	MVI	981	79	81	NA	NA	[100]

DWI: Diffusion weighted imaging; HCC: Hepatocellular carcinoma; LF: Liver fibrosis; MRE: Magnetic resonance elastography; MRI: Magnetic resonance imaging; MRI-PDFF: Magnetic resonance imaging proton density fat fraction; MRS: Magnetic resonance spectroscopy; MT: Malignant tumor; MVI: Microvascular invasion; NA: Not available; NAFLD: Non-alcoholic fatty liver disease; NPV: Negative predictive value; PDR: Perfusion-diffusion ratio; PPV: Positive predictive value; SPIO: Superparamagnetic iron oxide.

FOOTNOTES

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MINIREVIEWS

Application of single-cell omics in inflammatory bowel disease

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Abstract

Over the past decade, the advent of single cell RNA-sequencing has revolutionized the approach in cellular transcriptomics research. The current technology offers an unbiased platform to understand how genotype correlates to phenotype. Single-cell omics applications in gastrointestinal (GI) research namely inflammatory bowel disease (IBD) has become popular in the last few years with multiple publications as single-cell omics techniques can be applied directly to the target organ, the GI tract at the tissue level. Through examination of mucosal tissue and peripheral blood in IBD, the recent boom in single cell research has identified a myriad of key immune players from enterocytes to tissue resident memory T cells, and explored functional heterogeneity within cellular subsets previously unreported. As we begin to unravel the complex mucosal immune system in states of health and disease like IBD, the power of exploration through single-cell omics can change our approach to translational research. As novel techniques evolve through multiplexing single-cell omics and spatial transcriptomics come to the forefront, we can begin to fully comprehend the disease IBD and better design targets of treatment. In addition, hopefully these techniques can ultimately begin to identify biomarkers of therapeutic response and answer clinically relevant questions in how to tailor individual therapy to patients through personalized medicine.

Key Words: Single-cell omics; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Single cell RNA-sequencing; Precision medicine

Core Tip: Single-cell techniques and omics have taken off in the last few years and the ability to detect individual cellular transcript details has revolutionized the world of research. In the field of gastroenterology in just the last five years, several single-cell techniques have been applied to inflammatory bowel disease research with the identification of novel cellular immune players in the pathogenesis of both ulcerative colitis and Crohn's disease.

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INTRODUCTION

The gastrointestinal (GI) tract houses a complex network of immune cells, cellular signaling, and intestinal flora. By balancing host tolerance and microbial defense response, the mucosal immune system of the GI tract performs constant surveillance and sampling of antigens and microbes to induce either tolerance or mounts a vigorous immune response to pathogens[1]. Intestinal inflammation and disease occur when this delicate balance breaks down leading to over activation of mucosal immunity and aberrant response to host antigen and commensal organisms[2]. Microscopic disruption of the epithelial barrier, over activation of proinflammatory cells, and the lack of regulatory mechanisms are some pathways that present in clinical chronic GI disorders and distinguishes mucosal immunity from systemic immunity.

The ability to detect pathogenic pathophysiology is rooted in the organ, the GI tract, and may be missed through studying the systemic immune system. In order to understand the target organ and mucosal immune system, technical advances have been made in the last few years giving us the ability to interrogate down to the microscopic single cell level, thus revolutionizing GI research. Since its inception in 2009, single cell RNA-sequencing (scRNA-seq) has enhanced our ability to comprehensively map and resolve cell types, cellular subsets, and cells states present in both healthy GI tissue and diseased states[3]. The novelty of single-cell technologies *vs* previous technologies such as bulk-sequencing is the ability to detect rare subsets of cells that may be the aberrant drivers of disease[4]. The homogeneity of bulk-sequencing lacks the capacity to decipher cellular heterogeneity and loses dimmer signals in rare subsets that may be important in disease pathogenesis[4,5]. The inherent advantage of single-cell techniques has led to its continued popularity in research. Here, we aim to review the single-cell technologies that have emerged over the last decade and its published applications to GI disease, namely in inflammatory bowel disease (IBD).

IBD which includes both ulcerative colitis (UC) and Crohn's disease (CD) is a chronic complex autoimmune condition characterized by inflammation of the GI tract. The pathogenesis of IBD is thought to develop from an inappropriate immune response towards self-antigens and commensal microbiota in a genetically susceptible host. The advent of scRNA-seq has led to a boom in the number of publications with the application of single cell techniques in IBD research [6,7]. As a disclaimer, this is by no means a comprehensive review of all single-cell studies or single-cell techniques available as the technology has massively expanded in the last few years and will continue to grow in popularity in years to come.

OVERVIEW OF AVAILABLE SINGLE-CELL TECHNOLOGY

scRNA-seq

With the commercialization of products and increasing user compatibility, scRNA-seq can be done using a variety of platforms and through multiple approaches (10 × Genomics, Fluidigm, BD Rhapsody, SmartSeq, *etc.*) whether with multiple well plates, microfluidics, or drop-seq[8,9]. Single-cell data sets can also be created from the 3′ end or 5′ end of mRNA. The basis behind the technology is partitioning individual cells with one uniquely barcoded mRNA-capture medium which has unique molecular code tag for each cell and its contained transcripts. The cell is then lysed and the mRNA is captured by these coded tags and each cell's mRNA is reverse transcribed into uniquely barcoded cDNA. cDNA libraries are amplified, indexed and sequenced using next-generation sequencing platforms (NextGen). Using the unique molecular code tags, the cDNA can be linked to the cell of origin and abundance of transcripts can be deciphered using the number of copies of the certain cDNA (Figure 1).

With large sets of data, bioinformatics analysis can be challenging. To perform this analysis, FASTQ files are generated from binary base call output from sequencing using software programs that then align the reads from the FASTQ files to a human reference genome/transcriptome using STAR[10]. Gene-cell matrices with principle component analysis can then be used to quantify transcripts of interest using standard workflows to give traditional visualization of single-cell datasets in the form of uniform manifold approximation and projection algorithms or T-distributed stochastic neighbor embedding plots (Figure 1).

In some cases, studies are interested not only in the current state of the cell but what the possible future of how the cell may develop ("pseudotime"). Software programs including Monocle[11] and Census[12] offer toolkits of computational bioinformatic programs specifically designed for the analysis of scRNA-Seq data to look at the temporal resolution of

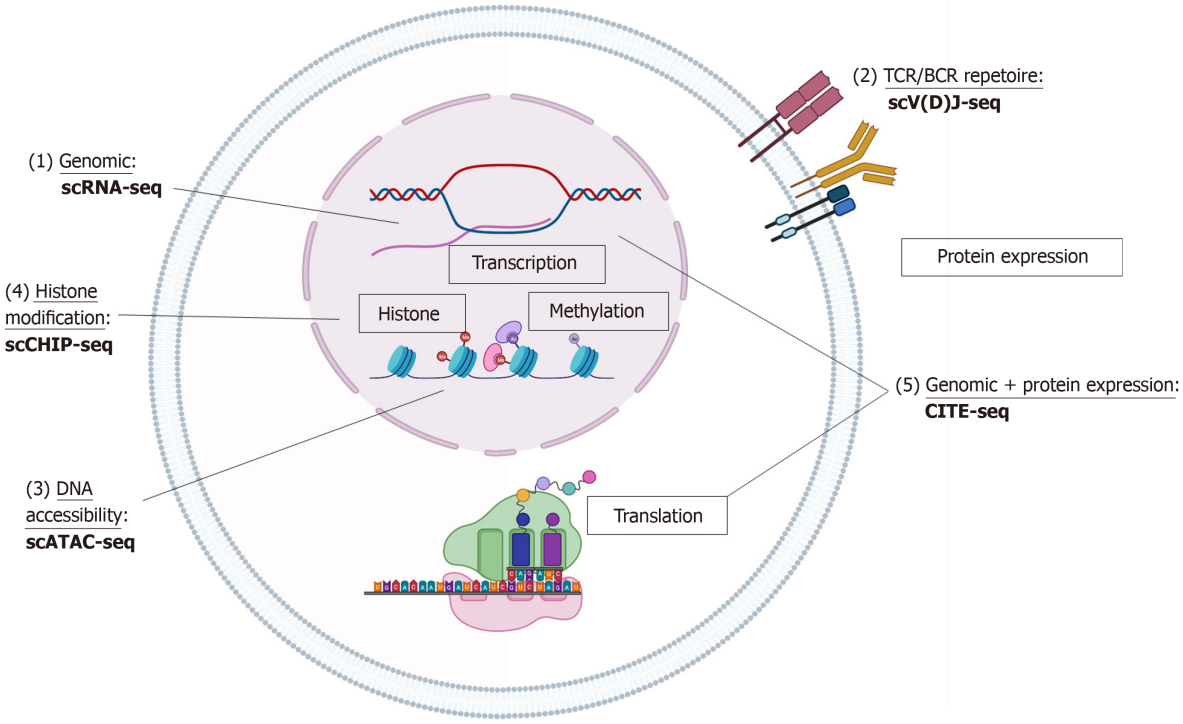

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Figure 1 Single-cell technologies. scRNA-seq: Single cell RNA-sequencing; TCR: T cell receptor; BCR: B cell receptor; scCHIP: Single-cell chromatin immunoprecipitation; scATAC: Single-cell assay for transposase-accessible chromatin; CITE: Cellular indexing of transcriptomes and epitopes. Created in biorender.com.

transcriptome dynamics using unsupervised algorithms on scRNA-Seq data collected at multiple time points. Thes programs can be used to recover single-cell gene expression kinetics from a wide array of cellular processes, including differentiation, proliferation and oncogenic transformation to identify branching patterns in the cells. The algorithm finds the longest path through the minimum spanning tree, one that corresponds to the longest sequence of transcriptionally similar cells and produces a 'trajectory' of an individual cell's progress through differentiation that is expressed in units of pseudotime (Figure 1).

T cell receptor/B cell receptor: scV(D)J- seq

T cell receptor (TCR) and B cell receptor (BCR) drive a range of antigen specific adaptive immune responses to pathogens with large highly diverse repertoires to allow for recognition of antigens[9]. The advent of 5′ single-cell sequencing allows researchers to better understand lymphocyte diversity and antigen specificity or essentially the clonality of T and B cells present in disease states. The technique of TCR/BCR is similar to scRNA-seq but utilizes a specialized switch oligo nucleotides and poly dT tail primers[9]. Untemplated C (cytosine) nucleotides to the 3′ end will pair with the end of switch oligo and reverse transcription occurs to capture the TCR/BCR sequences[9]. cDNA libraries of TCR/BCR are created and sequenced per usual protocol (Figure 1).

Single-cell assay for transposase-accessible chromatin

The modification in gene expression through epigenetics can also be studied down to a single-cell level. Through epigenetics, although the DNA sequence is unaltered, expression patterns can be affected by DNA methylation or chromatin structure. DNA chromatic accessibility is maintained by regulatory elements such as transcription factors, DNA methylation, and histone modification where DNA is wound around into nucleosomes. Single-cell assay for transposase-accessible chromatin-seq (scATAC-seq) takes advantage of the Tn5 transposase which is a bacterial enzyme primed to find and cut open DNA positions[13]. Through scATAC-seq, cells are lysed and nuclei are harvested and then undergo transposition where open DNA fragments are "cut and tagged" with adaptors[13]. Single nuclei run through a similar process as the scRNA-seq mechanism to create barcoded cDNA and sequenced and mapped to reference genome and accessible chromatic regions. These peak calling reads can then be linked to areas such as promoters and enhancers. The distribution of reads across the whole genome, functional analysis the genes associated with the peaks, and peak distribution on functional gene elements can also be done with further analysis[13] (Figure 1).

Single-cell chromatin immunoprecipitation-seq

Single-cell epigenetics in the measurement of transcription factor binding and histone modification can also be studied through single-cell chromatin immunoprecipitation-seq. Generally, cells are encapsulated and lysed within the droplets and chromatin is fragmented. DNA barcodes and chromatin fragments are merged *via* the microfluidics device[14]. DNA

barcodes are ligated to chromatin fragments and droplets are then immunoprecipitated with antibody with a carrier chromatin and library construction[14] (Figure 1).

Single-cell cellular indexing of transcriptomes and epitopes

Cell cellular indexing of transcriptomes and epitopes-seq (CITE)-seq combines traditional protein marker detection (such as flow cytometry) with scRNA-seq to provide phenotypic information such as cell-surface protein expression with transcript information[15]. Antibodies are conjugated to oligonucleotides with antibody specific barcodes using streptavidin-biotin interaction and cells are processed per scRNAseq technique[15]. The cells are then lysed and the Oligo-dT primers capture the oligonucleotides and mRNA to create cDNA[15]. cDNA is then processed into libraries and sequenced (Figure 1).

SINGLE-CELL STUDIES IN IBD

UC

Kinchen *et al*[16] published a study describing the activation of intestinal mesenchymal cells subpopulations in adult human UC and dextran-sodium sulfate colitis murine models. Through scRNA-seq, the authors identified SOX6, CD142, and WNT expressing colonic crypt mesenchymal cells consisting of fibroblasts subsets that when dysregulated can lead to impaired epithelial function and inflammation driving the UC state[16]. The human colonic epithelial layer in the UC state is again studied by Parikh *et al*[17] with further identification of various progenitor cells, colonocytes, and goblets cell also implicated in states of inflammation. The authors go on to describe a new subset of absorptive BEST4+ colonocytes cells expressing proton channel OTOP2 and uroguanylin implicated in sensing intraluminal pH and dysregulated in UC. Goblet cell expression of antiprotease molecule WFDC2 was found to be vital in bacterial defense and tight junction barrier function[17]. The study utilized inflamed human UC tissue, adjacent human UC noninflamed tissue, and healthy colonic tissue, and interestingly, the transcripts upregulated in inflamed tissue was also found to be upregulated to a lesser degree in noninflamed UC tissue implying that scRNA-seq is able to detect disease activity prior to tissue level damage[17]. Similarly, Smillie *et al*[18] described the changing landscape of inflammation in human adult UC with inflamed and noninflamed tissues. In addition, the authors describe the potential cellular players in anti-tumor necrosis factor (TNF) resistance in oncostatin M expressing monocytes and fibroblasts, and expanded on an intracellular circuitry of diseased state involving inflammatory fibroblasts, inflammatory monocytes, microfold-like cells, and CD8/interleukin (IL)17 T cells[18]. Uzzan *et al*[19] provided the first expanded adaptive immunity studies of the application of scRNA-seq in mucosal and circulating B cell and plasma cell subsets in human UC. The authors found that B cell response was dysregulated in UC through peripheral gut-homing plasmablasts that correlated with disease activity and expansion of naïve B cells and immunoglobulin (Ig)G+ plasma cells along with auto-reactive plasma cell from inflamed UC tissue[19].

Boland *et al*[20] integrated scRNA-seq with scTCR-seq and scBCR-seq to describe cellular states and clonal relationships of mucosal and peripheral adaptive immune cells in human adult UC. The authors describe an increase in IgG 1+ plasma cells and increased colonic ZEB2 transcription factor regulatory T cells in colonic tissue and gamma delta T cell subset enrichment in the peripheral blood. Of note, the authors also saw a skew in heterogeneity of CD8+ tissue-resident memory T cells in inflamed UC tissue indicating potential pathogenic role of these tissue-resident memory T cells[20]. In the same year 2020, Corridoni *et al*[21] utilized scRNA-seq, scTCR-seq, and CITE-seq to examine the repertoire and cellular subsets of tissue-resident memory CD8+ T cells in human adult UC. The study describes the heterogeneity of CD8+ T cells as both destructive expanded effector type that leads to a disease state with TNF-alpha production and post-effector type that seem to act in a more regulatory fashion through IL-26 production[21]. These studies exemplify the power and unbiased nature of single-cell technologies to understand pathogenic signatures and signify the complexity of human disease states.

CD

Martin *et al*[22] published one of the first CD scRNA-seq data sets linking anti-TNF therapy resistance with a cellular module they entitled GIMATS which stands for IgG plasma cells, inflammatory mononuclear phagocytes, activated T cells, and stromal cells. Through utilizing resected inflamed terminal ileum from adult human CD patients who did not respond to anti-TNF therapy along with uninflamed samples, the authors identified this unique cellular signature to potentially eventually develop biomarkers in prediction of therapy response[22]. Jaeger *et al*[23] further looked at the T cell composition of the terminal ileum from adult human patients with CD and resected terminal ileums, distinguishing between lamina propria and epithelial layers. Within the epithelial layer, intraepithelial lymphocytes from inflamed tissue included specific NKp30+ gamma delta T cells that expressed ROR gamma which produced IL-26 with an increased in active T helper 17 (Th17) cells[23]. The lamina propria layer also found a Th17 signature with increased CD8+ cells implicating the Th17 pathway in CD[23]. Yokoi *et al*[24] further classified T cell subsets in CD to identify CD4+ tissue-resident memory T cells that were increased in CD that expressed CD161, CCR5, and CD103 using scRNA-seq and CyTOF. Rosati *et al*[25] used both bulk TCR repertoire and scRNA-seq on peripheral blood in CD samples to identify a subpopulation of unconventional Crohn-associated invariant T (CAIT) cells with a distinctly unique TCR. These peripheral CAIT cells seem to show a gene expression similar to cells of the innate immune system and mucosal associated invariant T cells and NKT cells and seem to be present in peripheral blood in CD rather than UC[25]. Maddipatla *et al*[26] describes a pediatric CD scRNA-seq data set from treatment naïve pediatric patients, established CD patients in remission, and refractory patients. Patients in remission showed elevated apoliprotein and globlet cell trefoil factor though not in refractory Crohn's[26]. Cellular subsets of enterocyte, goblet cells and BEST4+ enterocytes, microfold and

tuft cells see, to undergo changes from treatment naïve to established CD[26].

Both UC and CD

Huang *et al*[27] describes a pediatric cohort of patients with UC, Crohn's colitis, and undefined pediatric IBD using scRNA-seq, scTCR-seq, and scBCR-seq. The authors describe a common pathway of impaired cyclic AMP-response signaling in all three pediatric cohorts along with infiltration of PDE4B-expresing and TNF-expressing macrophages within the mucosal samples. The authors also describe a decreased abundance of CD39 expressing intraepithelial T cells along with platelet aggregation and release of 5-hydroxytryptamine at the mucosal level[27]. Futhermore, they demonstrated the ability to improve clinical symptoms of some of the pediatric patients with colitis and IBD by using a drug (phosphodiesterase inhibitor dipyridamole) that targeted the pathways identified in a pilot study[27].

Mitsialis *et al*[28] also reports a study with scRNA-seq to confirm their mass cytometry (CyTOF) findings in patients with UC and CD. Within both the CD and UC cohorts, the authors found an expansion of HLA-DR+CD38+ T cells, CXCR+ plasmablasts, and IL1B+ macrophages and monocytes[28]. Expansion of IL17A+CD161+ effector memory T cells and IL17A+ T regulatory cells along with HLA-DR+CD56+ granulocytes was found within the UC cohort. Within CD, IL1B+HLA-DR+CD38+ T cells, ILB+TNF+IFNG+ naïve B cells, and IL1B+ dendritic cells, and IL1B+ plasmacytoid dendritic cells were expanded in the mucosal samples. Expanded IL1B+ T regulatory cells, IL-B+ dendritic cells, IL1B+ plasmacytoid dendritic cells, and IL1B+ monocytes were found in the peripheral blood of patients with CD but not UC [28].

FUTURE DIRECTIONS FOR SINGLE-CELL RESEARCH IN IBD

The future of single-cell technologies within gastroenterology research is already here in the ability to perform single-cell multi-omics and spatial transcriptomic on the target organ (GI tract) and peripheral blood. The ability to multiplex single-cell technologies is in development and the ability to use them on clinical samples will lead to even further growth in the field of IBD. The ability to combine transcriptomics (scRNA-seq), epitopes, (protein expression), and chromatin accessibility (scATAC-seq) from single cells was described by Swanson *et al*[29] on peripheral blood using transcriptomics, epitopes, accessibility-seq. These multimodal single-cell assays may provide a novel way to uncover and link gene expression, gene regulation, and phenotypic gene expression within a single cell and begin to fully cover genotype with phenotype in specific diseases such as IBD.

Another exciting technique that is more readily available now is spatial transcriptomics where architectural information is preserved with intact tissue[30,31]. The position of cellular location and interaction continues to be present and cell to cell interactions can be further interrogated[30,31]. Cell and tissue function can be probed to understand potential pathogenic cells and culprits of inflammation rather than bystander immune cells may be a detailed way to understand IBD. At the same time, as the GI tract is an immune active organ, spatial transcriptomics may be a way to detect the invasive pathogenic variants without losing more delicate cells in the tissue dissociation process.

SINGLE-CELL OMICS IN OTHER GI DISEASES

Given the limited scope of this minireview, we have described these technologies only in the field of IBD and did not elaborate into its use in other GI disorders such as GI cancers and other GI diseases such as allergies of the GI tract. Spatial transcriptomics and various single-cell techniques have been successfully applied to colorectal cancers using the GI cancer tissue and resections allowing researchers to better understand the tumor microenvironment to derive better chemotherapeutic targets[32-36]. Allergic disease of the GI tract such as eosinophilic esophagitis is also under interrogation using single-cell technologies[37]. The ability to study tissue level molecular changes across multiple disease is unparallel using single-cell technologies.

LIMITATIONS

The limitation to single-cell technologies is most importantly cost of reagents and cost of sequencing[38]. The ability to integrate multiple single-cell data across multiple platforms and techniques is also a challenge as there are many commercial products out in the market[38].

CONCLUSION

Single-cell techniques and omics have taken off in the last few years and the ability to detect individual cellular transcript details has revolutionized the world of research. In the field of gastroenterology in just the last five years, several single-cell techniques have been applied to IBD research with the identification of novel cellular immune players in the pathogenesis of both UC and CD. As we continue to develop further immunological techniques, we may begin to detect signals of treatment response in IBD and tailor therapies to immune signatures present in disease state.

FOOTNOTES

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MINIREVIEWS

Post-acute pancreatitis diabetes: A complication waiting for more recognition and understanding

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Abstract

Post-acute pancreatitis diabetes (PAPD) is the second most common type of diabetes below type 2 diabetes mellitus. Due to the boom in research on this entity carried out during the last decade, its recognition has increased. However, much of the medical community still does not recognize it as a medium and long-term complication of acute pancreatitis (AP). Recent prospective cohort studies show that its incidence is about 23% globally and 34.5% in patients with severe AP. With the overall increase in the incidence of AP this complication will be certainly seen more frequently. Due to its high morbidity, mortality and difficult control, early detection and treatment are essential. However, its risk factors and pathophysiological mechanisms are not clearly defined. Its diagnosis should be made excluding pre-existing diabetes and applying the criteria of the American Diabetes Association after 90 d of resolution of one or more AP episodes. This review will show the evidence published so far on the incidence and prevalence, risk factors, possible pathophysiological mechanisms, clinical outcomes, clinical characteristics and preventive and corrective management of PAPD. Some important gaps needing to be clarified in forthcoming studies will also be discussed.

Key Words: Acute pancreatitis; Diabetes mellitus; Chronic pancreatitis; Post-pancreatitis diabetes; Pancreatogenic diabetes

Core Tip: Post-acute pancreatitis diabetes (PAPD) is the second most common type of diabetes below type II diabetes mellitus. Its incidence is about 23% globally and 34.5% in severe acute pancreatitis (AP). With the overall increase in the incidence of AP this complication will also increase. Due to its high mortality, early detection and treatment are essential. Diagnosis should be made excluding pre-existing diabetes and applying the criteria of the American Diabetes Association after 90 d of resolution of AP episodes. This review will show published evidence on the incidence, risk factors, pathophysiology, clinical outcomes, clinical characteristics and preventive and corrective management of PAPD.

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INTRODUCTION

Diabetes of the exocrine pancreas (DEP) derives from the dysfunction of the exocrine component of the pancreas. It is the second most common type of diabetes after type 2 diabetes mellitus (T2DM)[1]. Its incidence has tripled in the last decade, reaching annual incidence increase of 2.8%[2]. It is associated with higher mortality compared to T2DM[3].

Despite the fact that the relationship between diabetes and diseases of the exocrine pancreas has been known for long time, there have been few advances in the knowledge of the epidemiology and pathophysiological mechanisms of the DEP, which has made it difficult to establish a clear classification of this entity[4].

Terms such as pancreatic, pancreatoprive, pancreatogenic, postpancreatectomy diabetes, and others have been used for this condition. The term "type 3c diabetes" has been attributed to the American Diabetes Association (ADA). The truth is that this nomenclature was not assigned as such to the DEP, but adopted this name because it appeared within the group "other specific types of diabetes" in subsection c of group 3 in the 1998 publication of the ADA on the classification of diabetes. In addition, the use of the term "type 3c diabetes" was not promoted in 2019 guidelines of the ADA[5]. In order to avoid more confusion, the term "diabetes of the exocrine pancreas" has been used more frequently since 2017[6-8]. There is evidence that an increase in the accumulation of intrapancreatic fat around the pancreatic islets of Langerhans may have pathophysiological role in three of the most frequent types of DEP: Post-pancreatitis diabetes (PPD), pancreatic cancer-related diabetes, and cystic fibrosis-related diabetes, but not on T2DM and T1DM[9,10]. However, each of this type of diabetes has different pathophysiology, so it is appropriate to address them separately.

On the other side, the pancreatitis giving rise to diabetes can be acute or chronic, so it is reasonable to make distinction between both: Post-acute pancreatitis diabetes (PAPD) and post-chronic pancreatitis diabetes. A simplified core classification of DEP is shown in Table 1.

Acute pancreatitis (AP) is one of the most common gastrointestinal causes of hospital admissions worldwide, accounting for more than 275000 cases per year. The global incidence rate of AP is increasing[11]. Because AP is the most common disease of the exocrine pancreas nowadays, it is probably the most common cause of DEP. However, this complication has been ignored by most physicians who care for patients recovering from AP[1]. In the last decade, epidemiological, clinical, and translational research on PAPD has boomed, and some important aspects of this pathology are now more clearly known[12,13].

In this review, the evidences published so far on the incidence, risk factors, possible pathophysiology, clinical outcomes, clinical characteristics, and management of PAPD will be discussed. However, the knowledge of a large part of these aspects is still incomplete, so some important gaps will be pointed out which will have to be clarified in future research studies.

EPIDEMIOLOGY

AP is an inflammatory disease of the exocrine pancreas whose global incidence is 34/100000 inhabitants per year, with some geographical differences[14]. This condition has mortality rate from 1 to 2/100000 person-years[15]. Biliary lithiasis, alcohol abuse, endoscopic retrograde cholangiopancreatography, hypertriglyceridemia, and some drugs are the most common causes. On the other hand, 80% of patients have mild pancreatitis associated with few complications and short hospital stay. However, up to 20% of patients may have severe or necrotizing pancreatitis, giving rise to local and systemic complications, increased mortality and long hospital stay[16].

Studies in general population have shown that an episode of AP confers at least twice the risk of subsequent diabetes compared with controls[1]. Two meta-analyses published in 2014 and 2019[17,18], (with 31 studies and 13894 adult patients with no history of DM or prediabetes), evaluated the prevalence of diabetes after one or more episodes of AP. Only 3 were case-control and 28 were non-comparative prospective cohort studies. The cumulative pooled incidence for diabetes was 23% (95%CI: 16%-31%). The diabetes incidence was higher in the populations that had severe AP than in those with mild AP (39% *vs* 14%). The case-control studies and 12 cohort studies had significant methodological shortcomings (few patients, short follow-up or deficient methods for defining diabetes). In the 16 remaining best-quality

Table 1 Classification of diabetes of exocrine pancreas

Type of diabetes	Definition
Post pancreatitis diabetes	Diabetes identified in a patient with pancreatitis without previous detection of diabetes
Post-acute pancreatitis diabetes	Diabetes identified after one or more episodes of acute pancreatitis
Post-chronic pancreatitis diabetes	Diabetes identified in a patient with diagnosis of chronic pancreatitis
Pancreatic cancer related diabetes	Diabetes associated to pancreatic cancer in a patient without history of diabetes
Cystic fibrosis related diabetes	Diabetes associated to cystic fibrosis in a patient without history of diabetes

studies, an overall incidence of PAPD of 27.8% (range 8% to 54%), and of 38.4% (range 16%-54%), only in the severe forms, was found. The cumulative incidence of diabetes reached up to 41% in studies with at least 5 years of follow-up [19-34] (Table 2). The wide range in diabetes incidence of these studies may be due to differences in methodological design, patient selection, and diabetes diagnostic methods.

The time at which diabetes appears after AP is unknown[35]. A recent prospective study, which assessed the course of glycemia over months, reported that the proportion of patients who developed diabetes after an AP episode was 3% at 6 mo, 7% at 12 mo, 9% at 18 mo, and 11% at 24 mo[36] (Figure 1).

RISK FACTORS AND PREDICTORS

Some authors have not found association between the severity of AP and the incidence of PAPD[37-39], while others have found strong relationship[28,40-43]. A higher prevalence of PAPD was found in patients with severe AP than with mild AP in the most recent meta-analysis[18]. In another study, intensive care stay during the AP episode was associated with higher risk of developing diabetes in the 2 years after discharge[44]. The differences in the prevalence of PAPD in the severe forms shown in these studies could be explained, by the different definitions of severity and the diversity of scales used for assessing severity, such as Ranson, APACHE II, BISAP, or the Atlanta classification[45]. It seems that the strongest risk factors in the development of PAPD are pancreatic necrosis and recurrent episodes of AP[40,46]. For recurrent AP, one study evaluated computed tomography evidence of pancreatic volume loss in patients with a single episode of AP compared with recurrent pancreatitis. The investigators found that total pancreatic volume was significantly reduced in those with recurrent AP and these patients also had a strong association with endocrine and exocrine insufficiency[46]. Diabetes in patients with severe and recurrent AP may be due to structural damage of the β cells of the pancreatic islets. Nevertheless, it is important to highlight that the increased risk of diabetes also in patients with mild AP (without necrosis) suggests that there could be other mechanisms involved in its pathophysiology.

Other studies have shown that advanced age and male gender are significant risk factors[47]. Additionally, the alcoholic etiology of pancreatitis seems to increase this incidence[18]. On the other hand, some parameters of metabolic dysfunction, such as obesity and dyslipidemia, could be important risk factors, including genetic factors, particularly in patients with family history of DM.

In total, PAPD risk factors have been poorly or incompletely studied, mostly in retrospective studies with unclear defining parameters. However, the definition of risk factors is important to predict the incidence of diabetes in order to adopt an effective screening strategy of diabetes in patients who recover from AP, particularly in the mild form which is the most frequently seen. Specially designed prospective studies are required in order to clear this issue.

In the other side, some biological markers with the aim of predicting the development of PAPD have been investigated. One study found that elevated plasma levels of interleukin (IL)-1β and interferon γ in individuals with AP and normal glycemia may predict the onset of de novo diabetes during follow-up[48]. Another study found that elevated basal insulin and glucagon plasma levels were associated with de novo diabetes post AP (OR: 1.99 and 3.44 respectively)[49]. Another prospective, longitudinal cohort study found that the variability of glucose plasma levels in the early stages of AP may predict the development of diabetes at 2-year follow-up[50]. Although the results of these studies may appear promising, the research on this field is still very limited and these findings need to be validated before being used in clinical practice.

PATHOPHYSIOLOGY

The pathophysiologic relationship between AP and diabetes seems to be bidirectional[51]. On the one hand, patients with T1DM and T2DM have higher risk of developing AP, as demonstrated in a meta-analysis with 5.7 million participants and 14124 cases. Patients with diabetes had higher risk of AP than individuals without diabetes (HR: 1.74)[52]. Likewise, other studies have reported that patients with diabetes develop more severe AP[42].

Despite it is accepted that AP can give rise to diabetes, the pathophysiological mechanisms are still unknown. In necrotizing AP, diabetes may be attributed to structural damage of pancreatic parenchyma. However, in the mild AP the involved mechanisms are less clear. Overall, it has been hypothesized that the pathophysiology of PAPD may be multifactorial, involving diverse mechanisms that could have effect at different levels of the glucose metabolism

Table 2 Prospective cohort studies with more than 12 mo of follow-up that determine the incidence of post-acute pancreatitis diabetes

Ref.	N	Follow-up, mo	AP grade, %	Definition of diabetes	Prediabetes, %	Diabetes, %
Johansen and Ornsholt[20], 1972	22	24	NS	OGTT	0	18
Olszewski *et al*[19], 1978	25	12	NS	OGTT, serum insulin	ND	28
Eriksson *et al*[21], 1992	36	74	S:56; M:44	OGTT	11	53
Angelini *et al*[22], 1993	118	53	S:70; M:30	OGTT	ND	8
Malecka *et al*[23], 2002	82	56	S:34; M:66	OGTT, serum insulin	2	16
Kaya *et al*[24], 2007	112	12	S:32; M:68	OGTT, fasting glucose, c-pep	24	21
Andersson *et al*[25], 2010	39	45	S:35; M:65	Serum insulin, OGTT	33	23
Garip *et al*[26], 2013	96	32	S:36; M:64	Fasting glucose, OGTT	ND	34
Vujasinovic *et al*[27], 2014	100	32	NS	Fasting glucose, OGTT	ND	14
Winter Gasparoto *et al*[28], 2015	16	38.4	NS	OGTT, HOMA	ND	31.2
Severe AP						
Doepel *et al*[29], 1993	37	74	S:100	Fasting glucose, C- pep, A1cHb, OGTT	ND	54
Yasuda *et al*[30], 2008	41	56	S:100	Fasting glucose	ND	39
Gupta *et al*[31], 2009	30	31	S:100	Fasting and postprandial glucose, OGTT, C-pep	20	20
Uomo *et al*[32], 2010	38	179	S:100	OGTT	ND	16
Chandrasekaran *et al*[33], 2015	35	26.2	S:100	OGTT	ND	48.5
Tu *et al*[34], 2017	113	42.9	S:90; M:10	OGTT A1cHb	ND	30

AP: Acute pancreatitis; NS: Not significant; ND: Non-determined; S: Severe; M: Mild and moderate; OGTT: Oral glucose tolerance test; C-pep: C-peptide; A1cHb: Glycosylated hemoglobin.

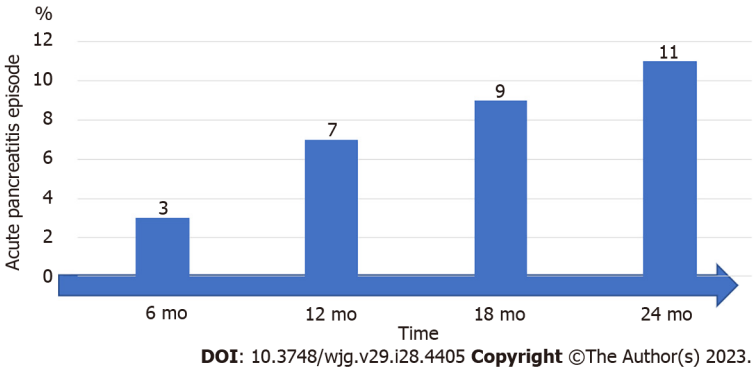

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Figure 1 Trajectory of glycemia after at least one episode of acute pancreatitis.

regulation pathways. It may be possible that one or more of these mechanisms may predominate in the different diabetes phenotypes. According to some evidence collected so far, some of the possible mechanisms are discussed in the following sections (Figure 2).

Pancreatic necrosis

This complication of AP has already been discussed previously in the above sections[28,40-42,53,54]. In a recent meta-analysis, patients who displayed pancreatic necrosis during the AP attack(s) had a higher frequency of diabetes than those without necrosis (37% *vs* 11%)[18]. In other series, the incidence exceeds 50% of the cases[29]. Notwithstanding, the relation between diabetes incidence and the extension and site of necrosis has not been completely defined. In a recent study with 109 patients with AP the incidence of de novo diabetes in patients with pancreatic necrosis demonstrated by contrasted computed tomography scan was higher (66.6%) than in those without necrosis (27.8%). However, no relationship was found between diabetes incidence and necrosis rate or site of necrosis (head, body or tail of pancreas)

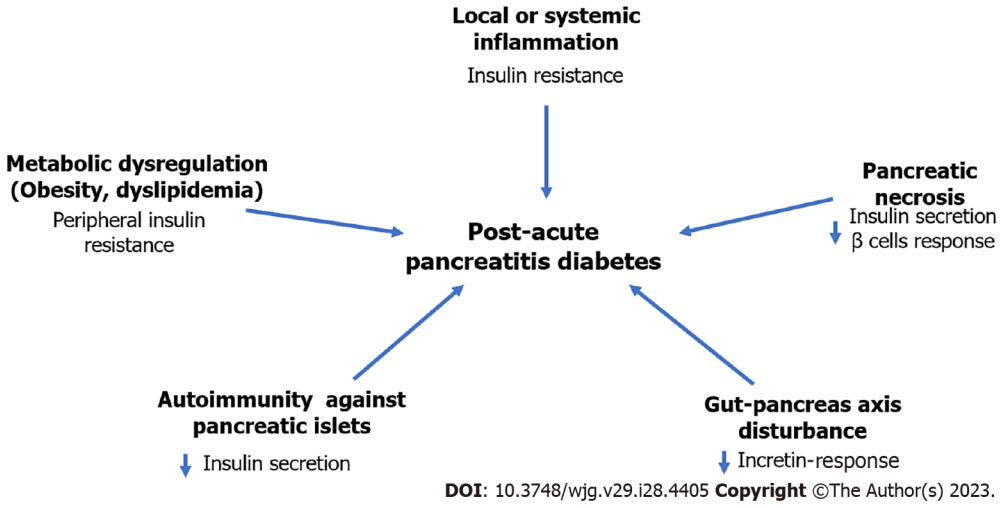

Figure 2 Possible mechanism involved in the pathophysiology of post-acute pancreatitis diabetes.

[26]. This may be explained because diabetes may be due, in addition to destruction of β cells of the pancreas, to insulin resistance. And also, because β cells are located homogeneously in the different segments of the pancreas.

Pancreatic necrosis may also induce exocrine pancreas insufficiency (EPI). From 15 to 30% of patients who have an episode of AP may have chronic pancreatitis after 3 years of follow-up[47].

Autoimmunity against β cells and other components of Langerhans islets

It has been speculated that the local and systemic inflammatory response occurring in AP patients may result in post-translational modifications of endogenous islet cell proteins, such as insulin, nucleic acids, and other proteins. Such modified neoepitopes may act as autoantigens, inducing an autoimmune response against components of the Langerhans islet[55]. To date, the frequency of autoimmunity during and after an AP episode has not been evaluated, particularly in patients who develop de novo diabetes.

Metabolic dysregulation

Obesity and hypertriglyceridemia are risk factors for the development of both T2DM and AP[56,57]. Their presence prior to AP may result in greater risk of developing diabetes and may accelerate the onset of this condition. Both factors are independently associated with increased risk of clinical severity of AP, which could explain the risk of diabetes[58,59]. In fact, hypertriglyceridemia is one of the most frequent causes of AP, only below biliary and alcoholic etiology. The impact that visceral obesity may have is unknown. However, there is evidence that increased accumulation of intrapancreatic fat around the islets of Langerhans may have pathophysiological role in acute and chronic PPD[9,10]. Insulin resistance may be the mechanism in these patients[60].

Local and systemic inflammatory response

During the course of AP, serum IL-6 levels increase as a consequence of the local and systemic inflammatory response. It has been hypothesized that this and other cytokines could favor the development of chronic hyperglycemia[61]. Multiple studies have found that the role of IL-6 on impaired glucose metabolism is primarily through insulin resistance[62,63].

Disturbance of the gut-pancreas axis

It has been suggested that of some of the pancreatic and intestine functional interconnections involved in the digestion, absorption, and utilization of nutrients which regulate glucose homeostasis may be disturbed in PAPD[53]. This assertion is based on the fact that 15% to 30% of patients who have AP show chronic pancreatitis 3 years after the acute episode[47,64]. The most common endocrine dysfunction results from decreased levels of insulin, glucagon, and pancreatic polypeptide[65]. Impaired secretion of enteral glucoregulatory hormones (incretins secreted by intestinal epithelium cells), such as glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) may also be observed. These peptides are associated with increased insulin secretion, glucagon secretion modulation and reduction of hepatic and peripheral insulin resistance[66]. It is important to underline that the reduction of glucagon secretion is the cause of severe hypoglycemia due to the loss of its counter-regulatory effect[67]. Therefore, the combined hormonal abnormalities that occur in PAPD result in a severely disrupted endocrine environment that is different from the pattern of abnormalities seen in T1DM or T2DM. Hence the need to understand and recognize these entities separately.

In summary, it is essential to determine the relative contribution of each of these pathophysiological mechanisms involved in PAPD, as well as their interrelationship with the possible genetic predispositions. In order to clarify this important issue, both clinical and basic studies in experimental models of PAPD are needed due to the difficulty in obtaining tissue samples from human beings.

DIAGNOSIS

For stablishing the diagnosis of PAPD it is important to rule out preexisting diabetes (mainly T2DM) which sometimes is difficult. The diagnostic criteria for PAPD proposed some years ago that included the demonstration of EPI as well as the absence of autoantibodies directed against β cells of the pancreas are not currently valid[68,69] because only 30% of patients show evidence of EPI 3 years after AP[64,70]. In addition, patients who develop EPI have increased risk of diabetes[71], so it should be considered as a risk factor and not as its defining characteristic. On the other hand, early and intermediate stages of EPI are difficult to demonstrate as functional tests and sophisticated imaging techniques are often not available in real life practice and are costly. In total, the term PAPD should be reserved specifically to de novo diabetes in individuals after AP with or without morphological or functional evidence of chronic pancreatitis and without the need to demonstrate the absence of anti-β cell antibodies.

The diagnosis of diabetes should be established based on the criteria recommended by the ADA: Glycosylated hemoglobin (HbA1c) ≥ 48 mm/moL or 6.5% and/or fasting glucose > 7 mmmol/L or 126 mg/dL[72], which must be performed more than 90 d after AP resolution. This is important because HbA1c levels reflect the mean plasma glucose concentration in the previous 8-12 wk and also due to the stress hyperglycemia that can occur before this period. It has been shown that the oral glucose tolerance test is better for detecting early-stage diabetes as it is not affected by stress and does not require 90 d to give reliable results.

Some clinical characteristics and biochemical markers can help to differentiate PAPD from T2DM. PAPD has greater glycemic variability and more difficult control, showing frequent hypoglycemic episodes and more insulin requirements [73]. From the biochemical point of view, PAPD patients have lower baseline and stimulated levels of insulin, glucagon and C-peptide[1]. Besides, pre and postprandial serum levels of oxyntomodulin (an intestinal peptide derived from proglucagon that participates in the regulation of the pancreatic exocrine function), have been found to be significantly higher in patients with PAPD compared to T2DM and healthy controls. This opens the possibility of being used as a specific biomarker[74]. For some, the presence of diagnostic autoimmune markers for T1DM (*i.e.*, islet cell antibodies or antibodies against glutamic acid decarboxylase, insulin, tyrosine phosphatase-like proteins, or zinc transporter)[75], rules out the diagnosis of PAPD. However, as already mentioned, an autoimmune component triggered by neoepitopes induced by the systemic inflammatory response of AP at the level of the β cells has not been ruled out as patho-physiological mechanism of PAPD in some patients[55]. Finally, it is also important to identify overlapping causes of DEP such as pancreatic surgery, cystic fibrosis, toxic pancreatic medications, hemochromatosis, and pancreatic cancer.

In summary, the diagnosis of PAPD should be based on the exclusion of any type of preexisting diabetes before AP and on identification of diabetes ninety days after AP based on the ADA criteria. For now, screening for PAPD should be performed in all patients who have had at least one episode of AP. The knowledge of the risk factors will contribute to selecting patients for screening in the future. Although there is no consensus on the frequency of screening, it is recommended to be carried out every 6 mo the first year and every year thereafter. Figure 3 shows a simplified PAPD diagnostic algorithm.

COMPLICATIONS

It has been demonstrated that PAPD has higher short- and long-term morbidity and mortality than T2DM[3]. A recent population-based study of 139843 individuals showed that those with PAPD had significantly higher risk of pancreatic cancer than those with T2DM or those with no history of pancreatitis (adjusted RR: 6.94; $P < 0.05$)[76]. It is important to underline that in patients recovering from AP, diabetes may be the clinical manifestation of pancreatic cancer, so early detection strategies for these neoplasms should be applied. Another recent study with 10549 individuals showed that patients with PAPD, compared to T2DM, had higher all-cause mortality (RR: 1.13), cancer (RR: 1.14), infections (RR: 2.52), and gastrointestinal disease (RR: 2.56). Likewise, the risk of rehospitalizations was significantly higher, which represented greater economic burden[77].

MANAGEMENT

The data so far available regarding the treatment of PAPD are very scarce, however, some rationale may be useful to guide treatment decisions with the understanding that refinement will be required based on the results of well-conducted future therapeutic studies. The management of PAPD should ideally be preventive and corrective.

Preventive management aims to reduce the incidence of diabetes which would be achieved if the risk factors and predictive clinical and biochemical markers were clearly known. It seems that pancreatic necrosis and recurrent episodes of AP are the most solid risk factors. Possibly in patients with these complications a more aggressive and earlier management of AP, the performance of early cholecystectomy in biliary AP and stopping alcohol consumption could have some beneficial impact. In this context, well-conducted studies are required in order to demonstrate this issue.

For corrective management it is suggested to apply the ADA recommendations for the treatment of T2DM and T1DM with some nuances[78]. It is important to be aware of the fragile stability of glycemia of these patients. This leads that a large part of patients be treated with insulin. In a large population-based study, higher proportion of patients with PAPD were already on insulin therapy within 5 years compared to T2DM (20.9% *vs* 4.1% respectively), and had poorer glycemic control (defined as HBA1c ≥ 7%)[1].

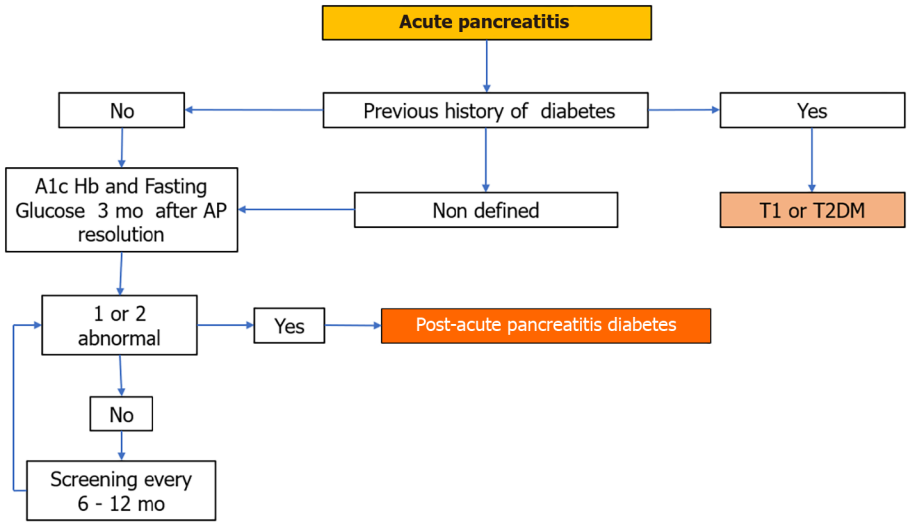

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Figure 3 Simplified algorithm for the diagnosis of post-acute pancreatitis diabetes. AP: Acute pancreatitis; T2DM: Type 2 diabetes mellitus.

It is important to remember that about 30% of patients with AP develop EPI[70]. At this point, one study reported increased postprandial responses of GLP-1 and GIP in patients with chronic pancreatitis and EPI following pancreatic enzyme substitution (PES). Concurrently, both plasma insulin, plasma C-peptide, and total insulin secretion increased after PES. These results suggest that secretion of GLP-1 and GIP is under influence of the digestion and absorption of nutrients in the small intestine and that PES increased insulin secretion[66]. Concomitant improvement of glycemic control was not assessed in diabetic patients from this study. As a result of these findings, the assessment of therapeutic effects of PES from the early stages of diabetes development may be warranted.

Finally, the management of PAPD is complex and requires a common approach, preferably by a medical team that includes gastroenterologists, endocrinologists, primary care physicians, nutritionists, and behavioral health specialists.

CONCLUSION

PAPD is currently the second most common type of diabetes. It is increasingly known as a result of the recently published research around this entity. However, much of the medical community still ignores its existence. With the increasing global incidence of AP, the frequency of this type of diabetes will certainly increase.

Due to its high morbidity, mortality and difficult treatment, its recognition as a complication of AP is of paramount importance. Pancreatic necrosis and recurrence seem to be the strongest risk factors. Its pathophysiological mechanisms and other risk factors are not yet clearly known. The diagnosis should be based on the exclusion of any type of preexisting diabetes and on identification of diabetes ninety days after AP based on the ADA criteria. Screening for diabetes should be performed in all patients who have had at least one episode of AP. Management is not yet standardized.

It was recently announced the launch of a multicenter clinical study designed to understand the frequency and phenotypes of this type of diabetes. This study has been called Diabetes RElated to Acute Pancreatitis and its Mechanisms and is supported by The National Institute of Diabetes and Digestive and Kidney Diseases[79]. In this research project, it is planned to study risk factors and some of the mechanisms possibly involved in the pathophysiology of PAPD[80-82].

Certainly, the results of this and other similar forthcoming studies will contribute to the clarification of some important gaps that still persist in the knowledge of PAPD making possible a more effective screening and better preventive and corrective management.

FOOTNOTES

Author contributions: García-Compeán D, Jiménez-Rodríguez AR, and Muñoz-Ayala JM made a bibliographic research and wrote sections of the manuscript; Villarreal-Pérez JZ, González-González JA, and Maldonado-Garza HJ critically reviewed the manuscript; García-Compeán D conceived and coordinated the whole project.

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γ-aminobutyric acid B2 receptor: A potential therapeutic target for cholangiocarcinoma in patients with diabetes mellitus

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Abstract

BACKGROUND

The association between diabetes mellitus (DM) and the increased risk and progression of cholangiocarcinoma (CCA) has been reported with unclear underlying mechanisms. Previous studies showed that γ-aminobutyric acid (GABA) B2 receptor (GABBR2) was upregulated in CCA cells cultured in high glucose (HG) conditions. Roles of GABA receptors in CCA progression have also been studied, but their association with DM and hyperglycemia in CCA remains unclarified.

AIM

To investigate the effects of hyperglycemia on GABBR2 expression and the potential use of GABBR2 as a CCA therapeutic target.

METHODS

CCA cells, KKU-055 and KKU-213A, were cultured in Dulbecco Modified Eagle's Medium supplemented with 5.6 mmol/L (normal glucose, NG) or 25 mmol/L (HG) glucose and assigned as NG and HG cells, respectively. GABBR2 expression in NG and HG cells was investigated using real-time quantitative polymerase chain reaction and western blot. Expression and localization of GABBR2 in CCA cells were determined using immunocytofluorescence. GABBR2 expression in tumor tissues from CCA patients with and without DM was studied using immunohistochemistry, and the correlations of GABBR2 with the clinicopathological characteristics of patients were analyzed using univariate analysis. Effects of baclofen, a GABA-B receptor agonist, on CCA cell proliferation and clonogenicity were tested using the MTT and clonogenic assays. Phospho-kinases arrays were used to screen the affected signaling pathways after baclofen treatment, and the candidate signaling molecules were validated using the public transcriptomic data and western blot.

RESULTS

GABBR2 expression in CCA cells was induced by HG in a dose- and time-dependent manner. CCA tissues from patients with DM and hyperglycemia also showed a significantly higher GABBR2 expression compared with tumor tissues from those with euglycemia ($P < 0.01$). High GABBR2 expression was significantly associated with a poorer non-papillary histological subtype but with smaller sizes of CCA tumors ($P < 0.05$). HG cells of both tested CCA cell lines were more sensitive to baclofen treatment. Baclofen significantly suppressed the proliferation and clonogenicity of CCA cells in both NG and HG conditions ($P < 0.05$). Phospho-kinase arrays suggested glycogen synthase kinase 3 (GSK3), β-catenin, and the signal transducer and activator of transcription 3 (STAT3) as candidate signaling molecules under the regulation of GABBR2, which were verified in NG and HG cells of the individual CCA cell lines. Cyclin D1 and c-Myc, the common downstream targets of GSK3/β-catenin and STAT3 involving cell proliferation, were accordingly downregulated after baclofen treatment.

CONCLUSION

GABBR2 is upregulated by HG and holds a promising role as a therapeutic target for CCA regardless of the glucose condition.

Key Words: Baclofen; Cholangiocarcinoma; Diabetes mellitus; Drug repurposing; Hyperglycemia; Gamma-aminobutyric acid

Core Tip: Diabetes mellitus is associated with an increased risk and progression of cholangiocarcinoma (CCA). The γ-aminobutyric acid (GABA) B2 receptor (GABBR2) was upregulated in CCA cells cultured in high glucose and in CCA tissues from patients with hyperglycemia. High GABBR2 expressions were significantly correlated with a non-papillary histotype and smaller sizes of CCA tumors. The treatment of baclofen, a GABA-B receptor agonist, significantly suppressed CCA cell proliferation and clonogenicity, suggesting that GABBR2 is a potential target for CCA treatment. Baclofen inhibited multiple kinases and signal transducers in CCA, resulting in downregulated downstream target proteins involving cell proliferation and suppression of CCA cell growth.

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INTRODUCTION

Diabetes mellitus (DM) is a global public health problem, and its incidence is increasing every year[1]. The associations between DM and cancers have been recognized for decades from epidemiological observations[2,3]; however, the molecular mechanisms underlying the linkage are not fully elucidated. Liver cancers, including hepatocellular carcinoma and cholangiocarcinoma (CCA), are among the high-risk malignancies developing in patients with DM[4-6]. DM is not only associated with an increased risk of CCA but also associated with a poor survival of patients who have a poorly controlled blood glucose level[7]. Individuals with DM infected with *Opisthorchis viverrine* (*O. viverrini*), a carcinogenic liver fluke, are at a greater risk of developing CCA compared to those either with liver fluke infection or having DM alone[8]. The hamster CCA carcinogenesis model also showed that hamsters with DM and *O. viverrini* infection had more hepatic morbidities compared with those infected with *O. viverrini* or having DM alone[9]. CCA once developed, itself, has a poor prognosis, and the 5-year survival rate of all types is approximately 10%[10]. The attempts to investigate novel medications, *e.g.*, targeted therapy[11] or immunotherapy[12-14], reported limited efficacy or benefit for a particular population. Other underlying factors of patients have been reported to influence the progression of CCA. In line with this, the study in patients with CCA who have DM also reported that DM is associated with a shorter overall survival and is an independent prognostic factor for patients with CCA[7].

Previous studies by the current authors found that diabetogenic glucose concentration is a promoting factor for CCA aggressiveness[15,16]. High glucose (HG) promotes CCA proliferation and metastatic potential by regulating several signaling pathways, such as the signal transducer and activator of transcription 3 (STAT3)[15,17] and the nuclear factor-kappa B pathways[18]. HG also promotes cell cycle progression by enhancing cell cycle machinery proteins, namely, cyclin D1, cyclin E, cyclin A, and cyclin-dependent kinase 2, suggesting potential targets for CCA treatment in patients with DM[19]. Transcriptomic analysis of CCA cells cultured in HG *vs* normal glucose (NG) also showed several potential targets for CCA under diabetogenic glucose conditions. Among the top 5 upregulated genes, γ-aminobutyric acid (GABA) B2 receptor (GABBR2) is one of the promising therapeutic targets that are striking[18]. Given that GABBR2 is upregulated in CCA cells cultured in HG, and both agonist and antagonist of this receptor are clinically available, GABBR2 is thus a great potential target for drug repurposing for CCA treatment.

GABBR2 is a G-protein coupled receptor for GABA, an inhibitory neurotransmitter abundantly found in the central nervous system[20-22]. GABBR2 is also expressed in the gastrointestinal tract and is speculated to be important for the differentiation of several gastrointestinal epithelial cells[23,24], including cholangiocytes[25]. The roles of GABBR2 outside the central nervous system, however, are not fully understood. As a G-protein coupled receptor, GABBR2 can interact with various adaptor proteins and then signal the different downstream pathways[23]. Previous studies in CCA tissues showed that only the GABA-B receptor was differentially expressed between CCA cells and adjacent normal cholangiocytes among all subtypes of receptors (GABA-A, GABA-B, and GABA-C receptors)[26], suggesting its potential as a therapeutic target. Treatment of CCA cells with GABA showed the suppression of growth and invasive ability[26-28]. Which GABA-B receptor subtypes are responsible for the suppression of CCA aggressiveness, however, remains unclear. In contrast, in some cancers, GABA exerted pro-tumor effects on cancer cells[29], and activating the GABA-B receptor can promote the migration of cancer cells[30]. These controversial findings and the finding that HG enhanced the expression of GABBR2 in CCA cells have led to the investigation of the roles of GABBR2 in CCA under diabetic conditions. Discovering the significance of GABBR2 in CCA, especially in patients with DM, might suggest the opportunities to improve the therapeutic outcome of CCA by repurposing GABA-B receptor agonists available in clinical practice.

MATERIALS AND METHODS

CCA tissues and clinicopathological data

CCA tissues were obtained from patients who underwent surgical resection at Srinagarind Hospital, Faculty of Medicine, and archived at the Cholangiocarcinoma Research Institute, Khon Kaen University. Clinical and pathological data, including preoperative fasting blood glucose (FBG) and diabetes status, were retrieved from the medical records of Srinagarind Hospital. Patients with DM and preoperative FBG ≥ 126 mg/dL were defined as having DM, while those who had FBG < 126 mg/dL were defined as having no DM[31]. Written informed consent was obtained from all participants. The protocol of the study has been reviewed and approved by the Khon Kaen University Ethics Committee for Human Research (approval No. HE641441), based on the Declaration of Helsinki and the ICH-Good Clinical Practice Guidelines.

CCA cell lines and cell culture

CCA cell lines, KKU-055 and KKU-213A, were established from Thai CCA patients[32] and obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Cells were cultured in Dulbecco Modified Eagle's Medium (DMEM, Gibco, Carlsbad, CA), supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotic-antimycotic (Gibco). Cells were cultured in DMEM containing 5.6 mmol/L glucose (NG) and 25 mmol/L (HG) for at least 5 passages and assigned as NG and HG cells, respectively[15,18]. Cells were subcultured when the confluence reached 80%. HG-induced GABBR2 expression was done by sequentially culturing NG cells in DMEM with 5.6, 15, and 25 mmol/L glucose for at least 5 passages, and cell lysates were collected timely for the experiments.

Reverse transcription-quantitative polymerase chain reaction

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to determine GABBR2 mRNA expression in CCA cells. Confluent CCA cells were lysed using TRIzol (ThermoFisher Scientific, Waltham, MA), and RNA extraction was performed according to the manufacturer recommendations. Total RNA was reverse-transcribed using the MultiScribe Reverse Transcriptase Kit (ThermoFisher Scientific) and qPCR was performed using LightCycler® 480 System (Roche Diagnostics, Rotkreuz, Switzerland). Primers for GABBR2 are: Forward, 5′-TGGAGGCGTCT-GTCCATCCGT-3′ and reverse, 5′-GTCTTGCGTCAGCGTGCCCA-3′. β2-microglobulin was used as the internal control, and the primer sequences are: Forward, 5′-AAGATGAGTATGCCTGCCG-3′ and reverse, 5′-CGGCATCTTCAAACCTCC-3′.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot analysis

Primary antibodies used to detect the proteins by western blot were: GABBR2 (1:500, Proteintech, Rosemont, IL), pSTAT3 (Y705) (1:500, Cell Signaling Technology, Danvers, MA), pSTAT3 (S727) (1:500, Cell Signaling Technology), STAT3 (1:1000, Cell Signaling Technology), p-glycogen synthase kinase 3 (GSK3)α/β (1:1000, Cell Signaling Technology), GSK3α /β (1:1000, Cell Signaling Technology), β-catenin (1:1000, Cell Signaling Technology), cyclin D1 (1:1000, Cell Signaling Technology), c-Myc (1:500, Santa Cruz Biotechnology, Dallas, TX), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:10000, Millipore Sigma, Burlington, MA).

Cell lysates were collected in radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitor (Sigma Aldrich, St. Louis, MO) and phosphatase inhibitor (Sigma Aldrich, St. Louis, MO). NG and HG cells of both cell lines were lysed after culture in their respective media at 72 h after incubation for determination of GABBR2 expression. Cells treated with baclofen (Sigma) were collected in the lysates at 24, 48, and 72 h after incubation with the drug. Total proteins (20 μg/well) were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Amersham-Hybond® polyvinylidene fluoride membrane (GE Healthcare, Buckinghamshire, United Kingdom). After blocking with 5% skim milk in Tris-buffered saline with 0.1% Tween-20 for 1 h at room temperature, the membranes were then incubated with the primary antibodies at 4 °C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Enhanced Chemiluminescence kit (Millipore Sigma, Burlington, MA) was applied to develop the signals, which were detected using ImageQuant® LAS 400 (GE Healthcare, Uppsala, Sweden). Band intensities of proteins were quantified using Image J (National Institute of Health, Bethesda, MD), in which the intensities of GAPDH were used as the loading controls.

Immunocytofluorescence

CCA (NG and HG) cells were seeded into 48 well plates at a density of 5000 cells/well and incubated for 72 h. After removing media and washing with phosphate-buffered saline, cells were fixed using 4% paraformaldehyde for 30 min at room temperature, and non-specific antigens were blocked using 5% skim milk. Anti-GABBR2 antibody (1:200) was then incubated at 4 °C overnight, and Alexa-Fluo-488 conjugated secondary antibody (Invitrogen, Waltham, MA) was applied and incubated at room temperature for 1 h. Fluorescent signals were detected using a fluorescent microscope (Nikon Ti-U Inverted Fluorescence Microscope, 20X) using NIS-Elements imaging software. Mean fluorescent intensities were calculated using Image J (National Institute of Health).

Immunohistochemistry

Immunohistochemical staining for GABBR2 was performed according to the standard immunohistochemistry protocol. Briefly, paraffin-embedded formalin-fixed CCA tissues (6 μm thickness) were rehydrated using serial-graded ethanol. Antigens were retrieved by heating the sample in 0.1 M citrate buffer (pH = 6.0) in a pressure cooker for 5 min. Endogenous peroxidase was blocked using 3% H_2O_2 in methanol, and non-specific antigens were blocked using 1% fetal bovine serum (Gibco). CCA tissues were then incubated with anti-GABBR2 antibody (1:100, Abcam, Cambridge, MA) at room temperature overnight in a moisture chamber. In the DAKO EnVision™ + System, horseradish peroxidase-conjugated secondary antibody (Dako, Carpinteria, Denmark) was then applied and further incubated at room temperature for 1 h. The signals were developed using 3,3′-diaminobenzidine (DAB, Sigma, St. Louise, MO) and graded as the IHC index (intensity × frequency; where intensity: No staining = 0, mild = 1, moderate = 2, strong =3; and frequency: 0% = 0, 0%-25% = 1+, 25%-50% = 2+, > 50% = 3+), by two researchers (CS and SaS). Microscopic photographs were obtained using Nikon NIS-Elements software (Nikon, Tokyo, Japan). High and low expression of GABBR2 in CCA tissues was classified by using a median score of IHC index as a cut-off point.

Baclofen treatment and proliferation assay

CCA (NG and HG) cells (1 x 10^3 cells/well) were seeded into 96 well plates and incubated overnight, and then cells were incubated with complete media containing varied concentrations of baclofen (Sigma), a GABA-B receptor agonist, for 72 h. Then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Invitrogen, Carlsbad, CA) was added at a final concentration of 0.5 mg/mL and further incubated for 4 h in a 37 °C incubator. Formazan crystals were dissolved in dimethyl sulfoxide, and OD540 was measured using a microplate reader (Tecan, Männedorf, Switzerland).

To determine the possible time-dependent effect of the GABA-B receptor agonist, NG and HG cells of KKU-055 were incubated with 1000 μM baclofen, and KKU-213A cells were incubated with 800 μM baclofen for 24, 48, and 72 h. MTT assay was then performed at each time point to determine the effect of baclofen on CCA cell growth in different glucose conditions.

Clonogenic assay

NG and HG cells of KKU-055 and KKU-213A in control media or media containing 800 μM baclofen were seeded into a 24-well plate at a density of 200 cells/well. Cells were allowed to grow and form the colonies for 7 d before being fixed with 4% paraformaldehyde. The colonies were stained with 0.5% crystal violet and counted under a light microscope. Only a cluster with > 50 cells was counted as a colony.

Phospho-kinase arrays

The Proteome Profiler Human Phospho-Kinase Array Kit (#ARY003B, R&D system, Minneapolis, MN) was used to screen the signaling pathways affected by baclofen. HG cells (5×10^5 cells) of KKU-213A were seeded into 6-cm dishes and allowed to adhere overnight. Then, cells were incubated with 800 μM baclofen for 24 h. Cell lysates (control and treatment) were collected using RIPA buffer containing protease and phosphatase inhibitors. Then, 200 μg of total proteins for each group were incubated with the membrane arrays overnight following the recommendation of the manufacturer. The membranes were then incubated with a cocktail-detection antibody and streptavidin-horseradish peroxidase. The signals were detected with Chemireagent provided in the same kit and quantified using an ImageQuant® LAS 400 (GE Healthcare). The candidate pathways were then selected and verified in individual cell lines cultured in different glucose conditions by western blot.

Transcriptomic analysis

The GEO datasets were retrieved using GEO2R[33]. The GSE89749 (n = 120)[34] dataset of Thai CCA cases was used for the analysis of the correlation between the expression levels of GABBR2, STAT3, GSK3B, CTNNB1, MYC, and CCND1 using Pearson's correlation coefficient.

Statistical analysis

All quantitative data, presented as the mean ± SD, were analyzed using student's t-test, one-way ANOVA, or two-way ANOVA with Tukey's multiple comparisons using IBM SPSS Statistics for Windows, version 28.0 (IBM, Armonk, NY). Univariate analysis was performed using the Chi-square test or Fisher's Exact test regarding the assumption of each model. Data visualization was done using GraphPad Prism 9 (Dotmatics, San Diego, CA). Statistical significance was considered at $P < 0.05$.

RESULTS

HG induces expression of GABBR2 in CCA cell lines

From the previous comparative transcriptomic analysis, GABBR2 is one of the top 5 upregulated genes in KKU-213A cells cultured in HG compared to those cultured in NG conditions[18]. To verify the results from the transcriptomics, determination of GABBR2 expression in different CCA cell lines was performed. GABBR2 expression was dose-dependently upregulated by various glucose concentrations ranging from 5.6 mmol/L to 25 mmol/L at both mRNA (Figure 1A) and protein levels (Figures 1B and C) in both KKU-055 and KKU-213A cells. As the expression of GABBR2 in both CCA cell lines was significantly different between those cultured in 5.6 mmol/L and 25 mmol/L ($P < 0.05$), these two glucose conditions were included in a further study and assigned as NG and HG cells, respectively.

Immunocytofluorescent staining for GABBR2 in both cell lines showed that GABBR2 was localized at membranous and cytoplasmic compartments of CCA cells (Figure 1D). Mean fluorescent intensities of GABBR2 in HG cells of both cell lines were significantly higher than those in NG cells ($P < 0.05$) (Figure 1E) and were concordant with the expression levels determined by RT-qPCR and western blot.

Hyperglycemia induces upregulation of GABBR2 in tumor tissues from CCA patients

To affirm that HG-induced GABBR2 expression in CCA cell lines is translatable to patients with CCA, the tumor tissues from patients with pre-operative hyperglycemia (FBG ≥ 126 mg/dL) and euglycemia (FBG < 126 mg/dL) were studied. Immunohistochemistry of CCA tissues revealed that GABBR2 was significantly upregulated in tumor tissues from patients with DM who had hyperglycemia compared to those without DM ($P < 0.01$) (Figures 1F-G). Cytoplasmic localization of GABBR2 was observed similarly to that found in CCA cell lines.

High GABBR2 expression is associated with a poor histological type but smaller sizes of CCA tumors

To investigate the clinical significance of GABBR2 expression, the associations between GABBR2 expression in CCA tissues and the clinicopathological characteristics of patients were analyzed by univariate analysis. High GABBR2 expression was significantly associated with a non-papillary histological type of CCA ($P < 0.05$). In contrast, high GABBR2 expression was significantly associated with CCA tumor sizes smaller than 7 cm in the longest diameter ($P < 0.05$) (Table 1), suggesting that GABBR2 is a potential target for CCA treatment.

HG cells of CCA are more sensitive to a GABA-B receptor agonist than NG cells

As GABBR2 was upregulated in HG cells and CCA tissues from patients with hyperglycemia, the therapeutic potential of using GABBR2 as a target was investigated. Baclofen, a GABA-B receptor agonist, was applied in NG and HG cells of both CCA cell lines with various concentrations and time points. The treatment of baclofen did not alter the expression levels of GABBR2 in both NG and HG cells (data not shown). The proliferation of both NG and HG cells of KKU-055 and

Table 1 Univariate analysis of γ-aminobutyric acid B2 receptor expression and clinicopathological characteristics of cholangiocarcinoma patients

Clinicopathological data	GABBR2 expression		*P* value
	High (IHC index ≥ 8)	Low (IHC index < 8)	
Diabetic status			
DM	13	2	0.109
Non-DM	8	7	
Sex			
Male	10	6	0.580
Female	8	3	
Survival			
≥ 5 mo	14	6	1.000
< 5 mo	7	3	
Histological type			
Papillary	4	6	0.030[a]
Non-papillary	17	3	
Histological grading			
Well differentiated	13	7	0.672
Moderately differentiated	5	1	
Poorly differentiated	3	1	
Tumor size (longest diameter)			
> 7 cm	6	7	0.020[a]
≤ 7 cm	15	2	
Regional lymph node involvement			
Yes	10	8	0.543
No	8	4	
Distant metastasis			
Metastasis	14	4	0.418
Non-metastasis	7	5	

[a]$P < 0.05$.
GABBR2: γ-aminobutyric acid B2 receptor.

KKU-213A was significantly suppressed in a dose- and time-dependent manner, compared with the control group (Figures 2A and B). HG cells of both cell lines showed a significantly higher sensitivity to baclofen treatment at every dosage of baclofen and at 48 and 72 h of the incubation (Figures 2A and B).

Baclofen suppresses the colony formation of CCA cells

Baclofen treatment not only suppressed the proliferation of CCA cells, but the ability to form colonies was also inhibited. Clonogenicity of both NG and HG cells of KKU-055 and KKU-213A was significantly inhibited by baclofen treatment compared with the control group (Figures 2C and D).

Baclofen inhibits multiple kinases and signal transducers in CCA cells

To unveil the underlying mechanisms in which baclofen inhibits the proliferation of CCA cells, the phospho-kinase arrays were used to screen HG cells of KKU-213A with and without baclofen treatment. Phosphorylation of several kinases and signal transducers was suppressed in KKU-213A-HG after being treated with baclofen (Figures 3A and B). The kinases and signal transducers belonging to the same pathways and reported for their roles in CCA progression were prioritized and included for further verification in downstream experiments. By these criteria, GSK3α/β, β-catenin, and STAT3 were then included for validation in the individual CCA cell lines in different glucose conditions.

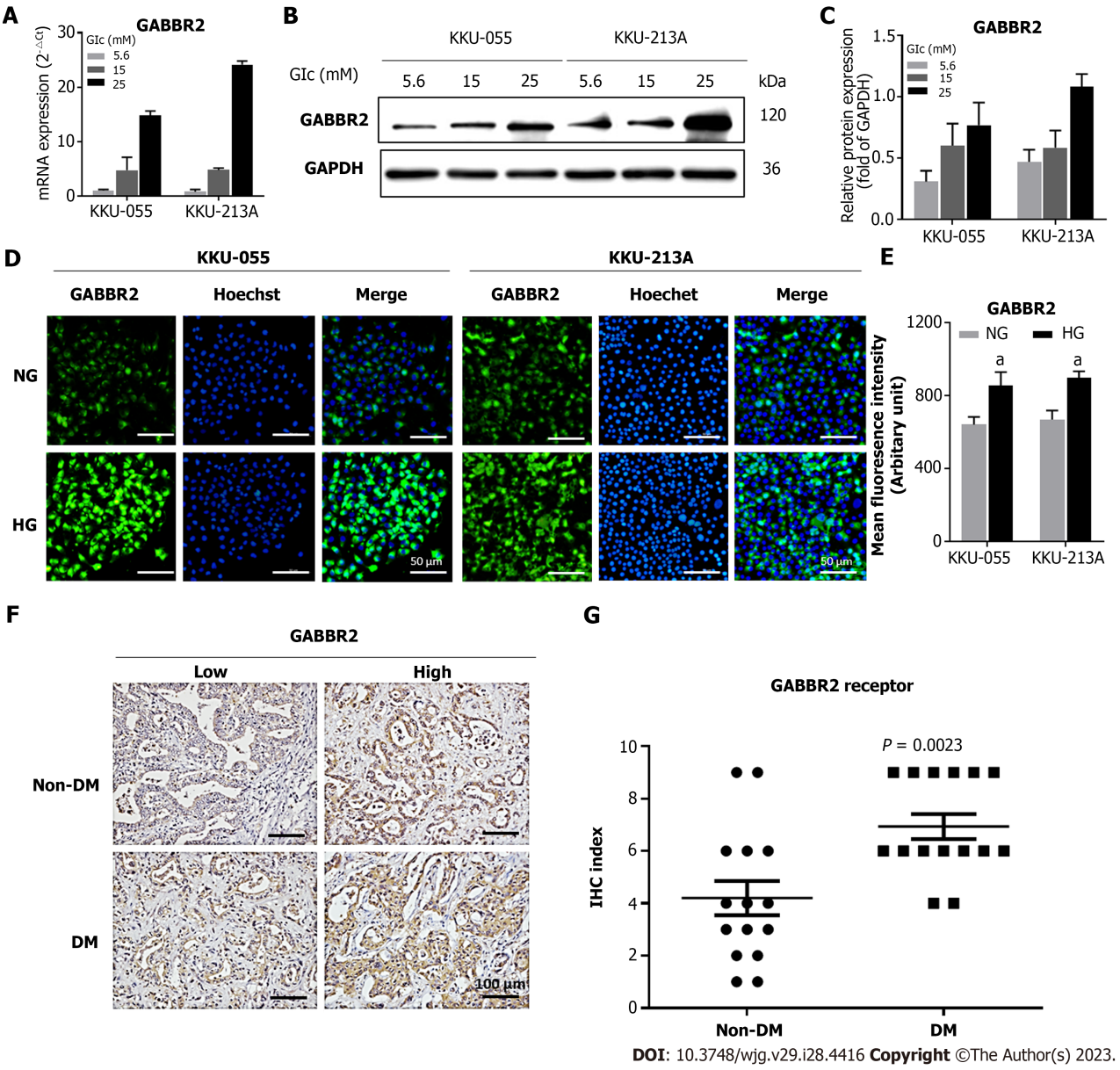

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Figure 1 High glucose induces upregulation of γ-aminobutyric acid B2 receptor in cholangiocarcinoma. A-C: The expression of γ-aminobutyric acid B2 receptor (GABBR2) in cholangiocarcinoma (CCA) cell lines, KKU-055 and KKU-213A, is dose-dependently upregulated by glucose at both RNA (A) and protein (B-C) levels; D and E: Immunocytofluorescence shows the membranous and cytoplasmic localizations of GABBR2 in CCA cells, and those cultured in high glucose show significantly higher mean fluorescent intensities; F-G: GABBR2 in tumor tissues from CCA patients with hyperglycemia is also upregulated compared with those from patients without diabetes mellitus. Presented data are the average of three biological replications with triplicated experiments. NG: Normal glucose; HG: High glucose; Glc: Glucose; DM: Diabetes mellitus; GABBR2: γ-aminobutyric acid B2 receptor; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Expression of GABBR2 significantly correlates with STAT3 expression

The associations between GABBR2 and its signal transducers suggested by the phospho-kinase arrays were first verified for their mRNA expression using public transcriptomic databases. The expression of *GABBR2* at the RNA level was significantly correlated with the expression of *STAT3* in a transcriptomic analysis of clinical CCA samples from Thai patients ($P < 0.05$) (Figure 3C).

Baclofen suppresses STAT3 and GSK3/β-catenin signaling pathways

The effects of baclofen on the STAT3 and GSK3α/β/β-catenin signaling pathways were then validated in the individual CCA cell lines, both in NG and HG cells. Baclofen suppressed STAT3 phosphorylation at both Y705 and S272 positions and NG cells were affected the most in both cell lines. Phosphorylation of GSK3α/β was also decreased time-dependently in HG cells, and the levels of β-catenin were then accordingly decreased in both cell lines. The GSK3α/β/β-catenin signaling pathway was also inhibited in NG cells of both cell lines as shown by the decreased phosphorylation levels of GSK3α/β. Total β-catenin proteins, the GSK3α/β downstream target, were also decreased starting 24 h after treatment (Figures 4A and B).

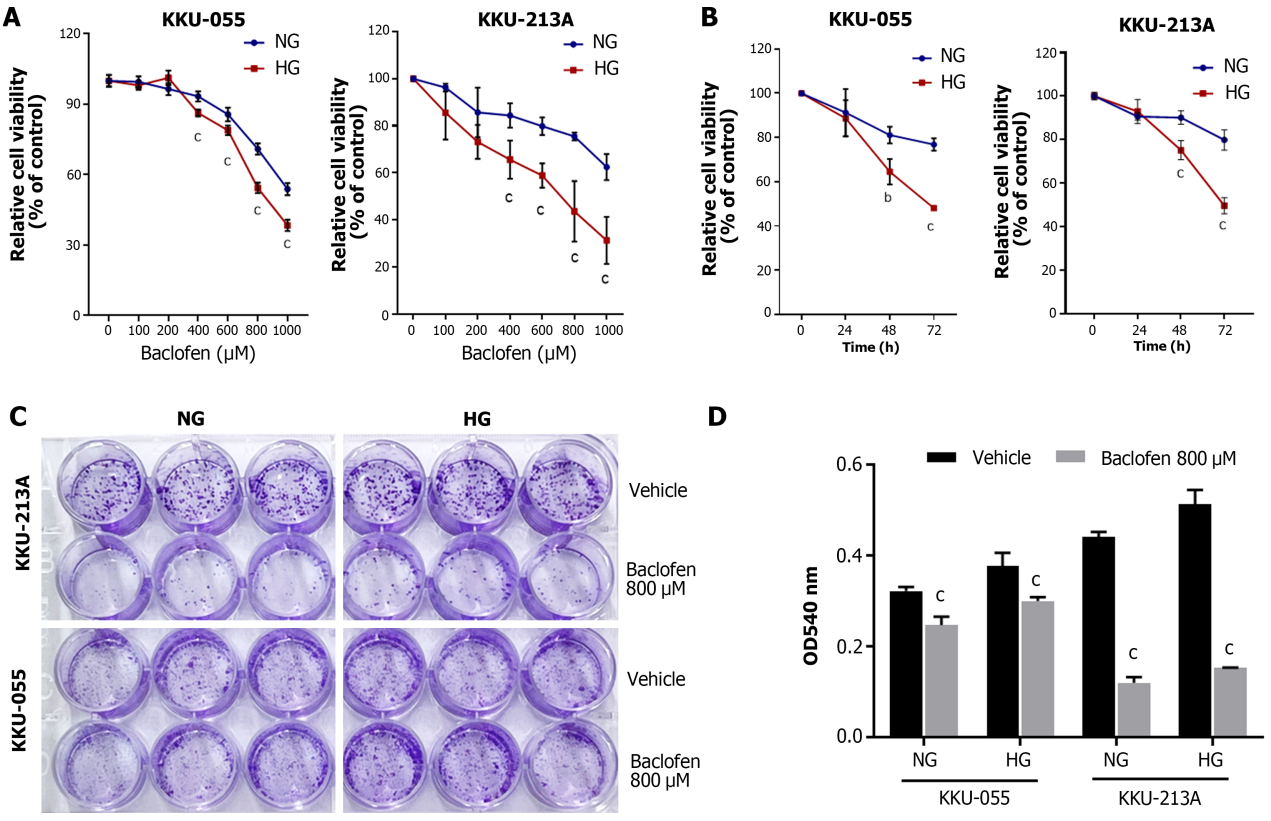

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Figure 2 Antiproliferative and anti-clonogenic effects of γ-aminobutyric acid-B receptor agonist. A and B: Baclofen, a γ-aminobutyric acid-B receptor agonist, exerts significant antiproliferative effects on cholangiocarcinoma (CCA) cells in a dose-dependent (A) and time-dependent (B) manner; C and D: CCA cells cultured in high glucose condition (HG) are more sensitive to baclofen than those cultured in normal glucose (NG). Baclofen also shows anti-clonogenic effects on both NG and HG cells of CCA. Presented data are representatives of three biological replications of each triplicated experiment with the same trends of results. $^{b}P < 0.01$, $^{c}P < 0.001$. NG: Normal glucose; HG: High glucose.

Baclofen suppresses c-Myc and cyclin D1 expression

According to the antiproliferative effects of baclofen *via* the suppression of GSK3α/β/β-catenin and STAT3 pathways, the common downstream targets of both pathways functioning in cell proliferation were then explored. Expression of c-Myc, a transcription factor involved in cell proliferation, and cyclin D1, a cell cycle regulatory protein, was examined for their correlation with GABBR2 at the mRNA and protein levels after baclofen treatment. Analyzing a public transcriptomic dataset, mRNA expression levels of *MYC* (encoding for c-Myc) and *CCND1* (encoding for cyclin D1) were not correlated with mRNA level of *GABBR2* (Supplementary Figure 1). However, both c-Myc and cyclin D1 expression at the protein level were suppressed at every time point after baclofen treatment in NG and HG cells of both CCA cell lines. Suppression of c-Myc was greater affected at the early time point in which cyclin D1 was gradually affected time-dependently in HG cells. The time-dependent suppression of c-Myc in NG cells after baclofen treatment was also observed (Figures 5A and B).

DISCUSSION

HG and hyperglycemia-induced aggressiveness of CCA cells has been reported with partially understood mechanisms [15,16,18,19]. The present study not only revealed the mechanisms by which HG activates the STAT3 and GSK3/β-catenin pathways, but also identified GABBR2 as the upstream receptor that is promising for CCA treatment, especially in patients with DM. Roles of GABA and its receptor have been studied in several types of cancer, including non-liver fluke-associated CCA[26-28,35]. The known mechanisms of actions of GABA and its receptor on CCA are by regulating several signaling pathways, *e.g.*, STAT3 and protein kinase A. This study, however, determined for the first time the association between hyperglycemia and the expression of GABBR2, and also identified GSK3/β-catenin signaling, an uncovered downstream pathway, that is modulated by GABBR2 in CCA. Roles of GABBR2 in the progression of CCA in both *in vitro* models and in the tumor tissues of CCA patients were also reported.

These current investigations first identified that GABBR2, a subtype of GABA-B receptor, was upregulated in CCA cells cultured in HG. This is of particular importance since approximately 60% of patients with CCA in Thailand had their blood glucose levels in a range of pre-DM and DM[36,37]. Targeting GABBR2 would then be beneficial to a substantial group of CCA patients. Univariate analysis revealed that high expression of GABBR2 in tumor tissues was associated

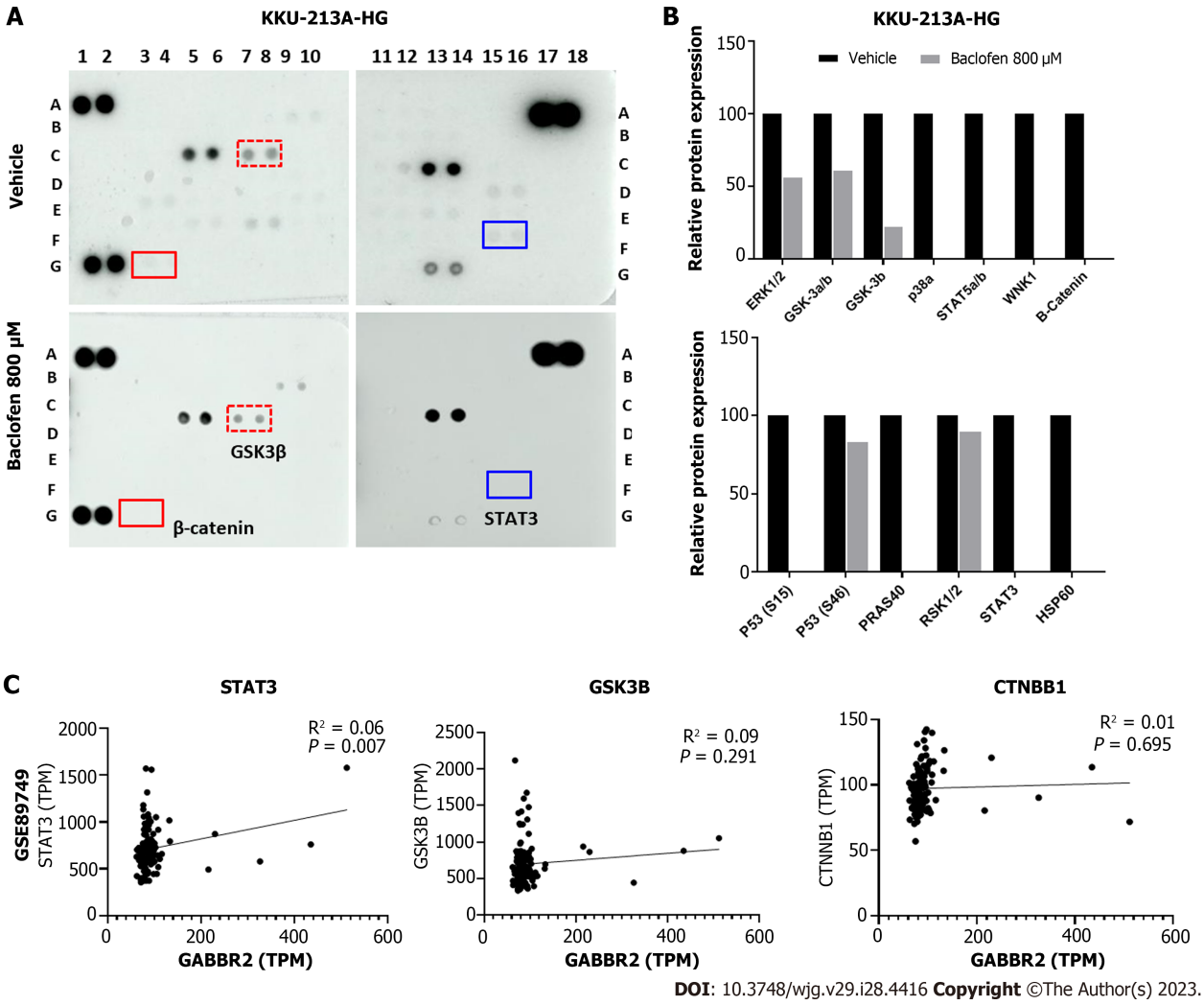

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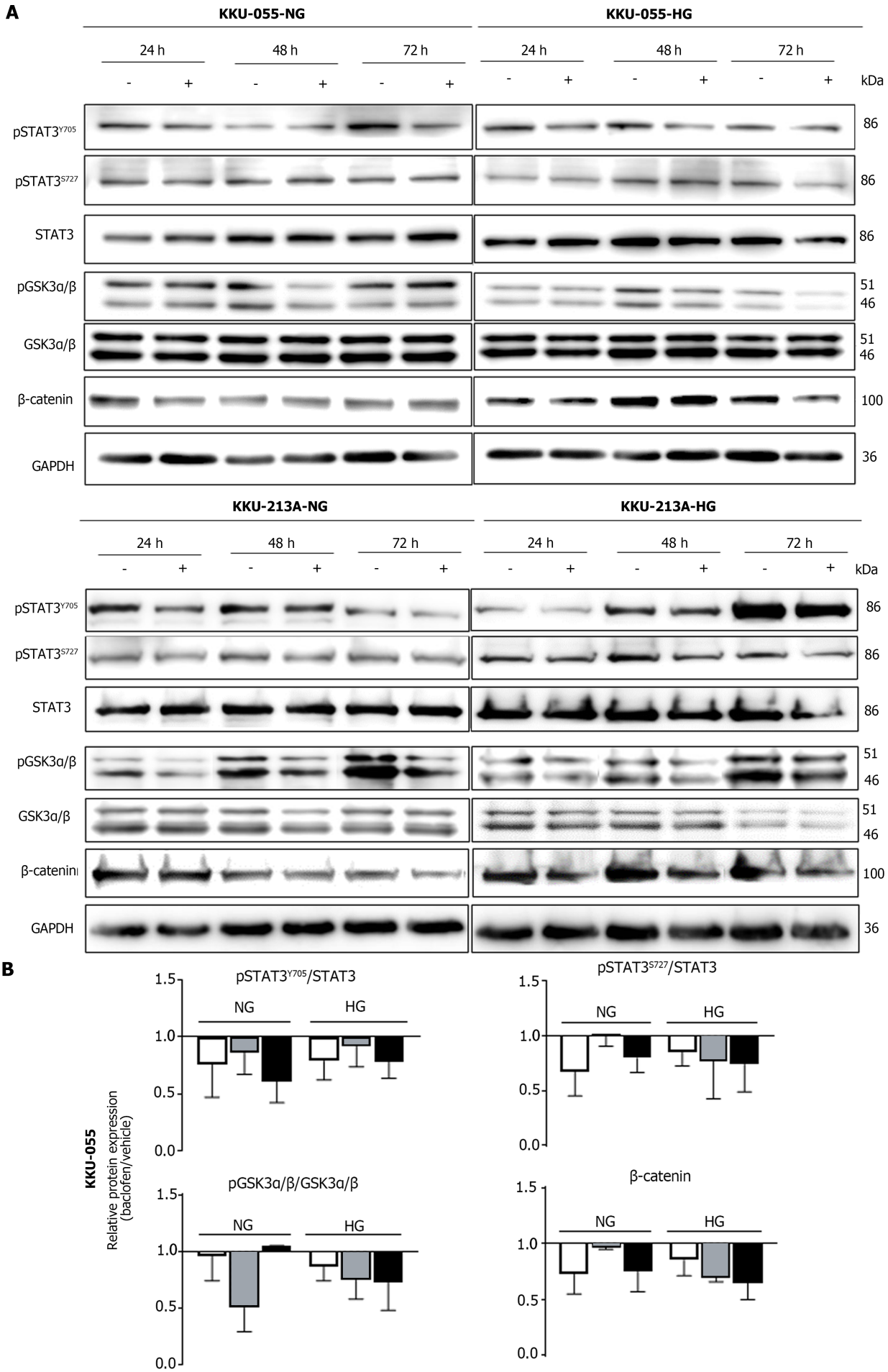
Figure 3 Baclofen inhibits the glycogen synthase kinase 3/β-catenin and signal transducer and activator of transcription 3 pathways. A and B: Phospho-kinase arrays reveal the inhibition of phosphorylation in multiple kinases and signal transducers; C: Glycogen synthase kinase 3 (GSK3)/β-catenin and signal transducer and activator of transcription 3 (STAT3) are included for further analysis as they are key pathways in cholangiocarcinoma (CCA) progression in which β-catenin and STAT3 are common targets of GSK3. RNA expression of γ-aminobutyric acid B2 receptor and that of STAT3 are significantly correlated in clinical CCA samples from Thai patients. HG: High glucose; GABBR2: γ-aminobutyric acid B2 receptor; GSK3: Glycogen synthase kinase 3; STAT3: Signal transducer and activator of transcription 3.

with a poorer histological subtype of CCA. High GABBR2 expression was associated with smaller tumor sizes compared with the group with low GABBR2 expression. These findings are in agreement with previous reports demonstrating that both high GABA-A or GABA-B receptors are associated with better prognostic outcomes for CCA patients[26,38]. The upregulated GABBR2 in CCA tissues of patients thus suggested a therapeutic strategy by using a natural GABA or even GABA receptor agonists. Patients with DM who have upregulated GABBR2 would, therefore, potentially benefit from targeting GABBR2 with available GABA or GABA analogs. The present study also found that HG cells of CCA with upregulated GABBR2 were more sensitive to baclofen, a GABA-B receptor agonist. This affirms the advantage of using GABA-B agonist as an add-on therapy for CCA treatment in patients with DM. As shown by western blot, the investigated signaling molecules were markedly suppressed in both NG and HG cells. This might be the result of the screening and recruitment of candidate pathways that were only from the HG cells whereas the signaling molecules in NG cells affected by baclofen were not screened and compared. Regardless of the glucose condition of the cell cultures, these results still verified that baclofen is effective for CCA treatment and is probably useful for CCA treatment in both patients with and without DM. As the available standard and developing treatment for CCA is of limited efficacy and needs more investigation[10-13], the findings in the present work may add value for alternative treatment of CCA. Especially, baclofen is a known drug in clinical practice in which its toxicity and therapeutic windows are known[39]. This medication is, therefore, highly promising for a repurposing aim. Of note, this study showed for the first time that a GABA receptor agonist is an alternative strategy to natural GABA treatment for CCA.

Suppression of GSK3/β-catenin, a new intracellular signaling mode under the regulation of GABBR2 in CCA, is shown in addition to the known affected STAT3 pathway[26]. Both GSK3/β-catenin and STAT3 are reported as crucial signaling pathways for CCA carcinogenesis and progression[40,41]. GSK3 is a kinase phosphorylating β-catenin for proteasomal degradation. The functions of GSK3 are inhibited by phosphorylation by its kinase regulators[42], hence decreased


A
KKU-055-NG
KKU-055-HG
24 h
48 h
72 h
kDa
pSTAT3Y705
pSTAT3S727
STAT3
pGSK3α/β
GSK3α/β
β-catenin
GAPDH
86
51
46
100
36
KKU-213A-NG
KKU-213A-HG
B
KKU-055
Relative protein expression (baclofen/vehicle)
pSTAT3Y705/STAT3
pSTAT3S727/STAT3
pGSK3α/β/GSK3α/β
β-catenin
NG
HG
1.5
1.0
0.5
0.0

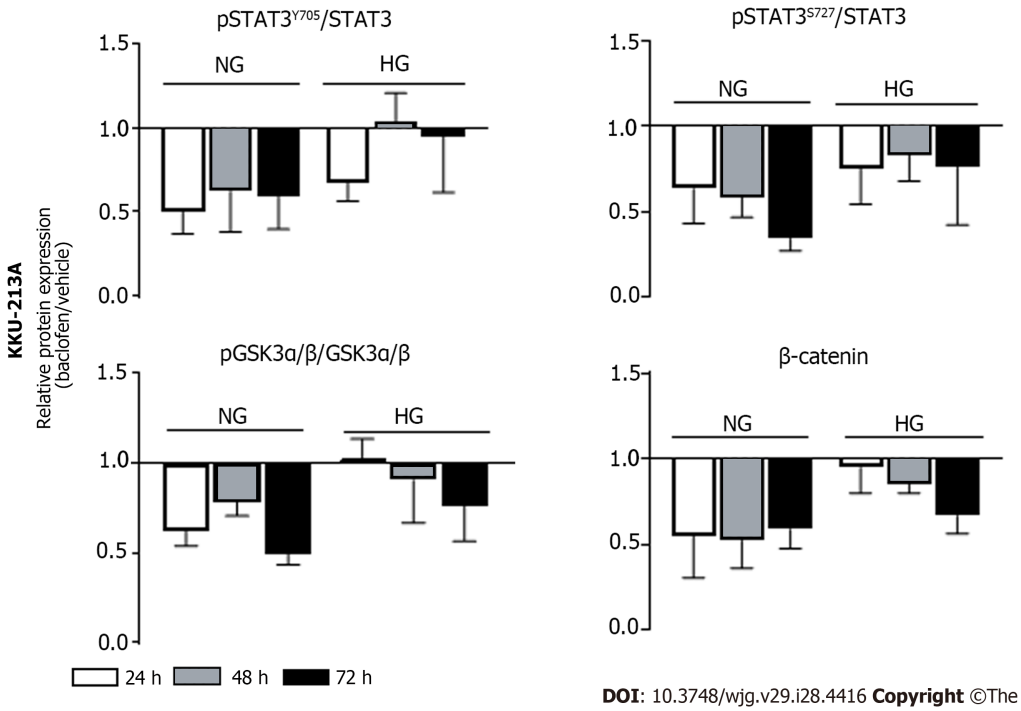


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Figure 4 Baclofen suppresses the glycogen synthase kinase 3/β-catenin and signal transducer and activators of transcription 3 pathways. A and B: Phosphorylation of glycogen synthase kinase 3 (GSK3) and signal transducer and activators of transcription 3 is decreased after baclofen treatment in both cholangiocarcinoma cell lines, both cultured in normal glucose and high glucose conditions. Total β-catenin protein is also decreased consistently with the decreased phosphorylated GSK3α/β. Western blots show the representative of three biological replications with the same trends of results. Band intensities are the average of three biological replications which are normalized using the intensities of glyceraldehyde-3-phosphate dehydrogenase for each experiment. The levels of phosphorylated forms are normalized with the total forms of their corresponding proteins. NG: Normal glucose; HG: High glucose; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GSK3: Glycogen synthase kinase 3; STAT3: Signal transducer and activator of transcription 3.

phosphorylated GSK3α/β in CCA cells after the baclofen treatment indicated the active state of GSK3. This is also evidenced by the decreased β-catenin, a GSK3 downstream target which is a known transcription factor for cell proliferation. Moreover, STAT3 phosphorylation was also inhibited after baclofen treatment, which is consistent with the previous findings in CCA cells treated with GABA. STAT3 has also been reported as an alternative downstream target of GSK3 phosphorylation[43]. The decreased levels of β-catenin and STAT3 phosphorylation in the present report might thus be a consequence of GSK3 inhibition by a GABBR2 agonist. As β-catenin and STAT3 control cell proliferation *via* the expression of c-Myc and cyclin D[44,45], these two common effector-proteins were also down-regulated after baclofen treatment. These suggested that the activation of GABBR2 protein by its natural or synthetic ligands might be a crucial step for the regulation of downstream signaling, although there is no correlation among *GABBR2*, *MYC*, and *CCND1* at the mRNA level. These findings not only add to the understanding of the molecular linkages among DM, hyperglycemia, and CCA progression but also imply the therapeutic strategies of the combined targeting multiple signaling pathways to improve CCA treatment outcomes. The schematic summary of the findings from the current study is depicted in Figure 6.

GABA is an inhibitory neurotransmitter mostly found in the central nervous system. It is a derivative of the non-essential amino acid glutamate and can be synthesized by converting glutamine as a precursor[29]. GABA functions by binding to its receptors which have three subtypes (GABA-A, GABA-B, and GABA-C receptors). Different GABA receptors are responsible for various physiological functions both within and outside the nervous system. While the GABA-A receptors are found to be important for hepatocytes[46], GABA-B receptors are also needed for the differentiation of biliary epithelial cells[25]. In addition to physiological roles, GABA and its receptors are also reported for both pro-tumor[29,30] and anti-tumor effects[28,47], depending on the cancer type. The previous study showed that all subtypes of GABA receptors were expressed in CCA cells[28]. Still, only GABA-B receptor was differentially expressed between CCA cells and normal adjacent cholangiocytes in tumor tissues from patients[26]. The GABA-B receptor then holds a high potential for targeting in CCA treatment. Treatment of GABA in CCA cells exerted antiproliferative[28] and anti-invasive effects[35] on CCA cells by inhibiting multiple pathways, *i.e.*, cyclic AMP–dependent regulation of the protein kinase A/extracellular signal-regulated kinase (PKA/ERK)[27,28] and STAT3[26,38] pathways. Low GABA-A and GABAB-B receptor expression was also associated with a poor prognosis in CCA patients[38]. The roles of GABA receptors under hyperglycemia in CCA patients with DM are for the first time reported in the present study. The study of a GABA-B receptor agonist, instead of using natural GABA, is also reiterated and suggested for a potential translation to a clinical study.

This study, nevertheless, has some limitations. First, all CCA tissues and CCA cell lines used in the study were derived from *O. viverrini*-associated CCA patients. As different molecular backgrounds of CCA in different areas have been noted [48,49], the current findings may be needed to verify in CCA from the non-endemic areas of liver fluke. Noteworthy, the

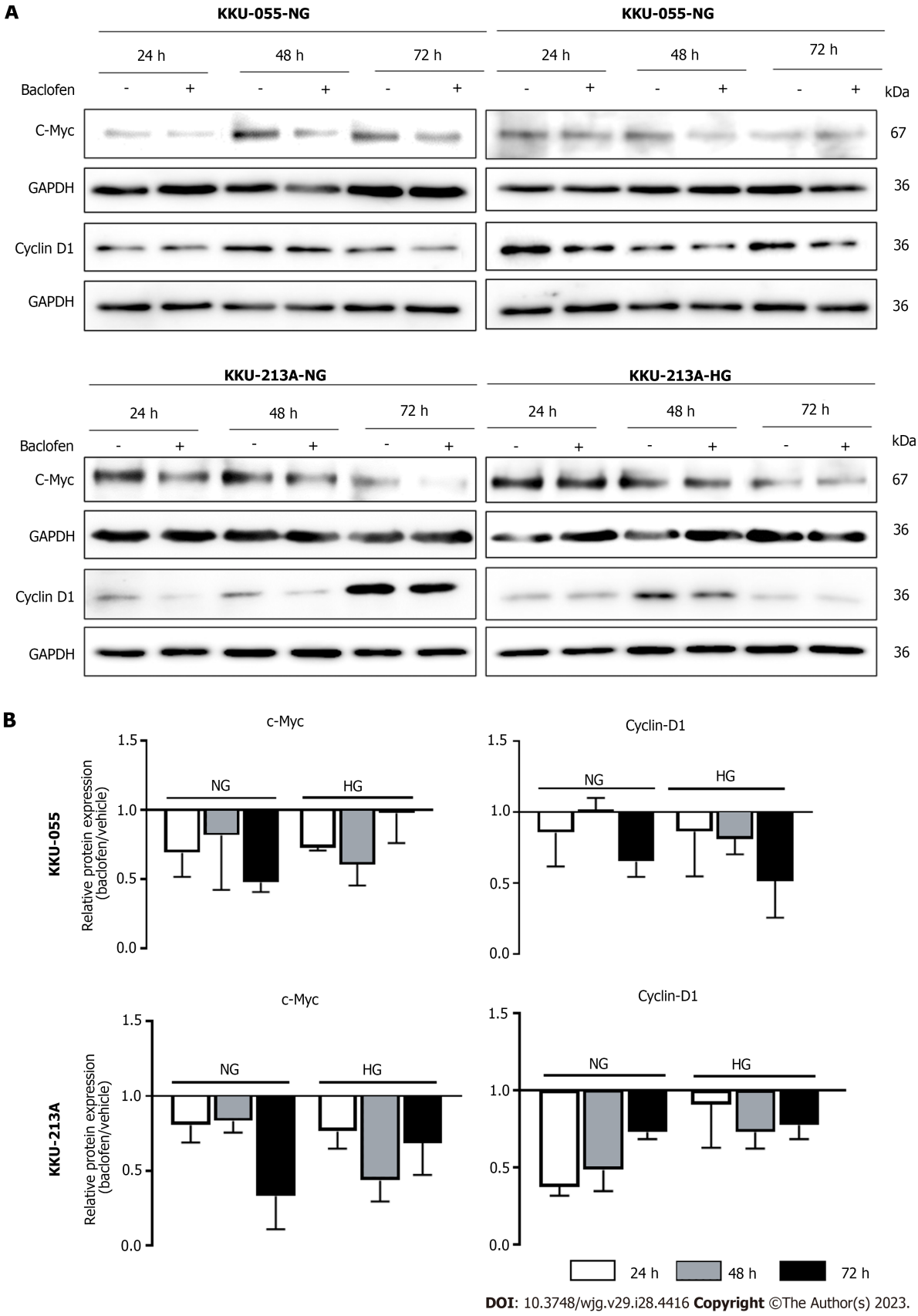

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Figure 5 Baclofen suppresses c-Myc and cyclin D1expression in cholangiocarcinoma cells. A and B: The expression of c-Myc and cyclin D1, key proteins for cell proliferation, is suppressed in both cholangiocarcinoma cell lines after baclofen treatment. Western blots show the representatives of three biological replications with the same trends of results. Band intensities are the average of three biological replications which are normalized using the intensities of glyceraldehyde-3-phosphate dehydrogenase for each experiment. NG: Normal glucose; HG: High glucose; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

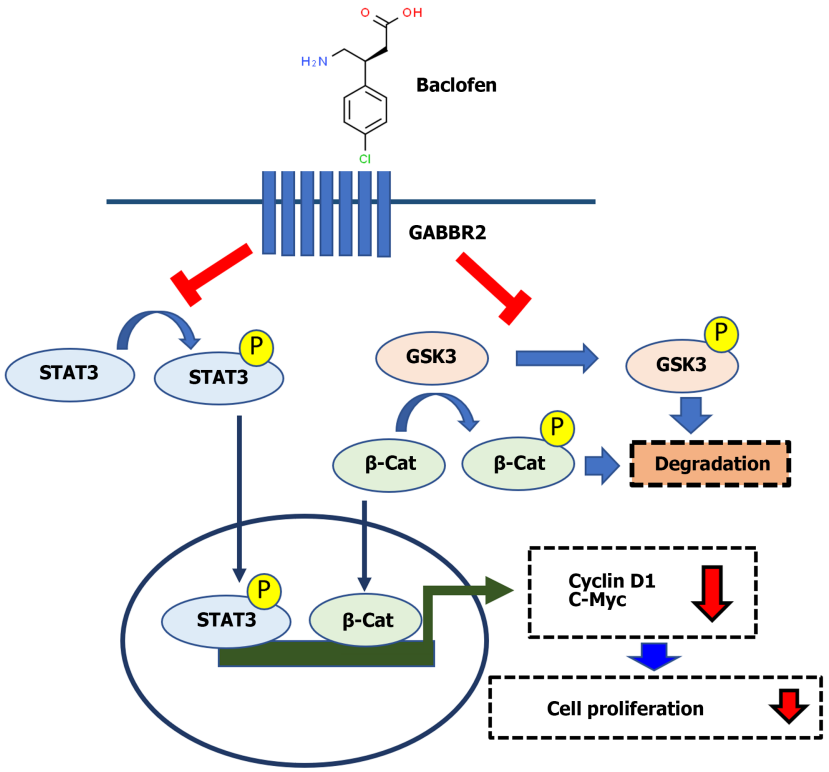

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Figure 6 Schematic summary of the effects of high glucose on γ-aminobutyric acid B2 receptor expression and the effects of baclofen on cholangiocarcinoma cells. High glucose induces the expression of γ-aminobutyric acid B2 receptor (GABBR2) in cholangiocarcinoma (CCA) cells. The treatment of baclofen, a GABBR2 agonist, to CCA cells inhibits phosphorylation of glycogen synthase kinase 3, resulting in the activation of the kinase activity which further phosphorylates β-catenin. Phosphorylated β-catenin is subjected to degradation preventing its function on promoting cell proliferation *via* c-Myc and cyclin D1 expression. On the other hand, activated GABBR2 by baclofen also inhibits phosphorylation of signal transducer and activator of transcription 3 (STAT3). The inhibition of STAT3 phosphorylation also suppresses its functions as a transcription factor for c-Myc and cyclin D1 expression. Activating GABBR2 by baclofen, thus, suppresses the proliferation of CCA cells. GABBR2: γ-aminobutyric acid B2 receptor; GSK3: Glycogen synthase kinase 3; STAT3: Signal transducer and activator of transcription 3.

correlations between GABBR2 and STAT3, GSK3B, and CTNNB1, were not observed in the analysis using a dataset from The Cancer Genome Atlas which included non-liver fluke-associated CCA[50].

Second, although the *in vivo* effect of natural GABA on CCA growth has been reported in several studies, the treatment of GABA receptor agonists in the animal model remains limited. A further *in vivo* study of GABA-B receptor agonists at an optimal therapeutic dosage in animal models should be certified. Third, the association of GABBR2 expression and nuclear localization of its downstream transcription factor, namely, β-catenin, needs further proof in clinical samples using appropriate techniques. Lastly, a combination of several modalities as the therapeutic regimen for CCA is recommended. The study of combining GABA-B receptor agonists with another standard or alternative modality will help translate these findings to clinical practice, especially in the treatment of CCA patients with DM who are likely suffering from a poor prognosis.

CONCLUSION

GABBR2 is upregulated by HG in both CCA cell lines and tumor tissues from patients. Targeting GABBR2 with GABA-B receptor agonists shows the potential of using GABBR2 as a therapeutic target and repurposing GABA-B receptor agonists for CCA treatment, especially for those patients with DM and hyperglycemia.

ARTICLE HIGHLIGHTS

Research background

The association between diabetes mellitus (DM) and cholangiocarcinoma (CCA) progression has been established with unclear mechanisms. Our previous study showed that γ-aminobutyric acid B2 receptor (GABBR2) is among the top 5 upregulated genes in CCA cells cultured in high glucose (HG). Thus, GABBR2 is highly potential for a repurposing aim in CCA treatment.

Research motivation

Approximately 60% of Thai patients with CCA had fasting blood glucose in a range of pre-diabetes or DM. Targeting the molecules underlying hyperglycemia-induced aggressiveness of CCA cells might improve the prognosis of CCA patients with DM.

Research objectives

This study aimed to investigate the effects of hyperglycemia on GABBR2 expression and the potential use of GABBR2 as a CCA therapeutic target.

Research methods

CCA cells cultured in normal glucose or HG conditions were used as models of *in vitro* euglycemia and hyperglycemia, respectively. Baclofen, a GABBR2 agonist, was used to study the functional roles of CCA cells. Western blot, immunocytofluorescence, and immunohistochemistry were used to study molecular mechanisms.

Research results

HG induced GABBR2 expression in both cell lines and in patients' CCA tissues. Baclofen treatment significantly suppressed CCA cell growth, while cells cultured in HG showed a significantly higher sensitivity. The effects of baclofen on CCA cell growth were achieved by the suppression of the signal transducer and activator of transcription 3 and glycogen synthase kinase 3/β-catenin pathways.

Research conclusions

The expression of GABBR2 in CCA is induced in hyperglycemic conditions. Baclofen significantly suppresses the growth of CCA cells and thus holds a high promise as a repurposing drug for CCA treatment.

Research perspectives

Investigating baclofen's effects at an optimal therapeutic dosage in *in vivo* models would verify the present work and facilitate the translation for clinical study in CCA cases.

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FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

F-box only protein 2 exacerbates non-alcoholic fatty liver disease by targeting the hydroxyl CoA dehydrogenase alpha subunit

Zhi Liu, Ning-Yuan Chen, Zhao Zhang, Sai Zhou, San-Yuan Hu

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Abstract

BACKGROUND

Non-alcoholic fatty liver disease (NAFLD) is a major health burden with an increasing global incidence. Unfortunately, the unavailability of knowledge underlying NAFLD pathogenesis inhibits effective preventive and therapeutic measures.

AIM

To explore the molecular mechanism of NAFLD.

METHODS

Whole genome sequencing (WGS) analysis was performed on liver tissues from patients with NAFLD (n = 6) and patients with normal metabolic conditions (n = 6) to identify the target genes. A NAFLD C57BL6/J mouse model induced by 16 wk of high-fat diet feeding and a hepatocyte-specific F-box only protein 2 (FBXO2) overexpression mouse model were used for *in vivo* studies. Plasmid transfection, co-immunoprecipitation-based mass spectrometry assays, and ubiquitination in HepG2 cells and HEK293T cells were used for *in vitro* studies.

RESULTS

A total of 30982 genes were detected in WGS analysis, with 649 up-regulated and 178 down-regulated. Expression of FBXO2, an E3 ligase, was upregulated in the liver tissues of patients with NAFLD. Hepatocyte-specific FBXO2 overexpression facilitated NAFLD-associated phenotypes in mice. Overexpression of FBXO2

aggravated odium oleate (OA)-induced lipid accumulation in HepG2 cells, resulting in an abnormal expression of genes related to lipid metabolism, such as fatty acid synthase, peroxisome proliferator-activated receptor alpha, and so on. In contrast, knocking down FBXO2 in HepG2 cells significantly alleviated the OA-induced lipid accumulation and aberrant expression of lipid metabolism genes. The hydroxyl CoA dehydrogenase alpha subunit (HADHA), a protein involved in oxidative stress, was a target of FBXO2-mediated ubiquitination. FBXO2 directly bound to HADHA and facilitated its proteasomal degradation in HepG2 and HEK293T cells. Supplementation with HADHA alleviated lipid accumulation caused by FBXO2 overexpression in HepG2 cells.

CONCLUSION

FBXO2 exacerbates lipid accumulation by targeting HADHA and is a potential therapeutic target for NAFLD.

Key Words: F-box only protein 2; Nonalcoholic fatty liver disease; The hydroxyl CoA dehydrogenase alpha subunit; Liver steatosis; Ubiquitination; Lipid accumulation

Core Tip: This study involves an assessment of the role of F-box only protein 2 (FBXO2) in non-alcoholic fatty liver disease (NAFLD). First, based on the whole genome sequencing analysis results of liver tissues from normal controls and patients with NAFLD, the expression of FBXO2 was found to be increased during NAFLD. Hepatocyte-specific FBXO2 overexpression facilitated NAFLD-associated phenotypes in mice. In contrast, the knockdown of FBXO2 expression of HepG2 cells significantly alleviated oleate-induced lipid accumulation. Mechanistically, the hydroxyl CoA dehydrogenase alpha subunit, a protein with known role in oxidative stress reaction, is one of the protein substrates of FBXO2-mediated ubiquitination.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), a clinicopathologic syndrome, is characterised by an excessive deposition of fat in hepatocytes [triglyceride (TG) content exceeding 5% of the liver weight] excluding alcohol and other liver-damaging factors[1-3]. With an increase in the incidence of obesity and its associated metabolic syndrome, more than one-third of adults are affected by NAFLD globally[4-6]. NAFLD includes simple fatty liver, non-alcoholic steatohepatitis, and related cirrhosis, which can eventually develop into decompensated cirrhosis and hepatocellular carcinoma[7,8]. The key issues affecting the treatment of patients with NAFLD are atherosclerotic cardio-cerebrovascular diseases, cirrhosis, and malignant tumours associated with the metabolic syndrome[9-11]. Since the pathogenesis of NAFLD remains unclear [12], a clinically effective drug to treat NAFLD is unavailable. A healthy diet and moderate exercise remain the main measures to treat NAFLD clinically[13].

The hydroxyl CoA dehydrogenase alpha subunit (HADHA), a fatty acid β-oxidation enzyme, has been identified as a key pathogenic regulator of metabolism-related diseases, such as NAFLD, obesity, and diabetes[14-16]. Previous studies have confirmed that the expression of HADHA is decreased in L02 cells treated with free fatty acids (FFA) and in mice fed with a high-fat diet (HFD). Knocking out HADHA aggravated liver steatosis, inflammation, and oxidative stress in FFA-treated L02 cells. In addition, oxidative stress and liver damage in NAFLD mice are alleviated by the up-regulation of HADHA[17]. Meanwhile, HADHA can be affected by the ubiquitin-proteasome system (UPS), which mediates important regulatory modifications. Ubiquitination and the degradation of HADHA were reported to be mediated by ubiquitin-conjugating enzyme E2 O, thereby modulating lipid metabolism[18]. However, it remains unclear if there is another regulatory factor that could control and mediate the ubiquitination of HADHA, thus affecting the pathogenesis of HADHA-mediated NAFLD. Therefore, confirming the regulatory signaling pathway in NAFLD that inhibits or activates HADHA is necessary.

The UPS comprises an evolutionarily conserved protein degradation mechanism, including ubiquitin activation (E1), ubiquitin coupling (E2), and ubiquitin ligase (E3) enzymes[19,20]. F-box only protein 2 (FBXO2), a well-known E3 ligase, plays an indispensable role in the UPS[21] and has unique functions in various pathological processes. For example, FBXO2 regulates signal transducer and activator of transcription 3 signaling responsible for modulating the proliferation and tumorigenicity of osteosarcoma cells[22], targets glycosylated SAD1/UNC84 domain protein-2 for ubiquitination and degradation to promote the development of ovarian cancer[21] and mediates the clearance of damaged lysosomes and alters the neurodegeneration phenotype of the brain in Niemann-Pick C disease[23]. In addition, the abnormal expression of FBXO2 in obese mice disrupts glucose homeostasis through ubiquitin-mediated insulin receptor degradation[24]. However, the role of FBXO2 in NAFLD pathology remains unclear.

In this study, we found that expression of FBXO2 was upregulated in the liver tissues of patients with NAFLD when compared with those in liver tissues of normal controls *via* the whole genome sequencing (WGS) analysis. These findings urged us to explore whether FBXO2 can promote the progression of NAFLD and its mechanism. Our findings can clarify whether FBXO2 is a feasible therapeutic target for NAFLD and related metabolism disorders.

MATERIALS AND METHODS

Human liver tissue samples

Liver tissue samples were collected from 10 patients with NAFLD, who underwent sleeve gastrectomy with liver biopsy at the Qilu Hospital of Shandong University, Shandong, China. Control liver tissue samples were obtained from 10 patients with normal metabolic conditions who underwent hepatic hemangioma resection at the same hospital.

The inclusion criteria were: Physical examination, laboratory investigation, ultrasound echography, and a liver biopsy consistent with the diagnosis of NAFLD, according to the Brunt criteria. Exclusion criteria were: Excess alcohol consumption (≥ 20 g/d for women and ≥ 30 g/d for men), the presence of hepatitis B virus surface antigen or hepatitis C virus antibodies in the absence of a history of vaccination, use of drugs linked to NAFLD, evidence of other specific liver diseases, such as autoimmune liver disease, and hemochromatosis[25].

A liver biopsy during surgery was performed to provide foundation to non-alcoholic steatohepatitis (NASH) score. Clinical data of the normal controls and patients with NAFLD are shown in Table 1. Written informed consent was obtained from the patients and their families. All procedures involving human liver samples were approved by the Ethics Committee of the Qilu Hospital and adhered to the principles of the Declaration of Helsinki (approval No. KYLL-2017-073).

WGS and result analysis

RNA extraction and whole genome library sequencing were performed in 12 liver tissues (NAFLD, $n = 6$; normal, $n = 6$) by LC-BIO TECHNOLOGIES (Hangzhou, China) CO. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analyses of the mRNA sequencing part were performed using the DAVID programme (https://david.ncifcrf.gov/). Bar, scatter, and Gene Set Enrichment Analysis result plots were drawn using the LC-BIO Technologies platform (https://www.omicstudio.cn/).

Animals

Eight-week-old pathogen-free C57BL/6J male mice (RRID:IMSR_JAX:000664) were purchased from SPF Biotechnology Co. (Beijing, China). Five mice were placed in each cage at a temperature of 23-26 °C with a 12 h light/dark cycle. Animals had free access to food and water. Rodent food was purchased from Xiao Shu You Tai Biotechnology (Beijing, China). The mice used in this investigation were divided randomly.

To induce NAFLD, 10 mice were fed an HFD (#D12492; Research Diets, New Brunswick, NJ, United States) for 16 wk. Control mice were fed with a standard normal chow diet (ND). After 16 wk of feeding an HFD, the weight of the mice in the HFD group exceeded that of mice in the ND group by 20%, and obvious lipid accumulation was observed in the liver. Serum intracellular TG and total cholesterol (T-Cho) levels also increased significantly in the HFD group ($P < 0.001$, Supplementary Table 1). Finally, eight mice met the experimental criteria. One underweight mouse and one mouse with no significant lipid deposition in the liver were excluded from any analysis.

To establish a liver-specific FBXO2 overexpression model, six mice were injected with adeno-associated virus serotype 8 (AAV8)-His-FBXO2 through the tail vein (100 μL of virus containing 5×10^{11} vg of vectors) after 6 wk of HFD feeding. An empty vector (AAV-His-vector) was injected into the corresponding control mice ($n = 6$). The virus-infected mice were fed with HFD for an another 16 wk. The AAV used in this study was purchased from WZ Biosciences Inc. (Shandong, China). The study was approved by the Institutional Animal Care and Use Committee of Shandong Provincial Qian Foshan Hospital (approval No. S453) in accordance with internationally accepted principles for the use of laboratory animals.

Cell lines and cell culture

Human hepatoma cells (HepG2, ATCC; HB-8065 BCRC, #CL-0103) and human embryonic kidney 293 cells (HEK293T, ATCC; CRL-3216 DSMZ, #CL-0005) were cultured in Dulbecco's Modified Eagle Medium, containing 10% foetal bovine serum and 1% penicillin-streptomycin, in a humidified chamber at 37 °C with 5% CO_2. HepG2 cells were exposed to odium oleate (OA) dissolved in 0.5% fatty acid-free bovine serum albumin (BSA) at a final concentration of 0.3 mmol/L for 24 h to establish an *in vitro* cell lipid deposition model. All cells were purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China). All cell lines were authenticated *via* STR identification.

Plasmid construction and transfection

FBXO2 or HADHA overexpression was achieved by transfecting cells with His-FBXO2 plasmid (#NM_012168CH895916, WZ Bioscience Inc., Shandong, China) or Myc-HADHA plasmids (#BC009235HG15019-NM, Sino Biological, Beijing, China), respectively, as per the respective manufacturer's instructions. His-vector (#66005-1-Ig, Proteintech) and Myc-vector plasmids (#16286-1-AP, Proteintech) were used as controls. FBXO2 knockdown was achieved by transfecting cells with Sh-FBXO2 plasmid (WZ Bioscience Inc). Sh-NC plasmids were used as a control. The DNA sequences of the plasmids used are shown in Supplementary material.

Table 1 Clinical information of normal controls and patients with non-alcoholic fatty liver disease

	Normal (*n* = 10)	NAFLD (*n* = 10)	*t* value	*P* value
Gender			-	-
Male, %	4 (40)	4 (40)		
Female, %	6 (60)	6 (60)		
Age, yr	33.30 ± 2.83	32.40 ± 7.09	0.373	0.714
Height, cm	169.10 ± 7.84	169.80 ± 10.38	0.170	0.867
Body weight, kg	70.95 ± 16.48	118.04 ± 28.87	4.480	< 0.001
Body mass index, kg/m^2	24.51 ± 4.02	40.41 ± 5.41	7.454	< 0.001
Waist circumference, cm	90.80 ± 11.33	128.5 ± 3.98	9.928	< 0.001
Systolic BP, mmHg	126.40 ± 6.33	134.80 ± 10.23	2.208	0.04
ALT, U/L	12.00 ± 6.77	69.00 ± 63.20	2.836	0.011
AST, U/L	15.40 ± 3.17	46.70 ± 29.74	3.309	0.004
GLDH, U/L	1.94 ± 1.22	7.74 ± 4.98	3.580	0.002
T-Cho, mmol/L	3.97 ± 0.52	4.47 ± 0.77	1.700	0.106
HDL, mmol/L	1.31 ± 0.20	0.97 ± 0.14	4.486	< 0.001
LDL, mmol/L	2.19 ± 0.37	2.83 ± 0.62	2.824	0.011
sdLDL, mmol/L	0.34 ± 0.14	1.11 ± 0.51	4.580	< 0.001
TG, mmol/L	0.62 ± 0.20	2.05 ± 0.67	6.483	< 0.001
NEFA, μmol/L	44.41 ± 29.72	55.20 ± 15.05	1.024	0.319
FPG, mmol/L	4.82 ± 0.33	4.81 ± 1.09	0.014	0.989
NASH score	0	5.10 ± 1.30	-	-

Data are presented as the mean ± SD. $P < 0.05$ was considered statistically significant. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BP: Blood pressure; FPG: Fasting plasma glucose; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; NEFA: Nonesterified fatty acid; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; sdLDL: Small and dense low-density lipoproteins; TG: Triglycerides; T-Cho: Total cholesterol.

Western blotting, co-immunoprecipitation, and mass spectrometry assays

Briefly, liver tissues and HepG2 cells were lysed with RIPA lysis buffer containing protease inhibitors. Western blotting was performed using standard procedures[26,27]. The primary antibodies used are listed in Supplementary Table 2. Image J (RRID:SCR_003070) and GraphPad Prism software (RRID:SCR_002798) were used to analyze the western blotting results.

For the co-immunoprecipitation (co-IP) assay, HepG2 cell protein lysate was prepared. The cracking solution was then washed with 1 mL of cracking buffer (containing 100 μL of protein A/G agarose beads) and the lysate was incubated overnight with the appropriate antibodies. Next, the beads were washed thrice with cracking buffer and then re-suspended in 2 × sodium dodecyl sulfate (SDS) loading buffer. The beads were incubated at 95 °C for 5 min to elute immunoprecipitated proteins. Finally, the proteins were separated using SDS-PAGE and immunoblotted for detection. Mass spectrometry (MS)-based proteomic analysis was performed by the Jinjie Bio company (Hangzhou, China).

Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from the liver tissues and HepG2 cells using TRIzol reagent (Invitrogen, #12183555). Complementary DNA (cDNA) was synthesised from the isolated RNA using a cDNA synthesis kit (Takara, #RR047A). Subsequently, SYBR Green Master Mix (Vazyme, Q111-02) and a quantitative reverse transcription polymerase chain reaction (qRT-PCR) system (LightCycle 480 II, Roche, Japan) were used to perform qRT-PCR and analyse the results. The primer sequences used in the study are listed in Supplementary Table 3.

Homeostasis model assessment of insulin resistance (HOMA-IR), oral glucose tolerance test (OGTT) and insulin tolerance test (ITT). Blood glucose levels were measured using a Glucometer Elite monitor (ACCU-CHEK, Roche, Germany) and serum insulin was measured using an insulin ELISA kit (D721159-0048, Sangon Biotech). The HOMA-IR index was calculated based on the fasting serum glucose and insulin levels. OGTT was performed *via* the administration of glucose (2 mg/g) after an overnight fast. ITTs were performed following the *i.p.* injection of 1 U/kg insulin after 4 h of fasting. OGTT and ITT were performed at 16 wk post adenovirus injection.

Mouse serum and cell cytosol lipid analysis

Blood was collected from the tail vein of mice. Serum was obtained after the blood was centrifuged at 8000 × g for 20 min at 4 °C. HepG2 cells were lysed using an ultrasonic crusher (SCINTZ-650E; Ningbo Scientz Biotechnology Co., Ningbo, China). Serum TG and T-Cho levels were measured using commercially available assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Histological analysis

Hematoxylin and eosin (H&E) staining was done to demonstrate the pattern of lipid accumulation. Oil Red-O staining was done to show the accumulation of fat droplets in the liver.

Immunohistochemical staining and immunofluorescence

For immunohistochemistry, paraffin sections of paired human or mouse livers were deparaffinized and rehydrated. Antigens were retrieved using sodium citrate buffer. After blocking with 1% BSA for 1 h at 25 °C, the sections were sequentially incubated with anti-FBXO2 (Proteintech Cat# 14590-1-AP, RRID:AB_2104394) and the corresponding secondary antibodies. The antibodies used in the study are listed in Supplementary Table 2.

For immunofluorescence staining, cells were fixed with 4% paraformaldehyde for 25 min at 25 °C. Cells were then washed thrice with phosphate-buffered saline (PBS) and lysed with 0.1% Triton X-100 in PBS for 20 min. After washing the cells with PBS three times, the cells were incubated in 1% BSA at 37 °C for 1.5 h followed by overnight incubation at 4 °C with rabbit anti-FBXO2 and anti-HADHA antibodies (Proteintech Cat#10758-1-AP, RRID:AB_2115593). After washing thrice with PBS, the cells were incubated with the appropriate secondary antibodies for 1 h at 37 °C. Finally, the cells were stained with 4′,6-diamidino-2-phenylindole. Images were captured using a confocal microscope (IX-73, Olympus, Japan).

Ubiquitination assay

The HADHA ubiquitination assay was performed by transfecting HEK293T cells with FBXO2, HADHA, HA-ubiquitin, or empty vectors using Lipofectamine 2000 (#11668, Invitrogen, United States). After 24 h, the cells were washed twice with PBS and lysed in RIPA lysis buffer. The lysates were then centrifuged to obtain cytoplasmic proteins. The obtained proteins were incubated overnight with anti-HADHA antibody followed by incubation with protein A/G agarose beads at 4 °C for an additional 4 h. After washing the beads three times with lysis buffer, the beads were boiled in 2 × SDS loading buffer, separated *via* the SDS-PAGE, and immunoblotted. All experiments were repeated three times independently.

Statistical analyses

The *t*-test was performed for comparisons between two groups. Statistical significance was set at $P < 0.05$.

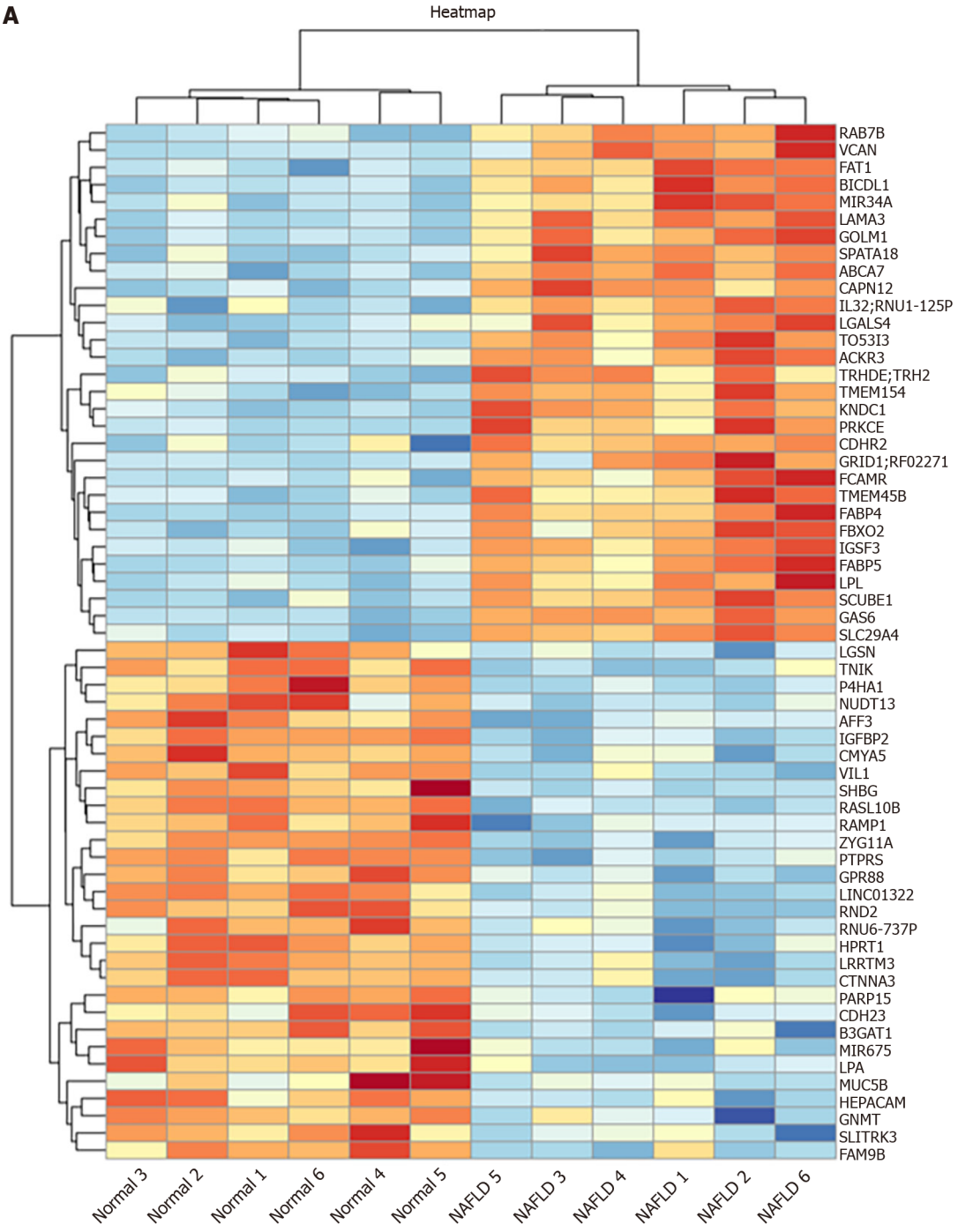
RESULTS

FBXO2 expression is upregulated in livers with hepatic steatosis

WGS was performed on the liver tissues of six patients with NAFLD and six normal controls. A total of 30982 genes were detected in the WGS analysis, of which 649 were up-regulated and 178 were down-regulated. The heat map in Figure 1A shows the 60 most significantly up- or down-regulated genes. The volcano plot in Figure 1B depicted an overview of the gene sequencing results [log2 (fold change) > 1; $P < 0.05$]. Some genes marked in our sequencing analysis were closely related to NAFLD[26,28-30], such as fatty acid synthase (FASN), growth differentiation factor 15 and insulin-like growth factor binding protein 2 (Figure 1B). GO and KEGG analyses were also performed (Figures 1C and D, Supplementary Figures 1A and B). GO-biological process analysis indicated that the differential genes were mainly involved in 'signal transduction', 'lipid metabolic process', and 'positive regulation of GTPase activity'. GO-cellular component analysis indicated that the differential genes were mainly associated with the cytoplasm, cytosol, and integral component of the membrane. GO-molecular function analysis indicated that the differential genes were mainly associated with protein binding, identical protein binding, and ATP binding processes. KEGG enrichment results revealed that the differential genes are largely enriched in 'metabolic pathways'.

Among the upregulated genes, the mRNA level of FBXO2 was significantly upregulated among patients with NAFLD. According to previous studies, the abnormal expression of FBXO2 in obese mice disrupts glucose homeostasis through ubiquitin-mediated insulin receptor degradation. We also found that sleeve gastrectomy could downregulate FBXO2 and activate in the phosphatidylinositol 3-kinase-protein kinase B (PI3K-AKT) pathway[31]. In our GO and KEGG analysis, some differential genes were enriched in the PI3K-AKT pathway. Hence, we speculated that FBXO2 may play a role in regulating lipid metabolism in NAFLD.

To investigate whether FBXO2 is involved in the occurrence and development of NAFLD, we analyzed its expression in liver tissues isolated from patients with NAFLD (Table 1, Figures 2A and B). We found that the mRNA and protein levels of FBXO2 were significantly higher in liver tissues of patients with NAFLD compared to that in control liver tissues *via* qRT-PCR analysis (Figure 2D, $P < 0.0001$), western blot analysis (Figure 2C, $P < 0.0001$), and immunohistochemical staining (Figure 2E). Moreover, we established a NAFLD mouse model by feeding C57BL6/J mice an HFD for 16 wk (Figures 2F and G, Supplementary Table 1). Similarly, we also found significantly higher FBXO2 mRNA and protein levels in liver samples obtained from HFD mice compared to ND mice *via* qRT-PCR (Figure 2I, $P < 0.0001$), western blot (Figure 2H, $P < 0.01$), and immunohistochemical staining (Figure 2J) analyses. Based on these results, we confirmed that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Heatmap
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VCAN
FAT1
BICDL1
MIR34A
LAMA3
GOLM1
SPATA18
ABCA7
CAPN12
IL32;RNU1-125P
LGALS4
TO53I3
ACKR3
TRHDE;TRH2
TMEM154
KNDC1
PRKCE
CDHR2
GRID1;RF02271
FCAMR
TMEM45B
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FBXO2
IGSF3
FABP5
LPL
SCUBE1
GAS6
SLC29A4
LGSN
TNIK
P4HA1
NUDT13
AFF3
IGFBP2
CMYA5
VIL1
SHBG
RASL10B
RAMP1
ZYG11A
PTPRS
GPR88
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PARP15
CDH23
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MIR675
LPA
MUC5B
HEPACAM
GNMT
SLITRK3
FAM9B
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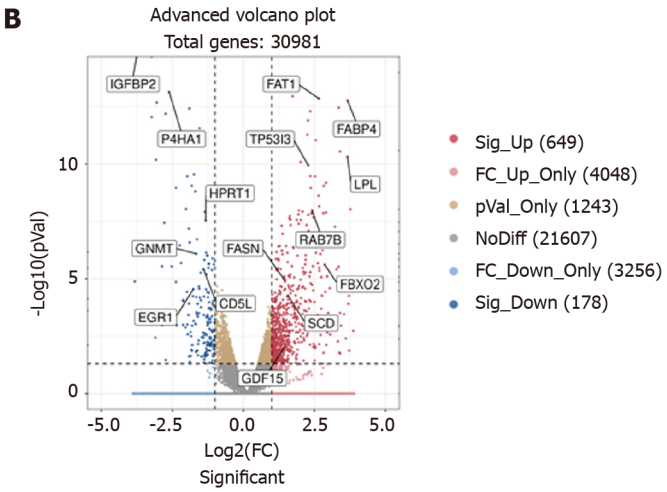
Normal 1
Normal 6
Normal 4
Normal 5
NAFLD 5
NAFLD 3
NAFLD 4
NAFLD 1
NAFLD 2
NAFLD 6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Advanced volcano plot
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FAT1
P4HA1
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LPL
HPRT1
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FBXO2
EGR1
CD5L
SCD
GDF15
10
5
0
-Log10(pVal)
-5.0
-2.5
0.0
2.5
5.0
Log2(FC)
Significant
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NoDiff (21607)
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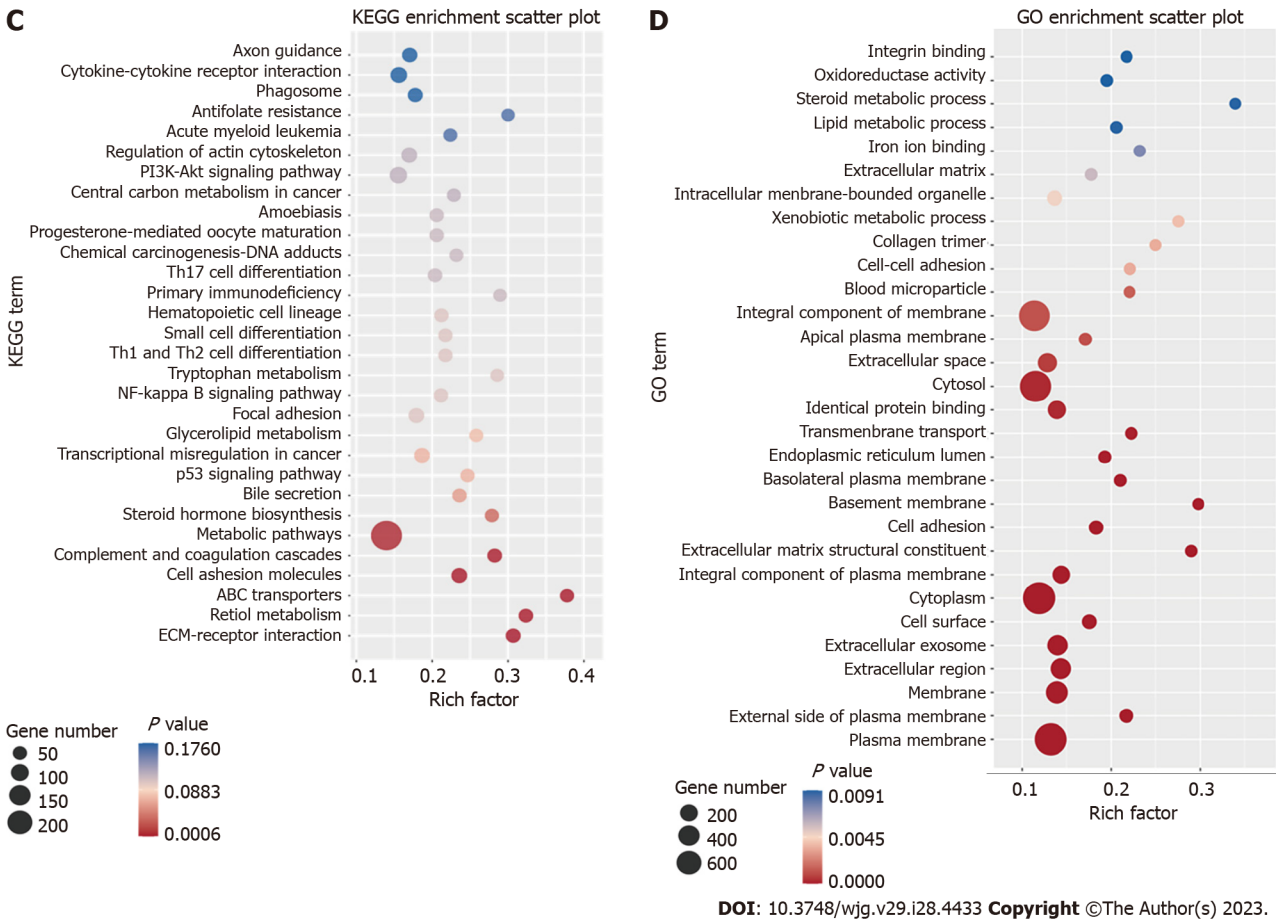


DOI: 10.3748/wjg.v29.i28.4433

Figure 1 Whole genome sequencing analysis of liver tissue from patients with non-alcoholic fatty liver disease and healthy controls. A and B: Heat map (A) and volcano plot (B) of the mRNA sequencing results; C: Scatter plot of the Kyoto Encyclopedia of Genes and Genomes enrichment analysis results; D: Scatter plot of the Gene Ontology enrich analysis results. FBXO2: F-box only protein 2; NAFLD: Non-alcoholic fatty liver disease; FC: Fold change; KEGG: Kyoto Encyclopedia of Genes and Genomes; GO: Gene Ontology.

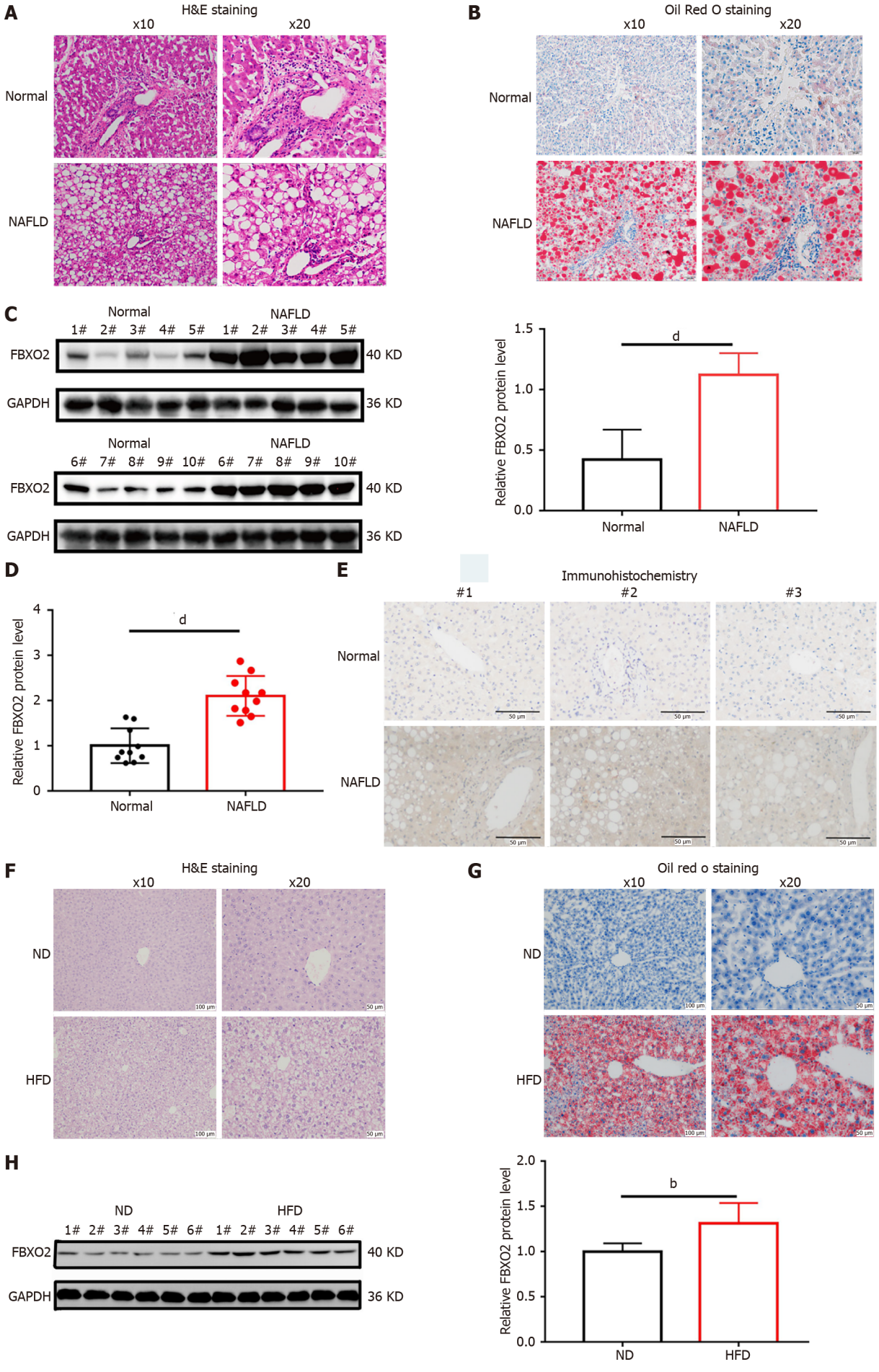
FBXO2 levels in the liver of patients with NAFLD and the HFD-fed mouse model were significantly increased compared to those in non-steatosis samples.

FBXO2 overexpression aggravates hepatic steatosis

Given the close correlation between FBXO2 expression and a fatty liver, we constructed a hepatocyte-specific FBXO2-overexpressing mouse model (Supplementary Figures 2A and B) to investigate the role of FBXO2 in exacerbating the HFD-induced liver steatosis, insulin resistance, and abnormal glucose metabolism. As expected, AAV-His-FBXO2-treated mice exhibited a significant increase in body weight (Supplementary Figure 2C), liver weight (Figure 3D), and the ratio of liver weight to body weight (Figure 3E) compared to AAV-His-vector-treated mice. The deposition of lipids in the liver was noticeably enhanced in AAV-His-FBXO2 mice, as determined by liver observations (Figure 3A), H&E staining (Figure 3B), Oil Red O staining (Figure 3C), and TG and T-Cho levels (Figures 3F and G). The overexpression of FBXO2 in the livers of AAV-His-FBXO2-treated mice significantly promoted the expression of lipogenesis genes [FASN and peroxisome proliferator-activated receptor gamma (PPARγ)] and fatty acid uptake genes [cluster of differentiation 36 (CD36) and fatty acid binding protein-1 (FABP1)] and suppressed the expression of fatty acid β-oxidation-related genes [acyl-CoA oxidase 1 (ACOX1), enoyl coenzyme A hydratase 1 (ECH1), medium-chain acyl-coenzyme A dehydrogenase (MCAD), and PPARα] (Figure 3H).

NAFLD is often accompanied by glucose metabolic disorders and insulin resistance. Unsurprisingly, FBXO2 overexpression significantly increased fasting blood glucose and insulin levels (Figures 3I and J). The HOMA-IR index was also increased accordingly (Figure 3K). Notably, there was no significant difference in food intake between AAV-His-FBXO2 mice and AAV-His-vector mice (Supplementary Figure 2D). In addition, the AAV-His-FBXO2 mice exhibited significantly higher blood glucose levels than AAV-His-vector mice, as determined from the OGTT and ITT data (Figures 3L-N).

We also investigated the effects of FBXO2 overexpression on lipid metabolic disorders *in vitro*. HepG2 cells were treated with OA to simulate the NAFLD phenotype. An increase in the mRNA and protein levels of FBXO2 was observed after 24 h of OA administration (Figures 4D and E). Oil Red O staining revealed that the intracellular lipid deposition (Figure 4A) and TG and T-Cho levels in HepG2 cells (Figures 4B and C) increased after OA treatment. Next, the His-FBXO2 plasmid was transfected into HepG2 cells. qRT-PCR and western blotting revealed increased FBXO2 expression (Supplementary Figures 3A and B). The effect of FBXO2 overexpression on lipid deposition was also observed in OA-


A
H&E staining
x10
x20
Normal
NAFLD
B
Oil Red O staining
x10
x20
Normal
NAFLD
C
Normal
NAFLD
1# 2# 3# 4# 5# 1# 2# 3# 4# 5#
FBXO2
40 KD
GAPDH
36 KD
Normal
NAFLD
6# 7# 8# 9# 10# 6# 7# 8# 9# 10#
FBXO2
40 KD
GAPDH
36 KD
Relative FBXO2 protein level
1.5
1.0
0.5
0.0
d
Normal
NAFLD
D
Relative FBXO2 protein level
4
3
2
1
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d
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NAFLD
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Immunohistochemistry
#1
#2
#3
Normal
NAFLD
F
H&E staining
x10
x20
ND
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G
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x10
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HFD
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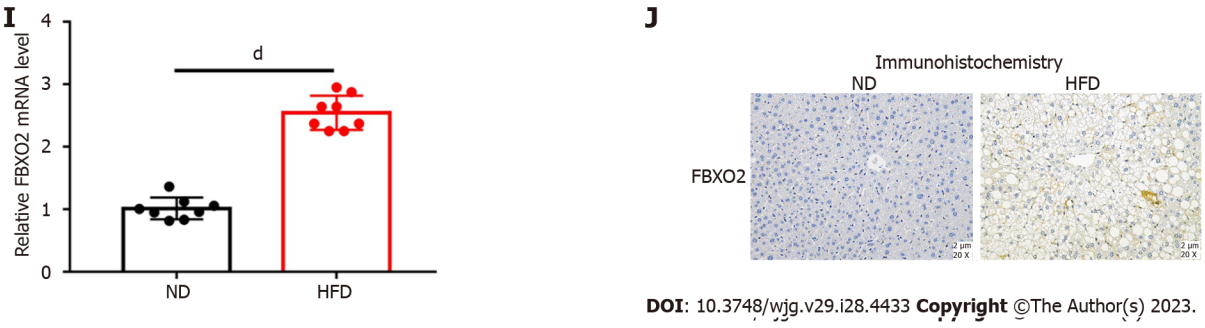

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Figure 2 F-box only protein 2 expression was up-regulated in livers of non-alcoholic fatty liver disease patients and high-fat diet mice models. A and B: Representative images of hematoxylin and eosin (H&E) and Oil Red O staining of liver tissues obtained from healthy controls and patients with non-alcoholic fatty liver disease (NAFLD). Scale bars, 50 μm and 100 μm; C: Western blot of F-box only protein 2 (FBXO2) expression in the liver tissues of healthy controls and patients with NAFLD (n = 10 liver samples per group); D: Expression of FBXO2 in liver tissue obtained from healthy controls and patients with NAFLD (n = 10 liver samples per group); E: Representative images of immunohistochemical staining of FBXO2 in liver tissue obtained from healthy controls and patients with NAFLD. Scale bars, 50 μm; F and G: Representative images of H&E and Oil Red O staining of liver tissue isolated from C57BL/6J mice treated with a normal chow diet (ND) or high-fat diet (HFD) for 16 wk. Scale bars, 50 μm and 100 μm; H: Representative images of FBXO2 western blots from liver tissue of C57BL/6J mice treated with an ND or HFD for 16 wk; I: Expression of FBXO2 in liver tissue obtained from C57BL/6J mice treated with an ND or HFD for 16 wk as detected using quantitative reverse transcription polymerase chain reaction (n = 8 liver samples per group); J: Representative images of immunohistochemical staining of FBXO2 in liver tissue obtained from C57BL/6J mice treated with an ND or HFD for 16 wk. Scale bars, 50 μm. $^{b}P < 0.01$, $^{d}P < 0.0001$. FBXO2: F-box only protein 2; NAFLD: Non-alcoholic fatty liver disease; HFD: High-fat diet; H&E: Hematoxylin and eosin; ND: Normal chow diet.

treated HepG2 cells. Specifically, FBXO2 overexpression after transfecting His-FBXO2 plasmids into HepG2 cells, greatly increased OA-induced lipid deposition (Figure 4F). FBXO2 overexpression also increased OA-induced cellular TG and T-Cho levels (Figures 4G and H). The overexpression of FBXO2 in OA-treated HepG2 cells also promoted the expression of FASN, PPARG, CD36, and FABP1 and suppressed the expression of ACOX1, ECH1, MCAD, and PPARα (Figure 4I). These data suggest that the up-regulation of FBXO2 expression in the liver exacerbated liver steatosis, HFD-triggered insulin resistance, and abnormal glucose metabolism.

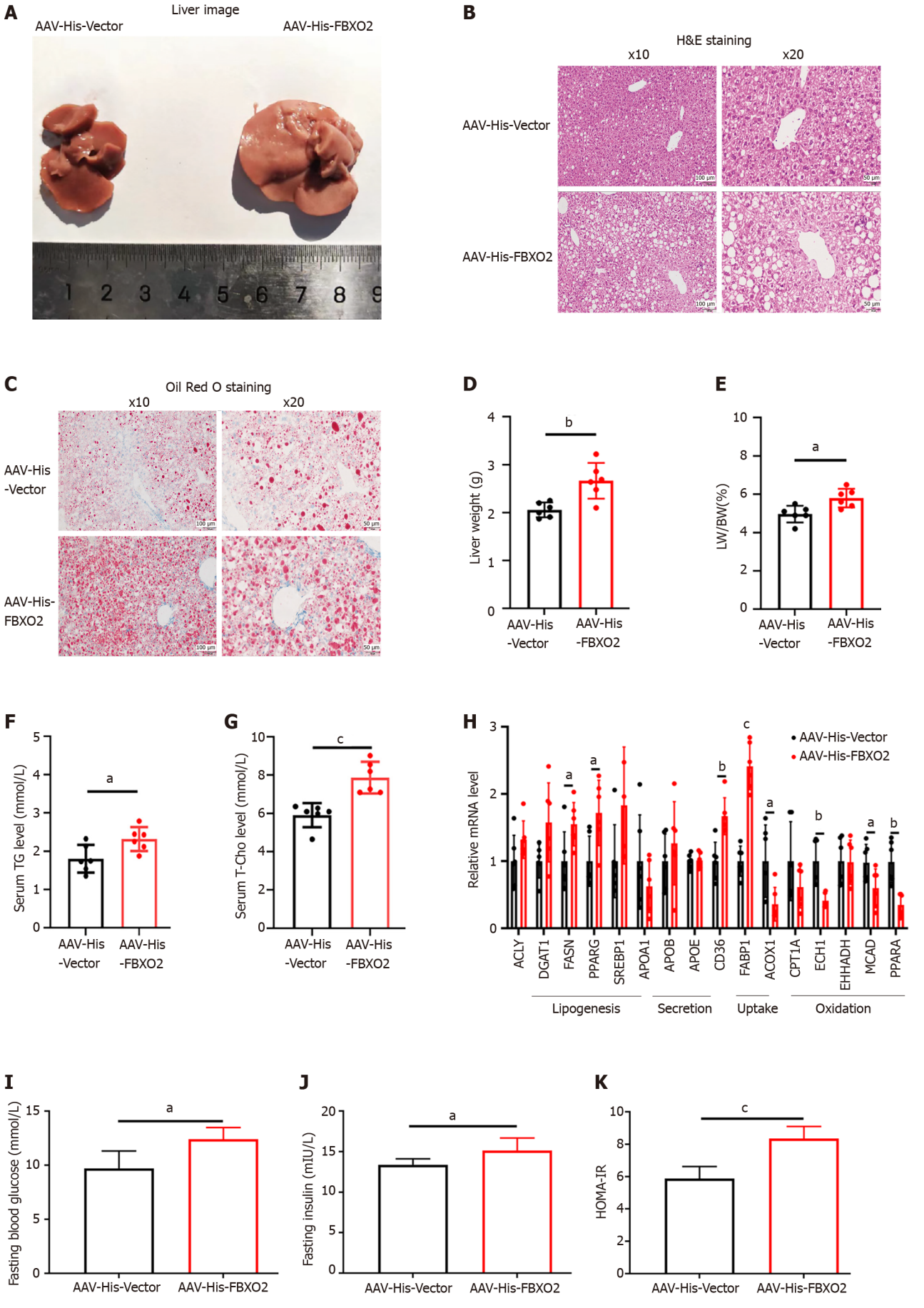
FBXO2 knockdown ameliorates lipid accumulation in hepatocytes

After confirming that the overexpression of FBXO2 exacerbated liver steatosis, we examined the effect of FBXO2 knockdown on lipid metabolic disorder in hepatocytes. To achieve this, the Sh-FBXO2 plasmid was transfected into HepG2 cells. Both qRT-PCR and western blot results indicated that FBXO2 expression levels decreased after transfection, confirming successful FBXO2 knockdown (Supplementary Figures 3C and D). FBXO2 knockdown decreased the OA-induced lipid deposition (Figure 4J) and regulated OA-induced TG and T-Cho secretion (Figures 4K and L). The knockdown of FBXO2 in OA-treated HepG2 cells inhibited the expression of lipogenesis genes (FASN and PPARγ) and fatty acid uptake genes (CD36 and FABP1) and promoted the expression of fatty acid β-oxidation related genes (ACOX1, ECH1, MCAD, and PPARα) (Figure 4M). Consistent with these data, *in vitro* gene therapy using Sh-FBXO2 plasmids further demonstrated that the knockdown of FBXO2 could significantly alleviate OA-induced lipid accumulation in hepatocytes (hepatic steatosis).

The up-regulation of FBXO2 expression promotes HADHA ubiquitination and degradation in HepG2 cells

We used a co-IP-based MS method to identify which proteins interact with endogenous FBXO2 in HepG2 cells. Based on peptide spectra matching protein score, HADHA was identified as a potential binding protein for FBXO2 in HepG2 cells (Figure 5A). *In vitro* interaction studies suggested that both exogenous and endogenous FBXO2 could bind directly to HADHA and vice versa. Additionally, the interaction between FBXO2 and HADHA was determined *via* a co-IP assay in HEK293T (Figures 5C and D) and HepG2 cells (Figures 5E and F). *In vitro* interaction tests suggested that both exogenous and endogenous FBXO2 could directly bind to HADHA and vice versa. Immunofluorescence staining also revealed the co-localization of FBXO2 and HADHA after the HEK293T cells were transfected with His-FBXO2 and Myc-HADHA plasmids (Figure 5B).

Next, we tested whether FBXO2 could activate the ubiquitination of HADHA. The expression of FBXO2 and HA-Ub together significantly enhanced the ubiquitination of HADHA in HEK293T cells, compared to control cells transfected with vectors expressing FBXO2 and HA-Ub alone (Figure 6A). Consistent with the results of the ubiquitination assays, gene expression analysis supported the reduction of HADHA protein levels in HepG2 cells and HFD-fed mouse models overexpressing FBXO2 (Figures 6B and D). Further, HADHA degradation was reduced after co-treatment with the proteasome inhibitor MG132 (SML1135; Sigma, United States) in HepG2 cells (Figure 6E). Moreover, FBXO2 knockdown increased the level protein of HADHA in HepG2 cells (Figure 6C). Notably, the mRNA levels of HADHA were not affected by FBXO2 overexpression or knockdown (Supplementary Figures 4A-C). Additionally, we analysed whether FBXO2 decreased the half-life of the HADHA protein. We transfected HepG2 cells with His-Vector or His-FBXO2 plasmids and treated them with the protein synthesis inhibitor cycloheximide (CHX) at different time points. The half-life of the HADHA protein was drastically reduced in FBXO2-overexpressing cells compared to the control cells (Figure 6F). These findings indicated that FBXO2 facilitates the degradation of HADHA *via* the ubiquitination machinery.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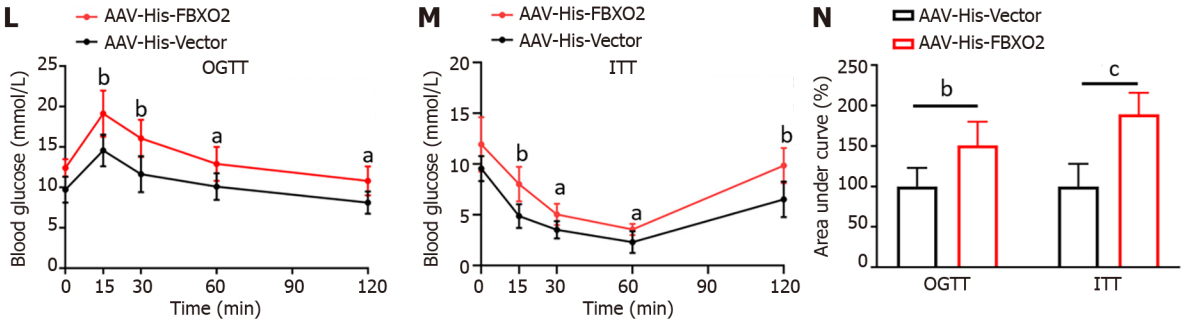

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Figure 3 Overexpression of F-box only protein 2 exacerbated high-fat diet-induced liver steatosis, insulin-resistance, and abnormal glucose metabolism. A: Representative images indicating histological changes in the livers of adeno-associated virus (AAV)-His-vector and AAV-His-F-box only protein 2 (FBXO2) mice after 16 wk of administering an high-fat diet (HFD); B and C: Representative images of hematoxylin and eosin and Oil Red O staining of liver tissue obtained from AAV-His-vector and AAV-His-FBXO2 mice after 16 wk of HFD. Scale bars, 50 μm and 100 μm; D-G: The liver weight, ratio of liver weight/body weight, serum triglyceride and total cholesterol levels in AAV-His-vector and AAV-His-FBXO2 mice after 16 wk of administering an HFD (n = 6 per group); H: quantitative reverse transcription polymerase chain reaction analysis of the expression of genes associated with lipogenesis, fatty acid secretion and uptake, and oxidation in AAV-His-vector and AAV-His-FBXO2 mice after 16 wk of administering an HFD (n = 6 per group); I: Fasting blood glucose levels; J: Fasting serum insulin levels; K: The homeostatic model assessment of insulin resistance; L: Oral glucose tolerance test (OGTT) scores; M: Insulin tolerance test (ITT); N: Calculated area under curve of OGTT and ITT in the AAV-His-vector mice and AAV-His-FBXO2 mice after 16 wk of HFD (n = 6 per group). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. AAV: Adeno-associated virus; FBXO2: F-box only protein 2; H&E: Hematoxylin and eosin; ITT: Insulin tolerance test; OGTT: Oral glucose tolerance test; LW: Liver weight; BW: Body weight; TG: Triglyceride; T-Cho: Total cholesterol; HOMA-IR: Homeostatic model assessment of insulin resistance; FASN: Fatty acid synthase; PPAR: Peroxisome proliferator-activated receptor; CD36: Cluster of differentiation 36; ACOX1: Acyl-CoA oxidase 1; ECH1: Enoyl coenzyme A hydratase 1; MCAD: Medium-chain acyl-coenzyme A dehydrogenase.

The above finding led us to determine whether HADHA alterations mediate the effect of FBXO2 on lipid accumulation in hepatocytes. First, FBXO2 overexpression increased OA-induced lipid deposition and TG and T-Cho secretion. Moreover, the overexpression of FBXO2 in OA- treated HepG2 cells enhanced the expression of FASN, PPARγ, CD36, and FABP1 and inhibited the expression of ACOX1, ECH1, MCAD, and PPARα. However, these effects were reversed after HepG2 cells were transfected with the Myc-HADHA plasmid to overexpress HADHA as indicated by the changes in lipid deposition (Figure 6G), TG and T-Cho secretion (Figures 6H and I), and mRNA expression of lipid metabolism-related genes (Figure 6J). FBXO2 and HADHA overexpression efficiency was also confirmed *via* western blot (Supplementary Figure 3E). Collectively, these indicated that the up-regulation of FBXO2 exacerbated OA-induced lipid accumulation by promoting the ubiquitination and degradation of HADHA in HepG2 cells.

DISCUSSION

FBXO2 is an E3 ligase that can regulate protein stability through ubiquitin-mediated protein-protein interaction and plays an indispensable role in the cell cycle regulation of the UPS[21]. It regulates cell proliferation, transcription, and apoptosis through the ubiquitination and degradation of target proteins, thereby affecting the occurrence and development of various diseases, such as malignant tumours, neuronal diseases, and inflammation[22,24,32,33]. However, the status of FBXO2 expression in NAFLD and its relationship with clinicopathological parameters has not been clarified. In the present study, we found that FBXO2 levels were significantly up-regulated in NAFLD liver tissues. Subsequently, we simulated NAFLD in HFD mice and OA-treated HepG2 cells, and the results were consistent with those obtained in human liver tissue. Our sequencing results of NAFLD liver tissue samples revealed changes in multiple metabolic pathways, for example the PI3K-AKT pathway. Recent studies conducted by our team found that the expression level of FBXO2 in NAFLD mouse models was downregulated after sleeve gastrectomy. Upregulation of FBXO2 could activate the PI3K-AKT pathway[31]. Based on the above results, we speculate that FBXO2 upregulation is related to NAFLD occurrence and development.

Next, we constructed a hepatocyte-specific FBXO2-overexpressing mouse model to further explore the relationship between FBXO2 and NAFLD. Lipid accumulation in the liver is a clinical feature of NAFLD and is caused by an imbalance between lipid input and output[34]. This imbalance is regulated by four pathways: Lipid lipogenesis, lipid uptake, lipid secretion, and fatty acid oxidation[27,35]. In the present study, we found that the deposition of liver lipids was visibly enhanced in hepatocyte-specific FBXO2-overexpressing mice models. FBXO2 overexpression in the liver significantly promoted the expression of lipogenesis genes (FASN and PPARγ) and fatty acid uptake genes (CD36 and FABP1) and inhibited the expression of fatty acid β-oxidation-related genes (ACOX1, ECH1, MCAD and PPARα) in AAV-His-FBXO2 mice. In contrast, FBXO2 knockdown in OA-treated HepG2 cells had the opposite effect on the expression of lipid accumulation and the expression of lipid metabolism-related genes. The alleviative effect of FBXO2 knockdown on lipid metabolism suggests its effect in treating NAFLD. In addition, combined with the effect of the overexpression and knockdown of FBXO2 on lipid metabolism, it is worth considering that FBXO2 may promote the ubiquitination of one or more proteins that can mediate lipid metabolism. In other words, the overexpression of FBXO2 can accelerate protein

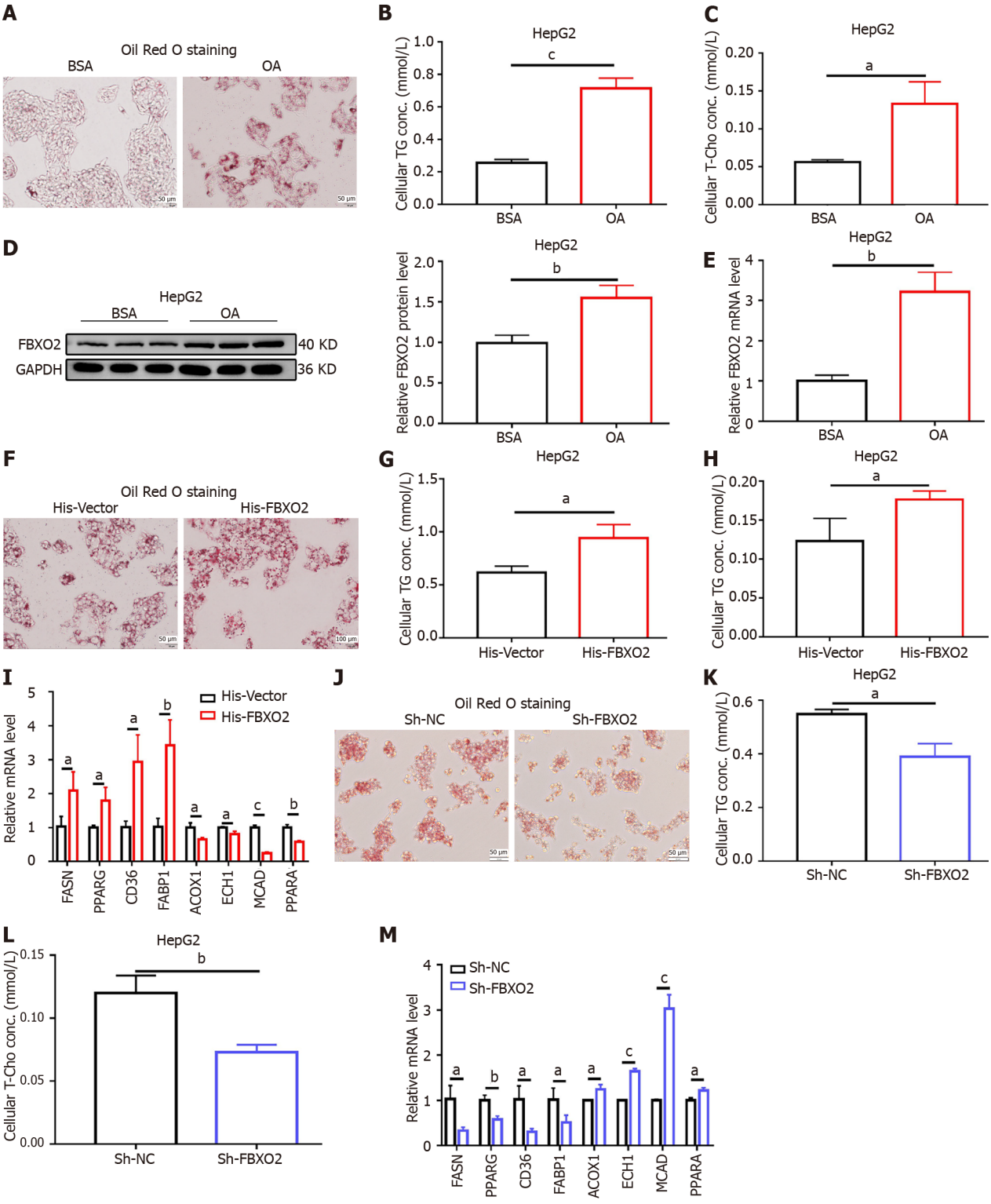

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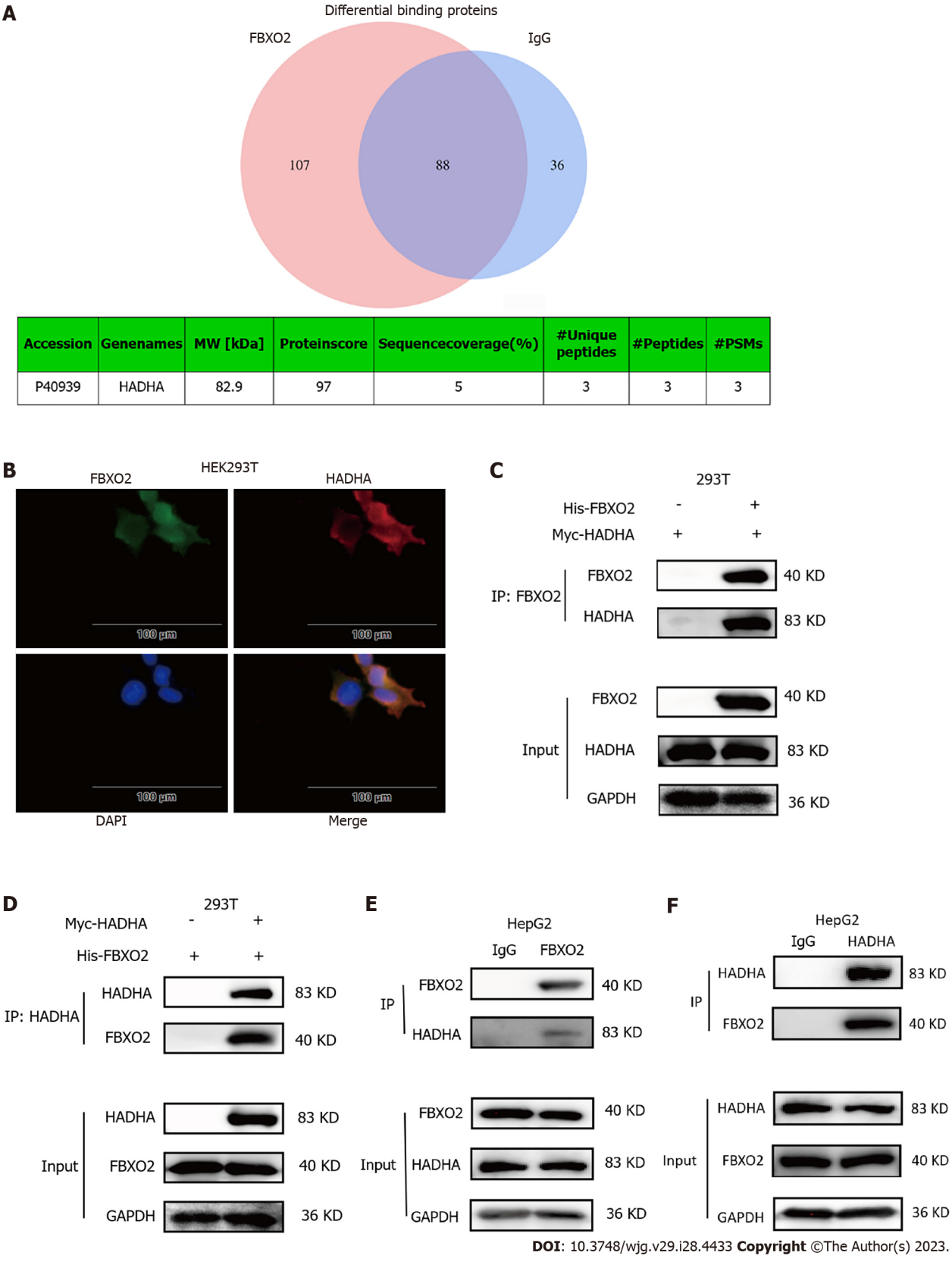
Figure 4 Effect of F-box only protein 2 overexpression or knockdown on lipid accumulation in hepatocytes. A: Representative images of Oil Red O staining of HepG2 cells treated with oleate (OA) for 24 h. Scale bar, 50 μm; B and C: Triglyceride (TG) and total cholesterol (T-Cho) levels in HepG2 cells treated with OA for 24 h; D and E: Western blot and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of F-box only protein 2 (FBXO2) expression in HepG2 cells treated with OA for 24 h; F-I: HepG2 cells transfected with His-FBXO2 plasmids to overexpress FBXO2, followed by OA treatment for 24 h; representative images of Oil Red O staining (F), TG (G) and T-Cho (H) levels; expression analysis of genes associated with lipogenesis, fatty acid uptake, and oxidation as detected using qRT-PCR (I); J-M: HepG2 cells were transfected with Sh-FBXO2 plasmids to knockdown FBXO2 expression, followed by OA treatment for 24 h; representative images of Oil Red O staining (J), TG (K) and T-Cho (L) levels; expression analysis of genes associated with lipogenesis, fatty acid uptake, and oxidation as detected using qRT-PCR (M). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. FBXO2: F-box only protein 2; BSA: Bovine serum albumin; OA: Oleate; TG: Triglyceride; T-Cho: Total cholesterol; FASN: Fatty acid synthase; PPAR: Peroxisome proliferator-activated receptor; CD36: Cluster of differentiation 36; ACOX1: Acyl-CoA oxidase 1; ECH1: Enoyl coenzyme A hydratase 1; MCAD: Medium-chain acyl-coenzyme A dehydrogenase; NC: Normal control.



Accession	Genenames	MW [kDa]	Proteinscore	Sequencecoverage(%)	#Unique peptides	#Peptides	#PSMs
P40939	HADHA	82.9	97	5	3	3	3

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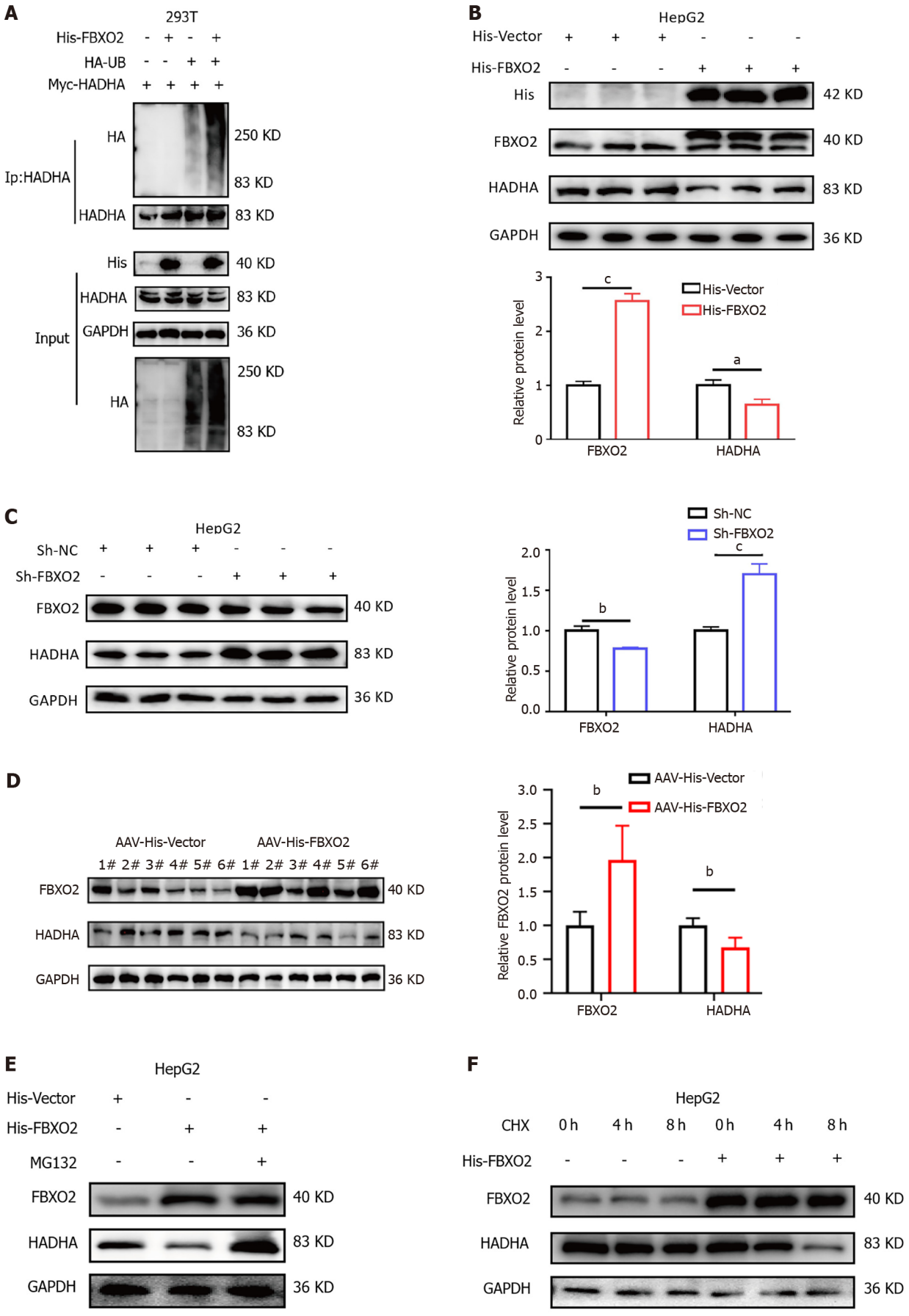
Figure 5 F-box only protein 2 directly binds to the hydroxyl CoA dehydrogenase alpha subunit and vice versa. A: Proteins interacting with endogenous F-box only protein 2 (FBXO2) in HepG2 cells as revealed using a co-immunoprecipitation (co-IP)-based mass spectrometry method; B: Immunofluorescence staining of HEK293T cells transfected with His-FBXO2 and Myc-the hydroxyl CoA dehydrogenase alpha subunit plasmids; C-F: Co-IP assays in HEK293T and HepG2 cells. FBXO2: F-box only protein 2; HADHA: Hydroxyl CoA dehydrogenase alpha subunit; IgG: Immunoglobulin G; DAPI: 4'-6-diamidino-2-phenylindole.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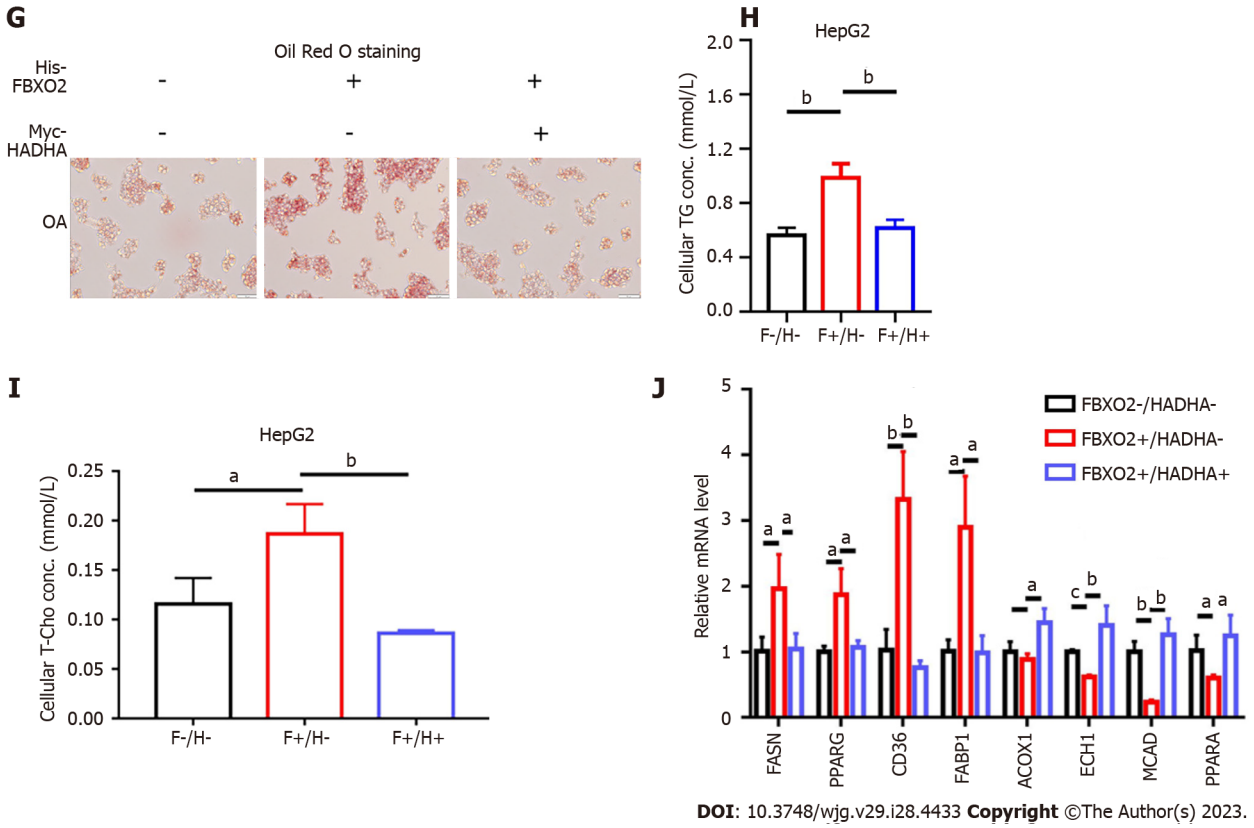

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Figure 6 F-box only protein 2 ubiquitinated and degraded the hydroxyl CoA dehydrogenase alpha subunit. A: The hydroxyl CoA dehydrogenase alpha subunit (HADHA) ubiquitination in HEK293T cells; B-D: Western blot of F-box only protein 2 (FBXO2) and HADHA expression in the His-Vector group *vs* His-FBXO2 group (B), Sh-NC group *vs* Sh-FBXO2 group (C), and adeno-associated virus (AAV)-His-vector mice *vs* AAV-His-FBXO2 mice (D); E: Western blot of FBXO2 and HADHA expression in HepG2 cells. HepG2 cells were transfected with His-FBXO2 plasmids for 24 h, followed by incubation with or without MG132 for 24 h; F: Western blot of FBXO2 and HADHA expression in HepG2 cells. HepG2 cells were transfected with FBXO2 adenovirus for 48 h, followed by incubation with cycloheximide (CHX) for 0, 4, or 8 h before sample collection; G-J: HepG2 cells were transfected with His-FBXO2 plasmids and Myc-HADHA plasmids to overexpress FBXO2 and HADHA, respectively, followed by treatment with oleate for 24 h; representative images of Oil Red O staining (G), triglyceride (H) and total cholesterol (I) levels; expression analysis of genes associated with lipogenesis, fatty acid uptake, and oxidation as detected using quantitative reverse transcription polymerase chain reaction (J). FBXO2: F-box only protein 2; HADHA: Hydroxyl CoA dehydrogenase alpha subunit; $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. AAV: Adeno-associated virus; OA: Oleate; TG: Triglyceride; T-Cho: Total cholesterol; FASN: Fatty acid synthase; PPAR: Peroxisome proliferator-activated receptor; CD36: Cluster of differentiation 36; ACOX1: Acyl-CoA oxidase 1; ECH1: Enoyl coenzyme A hydratase 1; MCAD: Medium-chain acyl-coenzyme A dehydrogenase; NC: Normal control.

degradation, thus aggravating NAFLD. On the contrary, the knockdown of FBXO2 protects lipid metabolising enzymes from degradation, thus maintaining normal lipid metabolism.

To further explore the mechanism through which FBXO2 affects NAFLD, we screened the proteins that could interact with endogenous FBXO2 in HepG2 cells using a co-IP-based MS method. The mitochondrial trifunctional enzyme subunit-alpha (HADHA) was identified as a potential binding protein for FBXO2. HADHA, a mitochondrial β-oxidation-associated enzyme, encodes the α-subunit of the mitochondrial trifunctional protein (hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase). This enzyme complex catalyses the three steps of the β-oxidation of fatty acids in the mitochondria[14]. HADHA deficiency in mice has been reported to aggravate NAFLD[36-38]. Further, HADHA knockout can lead to hyperglycemia and consequently induce liver lipid deposition[18,39]. *In vitro* interaction tests in the present study suggested that both exogenous and endogenous FBXO2 could directly bind to HADHA and vice versa. In addition, the results of our ubiquitination assays showed that FBXO2 facilitates the degradation of HADHA *via* the ubiquitination machinery. We further confirmed the interaction between FBXO2 and HADHA in hepatocytes using MG132 and CHX. We also found that FBXO2 overexpression during OA-induced liver steatosis was reversed after Myc-HADHA plasmids were transfected into HepG2 cells to overexpress HADHA.

Another interesting observation is that in addition to the exacerbation of steatosis, the targeted upregulation of FBXO2 expression in HFD-fed mouse livers led to a significant exacerbation of hyperglycemia and insulin resistance, which could be caused by various reasons. Previous studies have shown that FBXO2 targets insulin receptors to achieve ubiquitin-dependent degradation, thus regulating insulin signaling integrity[24]. The overexpression of FBXO2 can stimulate severe insulin resistance in the liver and inhibit its normal function, promoting adipogenesis. Therefore, increased insulin resistance leads to excessive lipid accumulation in the liver[34]. Recent studies have shown that HADHA knockdown augments the glucagon response[14]. This study speculates that a decrease in HADHA levels might induce an increase in blood glucose levels after the overexpression of FBXO2. In addition, the increased expression of lipogenesis- and fatty acid uptake-associated genes and decreased expression of fatty acid β-oxidation-related genes might also contribute to the observed effects on glycemic control[40-44].

CONCLUSION

Liver FBXO2 expression is upregulated in NAFLD and FBXO2 is correlated with metabolic disorders. Moreover, upregulation of FBXO2 exacerbates liver steatosis by promoting the degradation of HADHA. Finally, our findings suggest that FBXO2 can be used as a feasible therapeutic target for NAFLD.

ARTICLE HIGHLIGHTS

Research background

Non-alcoholic fatty liver disease (NAFLD) is a major health burden with an increasing global incidence. Unfortunately, the unavailability of knowledge underlying NAFLD pathogenesis inhibits effective preventive and therapeutic measures. The key issues affecting the treatment of patients with NAFLD are atherosclerotic cardio-cerebrovascular diseases, cirrhosis, and malignant tumors associated with the metabolic syndrome. The abnormal expression of F-box only protein 2 (FBXO2), an E3 ligase, in obese mice disrupts glucose homeostasis through ubiquitin-mediated insulin receptor degradation, however, the role of FBXO2 in NAFLD pathology remains unclear.

Research motivation

Recent studies conducted by our team found that the expression level of FBXO2 in NAFLD mouse models was downregulated after sleeve gastrectomy.

Research objectives

This study aimed to explore the molecular mechanism of NAFLD.

Research methods

Whole genome sequencing (WGS) analysis was performed on liver tissues from patients with NAFLD (n = 6) and patients with normal metabolic conditions (n = 6) to identify the target genes. A NAFLD C57BL6/J mouse model induced by 16 wk of high-fat diet (HFD) feeding and a hepatocyte-specific FBXO2 overexpression mouse model were used for *in vivo* studies. Plasmid transfection, co-immunoprecipitation-based mass spectrometry assays, and ubiquitination in HepG2 cells and HEK293T cells were used for *in vitro* studies.

Research results

A total of 30982 genes were detected in WGS analysis, with 649 up-regulated and 178 down-regulated. Expression of FBXO2 was upregulated in the liver tissues of patients with NAFLD. Hepatocyte-specific FBXO2 overexpression facilitated NAFLD-associated phenotypes in mice. Overexpression of FBXO2 aggravated odium oleate (OA)-induced lipid accumulation in HepG2 cells, resulting in an abnormal expression of genes related to lipid metabolism, such as fatty acid synthase, peroxisome proliferator-activated receptor alpha, and so on. In contrast, knocking down FBXO2 in HepG2 cells significantly alleviated the oleate-induced lipid accumulation and aberrant expression of lipid metabolism genes. The hydroxyl CoA dehydrogenase alpha subunit (HADHA), a protein involved in oxidative stress, was a target of FBXO2-mediated ubiquitination. FBXO2 directly bound to HADHA and facilitated its proteasomal degradation in HepG2 and HEK293T cells. Supplementation with HADHA alleviated lipid accumulation caused by FBXO2 overexpression in HepG2 cells.

Research conclusions

FBXO2 exacerbates lipid accumulation by targeting HADHA.

Research perspectives

Our results shed light on the role of FBXO2 not only in NAFLD but also in other metabolic diseases involving lipid accumulation, supporting further investigation of the therapeutic potential of this molecule in further studies.

FOOTNOTES

Author contributions: Hu SY and Liu Z contributed equally to this work; Hu SY and Liu Z designed the research study; Liu Z, Chen NY, Zhang Z, and Zhou S performed the research; Liu Z and Chen NY contributed new reagents and analytic tools; Liu Z and Chen NY analyzed the data and wrote the manuscript; and all authors have read and approve the final manuscript.

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Institutional animal care and use committee statement: All animal experiments conformed to the internationally accepted principles for the care and use of laboratory animals (license No. S453, Institutional Animal Care and Use Committee of Shandong Provincial Qian

Foshan Hospital).

Informed consent statement: Written informed consent was obtained from the patients and their families.

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ORIGINAL ARTICLE

Clinical Trials Study

Efficacy and dose response of *Lactiplantibacillus plantarum* in diarrhea-predominant irritable bowel syndrome

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Abstract

BACKGROUND

Probiotics have shown promise in alleviating symptoms of diarrhea-predominant irritable bowel syndrome (IBS-D); however, the certainty of evidence is low. Well-powered randomized controlled dose-ranging trials are warranted on promising single-strain candidates.

AIM

To investigate the clinical efficacy of *Lactiplantibacillus plantarum* (*L. plantarum*) Lpla33 (DSM34428) in adults with IBS-D.

METHODS

This is a randomized, double-blind, placebo-controlled, multi-center, and dose-ranging study. Three hundred and seven adults, 18-70 years of age, with IBS-D, according to Rome IV criteria, were allocated (1:1:1) to receive placebo or *L. plantarum* Lpla33 at 1×10^9 (1B) or 1×10^{10} (10B) colony-forming units/d over an 8-wk intervention period. The primary outcome was the change in IBS severity scoring system (IBS-SSS) total score after 8 wk, while secondary and exploratory outcomes included abdominal pain severity, IBS related quality of life, stool and microbial profile, and perceived stress.

RESULTS

IBS-SSS was significantly reduced, after 8 wk, in participants receiving *L. plantarum* 1B (-128.45 ± 83.30; $P < 0.001$) and *L. plantarum* 10B (-156.77 ± 99.06; $P < 0.001$), compared to placebo (-58.82 ± 74.75). Further, a dose-ranging effect was observed, with a greater absolute reduction in the *L. plantarum* 10B group ($P <$

0.05). A reduction in sub-scores related to abdominal pain, abdominal distension, bowel habits, and quality of life was observed in both *L. plantarum* groups compared to placebo ($P < 0.001$). Further, 62.5% and 88.4% of participants administered *L. plantarum* 1B and 10B, respectively, were classified as stool consistency responders based on a reduction in diarrheal stool form, as compared to 26.3% in the placebo group ($P < 0.001$). In contrast, no significant shifts were observed in microbial diversity.

CONCLUSION

L. plantarum Lpla33 (DSM34428) is well tolerated and improves IBS symptom severity with a dose-ranging effect and a corresponding normalization of bowel habits in adults with IBS-D.

Key Words: Randomized clinical trial; Diarrhea-predominant irritable bowel syndrome; Microbiome; Probiotic; *Lactiplantibacillus plantarum*

Core Tip: The current study is unique in its dose-ranging assessment of a *Lactiplantibacillus plantarum* (*L. plantarum*) probiotic strain in a well-powered multi-center randomized controlled trial in adults with diarrhea-predominant irritable bowel syndrome (IBS-D). *L. plantarum* Lpla33 (DSM34428) was well tolerated and significantly improved global IBS symptom scores as compared to placebo, both at an absolute level and in the number of clinically relevant responders. Further, a dose-ranging effect was observed in global IBS symptom scores, abdominal pain severity, quality of life, and normalization of diarrheal stool type.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a chronic disorder of gut-brain interaction, characterized by abdominal pain in association with altered bowel habits[1]. Current estimates report a global prevalence of 4% to 9%, based on Rome IV or Rome III diagnostic criteria, respectively[2]. IBS is further classified according to the predominant stool pattern, with diarrhea predominant subtype (IBS-D) reported to be the most common, affecting approximately 40% of adults with IBS [3]. Pathophysiology is multi-factorial and may include altered gastrointestinal (GI) microbiota, motility or barrier function, immune activation, neurotransmitter imbalance, or visceral hypersensitivity[4,5]. In addition to GI symptoms, IBS significantly impacts quality of life (QoL) and is associated with work absenteeism and avoidance of daily activities [6].

IBS-D treatment options include United States Food and Drug Administration approved pharmacologic therapies such as rifaximin, eluxadoline, and alosetron[4]. However, clinical management is challenging due to chronic daily administration, safety concerns, and the heterogeneity of symptoms to address. Increasing evidence supports a link between psychological processes and IBS symptomology *via* altered gut-brain interaction, and there is a growing application of psychological interventions for IBS[7]. Within the gut, dysbiosis, including reduced abundance of lactobacilli and *Bifidobacterium*[8,9], has been reported and is linked to features of IBS-D such as intestinal permeability and low-grade inflammation[6,10]. There are conflicting reports regarding the efficacy of fecal microbiota transplantation in IBS, with enriched lactobacilli, *Streptococcus, Dorea*, and *Ruminococcaceae* reported as favorable bacterial profiles for donors[11].

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit to the host[12]. Select strains are hypothesized to alleviate symptoms of IBS by promoting intestinal barrier function, modulating markers of inflammation, regulating immune cell balance, preventing pathogen adherence, or producing bioactive molecules such as short chain fatty acids (SCFAs), neurotransmitters, or their precursors[5]. A recent meta-analysis of probiotics for adults with IBS-D assessed ten randomized controlled trials evaluating 943 participants[13]. Probiotics were deemed safe and superior to placebo regarding global IBS-D symptoms, abdominal pain, and abdominal distension, but had no beneficial effect on QoL, stool frequency, or flatulence[13]. Additionally, the certainty of evidence was very low to low, due to heterogeneity between studies, and differences in study population, strain selection, dose, dosage form, and duration of intervention[13]. Another recent systematic review and meta-analysis accounted for probiotic strain-specific efficacy in IBS and included 42 randomized controlled trials evaluating 3856 participants[14]. The meta-analysis highlighted single-strains and combinations showing efficacy in at least one IBS outcome measure; however, most studies did not assess outcomes by IBS subtype[14]. As a result, there is a need for well-powered randomized controlled trials on promising single strain candidates with an IBS-D focus.

The present study investigated a *Lactiplantibacillus plantarum* (*L. plantarum*) strain with several relevant attributes, including GI persistence, maintenance of intestinal barrier function, and anti-pathogenic, anti-inflammatory, and bile salt hydrolase activity (Chr. Hansen internal data). The objective was to assess the efficacy and tolerability of *L. plantarum* Lpla33 (DSM34428), with respect to IBS symptomology in a randomized, double-blind, placebo-controlled, multi-center, dose-ranging study in adults with IBS-D.

MATERIALS AND METHODS

Study population and setting

Females and males, ages 18 to 70 years, meeting Rome IV diagnostic criteria for IBS-D were recruited from 12 gastroenterology specialized centers across India. As per Rome IV, included participants had recurrent abdominal pain, at least once a week for the last 3 mo, with onset of symptoms for at least 6 mo, and with at least two of the following criteria: Pain related to defecation or associated with change in stool frequency or stool form. Participants also had a history of abnormal bowel movements wherein diarrhea (type 6 or 7) was the predominant (> 25%) stool pattern on the Bristol stool scale (BSS). Further, an electronic diary was used during a 14-d placebo run-in period, wherein it was re-confirmed that predominant stool type was type 6 or 7 on the BSS. Additionally, participants had an abdominal pain severity-numeric rating score (APS-NRS) of ≥ 4 (on an 11-point scale) and an IBS severity scoring system (IBS-SSS) total score of > 175 (on a 500-point scale) prior to and after a 14-d placebo run-in period. Dietary preferences of all participants included non-vegetarian options.

The exclusion criteria included a history of organic gastrointestinal disease, including inflammatory bowel disease or ischemic colitis, a history of surgical resection of the GI tract, acute gastroenteritis or complications from infectious enteritis, a recent diagnosis of *Helicobacter pylori* infection, a history of gluten or lactose intolerance, a history of malignant tumors, or a history of neurological or psychiatric conditions. A current or recent history (*i.e.*, past 2 years) of smoking as well as heavy drinking, defined as more than 14 or 7 units per week, for males and females, respectively, was also an exclusion criterion. Additionally, individuals with type I or II diabetes, or uncontrolled hypertension were excluded, as well as individuals with abnormal thyroid-stimulating hormone (< 0.35 mIU/L or > 4.94 mIU/L) or hemoglobin (< 6.21 mmol/L) at screening. The use of probiotic/prebiotic supplements or herbal medicines for gut health, was not permitted within 4 wk prior to screening or during the study. Prohibited medications during the study period included antibiotics, antidiarrheals, or other medications affecting gastric motility and anti-depressants, among others. Lastly, participants who were pregnant or intended to get pregnant or breastfeeding were excluded. All participants provided their voluntary, written, informed consent prior to their inclusion.

Study design and procedures

The study was conducted in accordance with the ethical principles that have their origins in the current version of the Declaration of Helsinki, the International Council on Harmonization E6 Good Clinical Practice, and all applicable local regulatory requirements. The study was approved and monitored by an independent ethics committee (Approval No: VED/P-20/22/JUL/2021; Address: ACEAS, Ahmedabad, Gujarat, India). The trial was prospectively registered on clinicaltrials.gov under study number NCT04950296.

This is a prospective, randomized, double-blind, placebo-controlled, multi-center, and parallel-arm study, including two doses of the probiotic product and one placebo arm. The study population was recruited from physician databases of individuals with IBS symptoms within the gastroenterology specialized centers. Participants visited the clinical sites for screening at least 14 d prior to planned randomization. At screening, the study was explained to participants in their local language, following which written informed consent was obtained. Participants were assessed for inclusion-exclusion criteria; a clinical examination was performed, and medical history, concomitant medication, demographic details, and vitals were recorded. Participants meeting the screening criteria underwent a 2-wk placebo run-in period to observe pre-study symptomology and to identify placebo responders, defined as a decrease of more than 25% in APS-NRS. Participants not meeting the placebo responder criteria were eligible for randomization (day 0) and subsequent visits at day 28 ± 2 and day 56 ± 2 over an 8-wk intervention period.

Participants were randomized (1:1:1), without stratification, to receive either placebo, *L. plantarum* (1B), or *L. plantarum* (10B). Block randomization (block size of six) was performed using Stats Direct software (Version 3.1.17), generating distinct alphanumeric codes, with every block containing two of each arm in random sequence. The study sites were allocated randomization codes in series, and the sites dispensed blinded product and corresponding randomization code, in sequential order of participant qualification. The randomization codes were concealed from site investigators, as well as site and study teams involved in study conduct and evaluation, with blinding codes secured in tamper-evident sealed envelopes.

Study products

Placebo and *L. plantarum* capsules were prepared in compliance with standard operating and quality control procedures at Chr. Hansen Inc. (Wausau, WI, United States). Microbiological analyses were confirmed following production. *L. plantarum* 1B and 10B capsules were formulated with the lyophilized strain and microcrystalline cellulose and contained a potency of not less than (NLT) 1×10^9 colony-forming units (CFU)/capsule or 1×10^{10} CFU/capsule, respectively. Placebo capsules were formulated with microcrystalline cellulose. All capsules were size 1 hypromellose, contained minimal but identical quantities of magnesium stearate and silica as flow aids, were identical in mass, appearance, and taste, and were bottled in identical sealed 112 mL CSP bottles (Aptar CSP Technologies Inc., Auburn, AL, United States) containing 35

capsules per bottle. Participants took the allotted study product orally, one capsule daily before lunch, with a glass of water. Compliance was assessed *via* participants' investigational product diary and confirmed by examining unused study product at each visit.

Outcome measures

On days 0, 28, and 56 visits, study outcomes were assessed, a clinical examination was conducted, study product was dispensed or collected, and concomitant medication usage was recorded. Metronidazole (400 mg/d) was provided as a rescue medication in the case of severe pain and/or frequent loose stool, as it has been shown to provide symptom relief in IBS without affecting rectosigmoid motility[15]. Study product compliance was assessed *via* participants' investigational product diary and confirmed by examining unused study product at each visit. A dietary diary was completed by participants over the 14 d prior to each study visit, recording dietary intake for two weekdays and one weekend day. Dietary information was processed *via* HealthifyMe software[16] for average daily intake of calories and macronutrients over the study period.

The pre-specified ranking of primary and secondary outcomes is listed in Supplementary Table 1. The primary outcome was the change in IBS symptom severity, assessed *via* the IBS-SSS total score[17] from baseline to day 56. Scores on the IBS-SSS range from 0 to 500, with five domains related to abdominal pain severity and frequency, abdominal distention, dissatisfaction with bowel habits, and interference with QoL. A decrease of 95 points has been associated with clinically meaningful improvement[18], and was used for classification of IBS-SSS responders.

Among secondary outcomes, abdominal pain severity was assessed *via* an 11-point numeric rating scale (APS-NRS) [18], with 10 representing most severe pain and 0 representing no pain. Post-screening APS-NRS scores were submitted using a digital diary on a weekly basis. An average of the prior weeks' score was considered as the score for the corresponding visit.

Stool consistency was assessed *via* the BSS, a validated ordinal scale of stool types[19] ranging from 1 through 7, with types 6-7 being indicative of diarrhea. Stool consistency responders were defined as a decrease of 50% or more *vs* baseline in the number of days per week with at least one types 6-7 bowel movement[20].

IBS related QoL was assessed *via* the IBS-QoL, a 34-item questionnaire wherein the individual responses are summed and transformed to a 0-100 scale, with increasing scores indicating improved IBS specific QoL[21]. Lastly, mental stress was assessed using the perceived stress scale (PSS), consisting of 10 items and a total score ranging from 0-40, with higher scores indicating higher perceived stress[22].

Microbial profiling

Participants collected fecal samples, as close as possible but prior to the day 0 and day 56 visits, inside a biome collector device and ultimately a barcoded stool collection tube with preservation buffer. Stool collection tubes were stored at the individual sites, with regular shipments to a central laboratory, wherein they were shipped approximately every 8 wk to CosmosID (Germantown, MD, United States) for analysis. Cold chain (2-8 °C) was maintained during the entire process with data loggers used to confirm temperature variations during shipment.

Sample DNA isolation and quantification, library preparation, and shotgun sequencing of fecal samples were performed by CosmosID. DNA from samples was isolated using the QIAGEN DNeasy PowerSoil Pro Kit, according to the manufacturer's protocol. DNA samples were quantified using the GloMax Plate Reader System (Promega) using the QuantiFluor® dsDNA System (Promega) chemistry. DNA libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina) and IDT Unique Dual Indexes with total DNA input of 1 ng. Genomic DNA was fragmented using a proportional amount of Illumina Nextera XT fragmentation enzyme. Unique dual indexes were added to each sample followed by 12 cycles of PCR to construct libraries. DNA libraries were purified using AMpure magnetic beads (Beckman Coulter) and eluted in QIAGEN EB buffer. DNA libraries were quantified using Qubit 4 fluorometer and Qubit dsDNA HS Assay Kit. Libraries were then sequenced on an Illumina NextSeq 2000 platform (2 × 150 bp).

Statistical analysis

The sample size calculation was based on the primary outcome. A reduction in IBS-SSS score of at least 15% in the *L. plantarum* 1B group as compared to placebo, was considered a relevant treatment effect[23]. The standard deviation of the primary outcome was estimated at 30%. A placebo effect was estimated at 20%-25%. Assuming a 90% study power and a two-sided statistical significance level of 5%, the sample size was estimated to be approximately 86 participants per group. With an estimated 15% drop-out rate, 100 participants per group were planned to be randomized.

The primary and secondary outcomes (Supplementary Table 1) were assessed on the intention-to-treat (ITT) population. Descriptive statistics are presented as the mean (SD) for continuous variables or as a percentage for qualitative variables. Data normality was assessed using the Shapiro-Wilk test. Differences between groups for demographic and clinical characteristics at baseline were analyzed using one-way ANOVA for continuous variables or Pearson chi-square test for categorical variables. An analysis of covariance (ANCOVA) was assessed for continuous outcomes on the absolute change from baseline to subsequent visit. Dunnett's test for multiple comparisons was applied to assess statistical significance for each *L. plantarum* dose group as compared to placebo. A two-sample *t*-test was used to compare the difference between the two *L. plantarum* dose groups. Categorical outcomes were assessed *via* a Pearson chi-square test. The associations between efficacy parameters were assessed *via* Pearson correlation coefficients. Descriptive and inferential statistics were performed using Statistical Package for the Social Sciences (SPSS, IBM®) Python 3.0 (Armonk, NY, United States).

Microbial profile stacked bar figures were generated using the R package ggpubr. Linear Discriminant Analysis Effect Size (LEfSe) was calculated with a Kruskal-Wallis alpha value of 0.05, a Wilcoxon alpha value of 0.05, and a logarithmic

LDA score threshold of 2.0.

RESULTS

Study parameters

A total of 333 screened participants were assessed for eligibility and 314 participants were randomized in the study (Figure 1). Enrolment and intervention occurred continuously from September 2021 through May 2022. Seven participants were incorrectly enrolled, without meeting entry criteria, and were excluded. Three hundred and seven participants were randomized as part of the ITT population, with 104, 104, and 99 allocated to the placebo, *L. plantarum* 1B, and *L. plantarum* 10B groups, respectively. A total of 298 participants completed the first follow-up visit, while nine participants were lost to follow-up with no post-baseline efficacy data. Study attrition rates over the intervention period were 5 (4.8%), 2 (1.9%), and 4 (4.0%) for the placebo, *L. plantarum* 1B, and *L. plantarum* 10B groups, respectively. A total of 294 participants completed the study per protocol.

Baseline characteristics

The screening and baseline characteristics of the ITT population are presented in Table 1. The three groups had similar demographic and clinical characteristics with no differences between groups ($P > 0.05$). The mean ages of participants were 39.5, 38.7, and 40.5 years across the three groups with a nearly even distribution of females and males. Participants met Rome IV criteria for IBS-D and demonstrated similar baseline mean IBS-SSS scores of 308.7, 315.2, and 302.9 for the placebo, *L. plantarum* 1B, and *L. plantarum* 10B groups, respectively ($P > 0.05$). At baseline, 43.4%, 41.4%, and 47.4% of participants in the placebo, *L. plantarum* 1B, and *L. plantarum* 10B groups, respectively, had moderate (175-300) IBS-SSS profiles, while 56.6%, 58.7%, and 52.6% of participants in the placebo, *L. plantarum* 1B, and *L. plantarum* 10B groups, respectively, had severe (> 300) IBS-SSS profiles.

IBS symptom severity

A significant between-group difference was observed in the primary outcome, change in IBS-SSS total score after 56 d, for both *L. plantarum* groups when compared to placebo (Table 2). On day 56, a greater reduction in IBS-SSS total score was observed in the *L. plantarum* 1B (-128.45 ± 83.30) and *L. plantarum* 10B (-156.77 ± 99.06) groups, as compared to placebo (-58.82 ± 74.75) ($P < 0.001$). Similarly, a greater reduction in IBS-SSS total score was observed on day 28 in the *L. plantarum* 1B (-66.37 ± 72.27) and *L. plantarum* 10B (-85.80 ± 89.53) groups, as compared to placebo (-35.68 ± 60.81) ($P < 0.01$). Comparing the two dose groups, the *L. plantarum* 10B group demonstrated a significantly greater reduction in IBS-SSS total score at day 56 ($P < 0.05$). End-of-study IBS-SSS profiles were considered either in remission or mild (< 175) in 48.1% and 72.6% of participants in the *L. plantarum* 1B and *L. plantarum* 10B groups, respectively. as compared to 11.1% in the placebo group ($P < 0.001$).

Participants receiving *L. plantarum* 1B capsules reported significant reductions in the scores of IBS-SSS individual domains over the intervention period, as compared to placebo, including abdominal pain severity (-28.91 ± 20.36), abdominal pain duration (-15.29 ± 13.72), abdominal distension (-29.33 ± 21.64), bowel habits (-27.73 ± 19.54) and QoL (-27.19 ± 19.19) ($P < 0.001$). Similarly, participants receiving *L. plantarum* 10B capsules reported significant reductions in IBS-SSS domain specific scores, as compared to placebo, including abdominal pain severity (-36.52 ± 24.39), abdominal pain duration (-19.68 ± 14.91), abdominal distension (-35.45 ± 24.00), bowel habits (-32.31 ± 25.12), and QoL (-32.81 ± 23.66) ($P < 0.001$). Comparing the two dose groups, the *L. plantarum* 10B group demonstrated a greater reduction in abdominal pain severity and duration scores over the intervention period ($P < 0.05$).

After 56 d, 59.6% and 72.6% of participants met 95-point reduction thresholds[18] in the *L. plantarum* 1B and *L. plantarum* 10B groups, respectively, as compared to 26.3% in the placebo group ($P < 0.001$) (Figure 2A). A post-hoc assessment showed 83.7% and 87.4% of participants met 50-point IBS-SSS reduction thresholds[17] in the *L. plantarum* 1B and *L. plantarum* 10B groups, respectively, as compared to 46.5% in the placebo group ($P < 0.001$). Additionally, 2.9% and 2.1% of participants demonstrated an increase in IBS-SSS score in the *L. plantarum* 1B and *L. plantarum* 10B groups, respectively, as compared to 18.2% in the placebo group ($P < 0.001$).

Abdominal pain severity, quality of life, and perceived stress

Baseline APS-NRS scores were 7.14 ± 0.69, 7.14 ± 0.77, and 7.17 ± 0.86 for the placebo, *L. plantarum* 1B, and *L. plantarum* 10B groups, respectively ($P > 0.05$). On day 56, a greater reduction in APS-NRS score (Table 3) was observed in the *L. plantarum* 1B (-1.83 ± 1.38) and *L. plantarum* 10B (-2.39 ± 1.47) groups, as compared to placebo (-0.94 ± 1.36) ($P < 0.001$). The mean reduction in the *L. plantarum* 10B group exceeded a clinically meaningful threshold of 30%[20] over the intervention period.

On day 56, a significant increase in IBS-Qol score (Table 3) was observed in the *L. plantarum* 1B (17.47 ± 19.54) and *L. plantarum* 10B (28.78 ± 23.64) groups, as compared to placebo (4.90 ± 14.29) ($P < 0.001$). Similarly, a greater improvement in total IBS-QoL score was demonstrated on day 28 in the *L. plantarum* 1B (8.82 ± 17.38) and *L. plantarum* 10B (17.65 ± 19.47) groups, as compared to placebo (4.20 ± 14.19) ($P < 0.01$). Comparing the two dose groups, the *L. plantarum* 10B group demonstrated a greater improvement in IBS-QoL at both timepoints ($P < 0.001$).

Perceived stress, assessed *via* the PSS, was similarly improved in the *L. plantarum* 1B and *L. plantarum* 10B groups, as compared to placebo on both day 28 and day 56 (Table 3). On day 56, a significant reduction in PSS score was observed in the *L. plantarum* 1B (-4.33 ± 6.51) and *L. plantarum* 10B (-5.59 ± 6.43) groups, as compared to placebo (-0.46 ± 4.14) ($P <$

Table 1 Baseline demographics and clinical characteristics of the intention-to-treat population, *n* (%)

	Placebo 1B (*n* = 104)	*L. plantarum* 1B (*n* = 104)	*L. plantarum* 10B (*n* = 99)	*P* value
	mean (SD) or *n* (%)	mean (SD) or *n* (%)	mean (SD) or *n* (%)	
Age (yr)	39.50 (13.26)	38.66 (11.25)	40.46 (11.15)	0.5619[1]
Female allocation	46 (44.23)	50 (48.08)	44 (44.44)	0.8232[2]
Height (m)	1.60 (0.07)	1.59 (0.08)	1.61 (0.07)	0.1775[1]
Weight (kg)	63.57 (9.10)	63.09 (9.01)	63.25 (8.50)	0.9256[1]
BMI (kg/m^2)	24.83 (3.15)	25.01 (3.31)	24.46 (2.92)	0.4506[1]
Systolic BP (mmHG)	119.68 (8.27)	119.76 (8.68)	118.88 (8.79)	0.7223[1]
Diastolic BP (mmHG)	79.29 (5.06)	79.21 (6.30)	78.75 (4.62)	0.7429[1]
Fasting glucose (mmol/L)	5.16 (0.66)	5.01 (0.65)	5.13 (0.62)	0.1961[1]
IBS-SSS aggregate score	308.70 (70.91)	315.20 (62.79)	302.85 (61.06)	0.4090[1]
APS-NRS score	7.14 (0.69)	7.14 (0.77)	7.17 (0.86)	0.9648[1]
IBS-QoL aggregate score	36.19 (17.97)	37.83 (17.25)	37.54 (17.02)	0.7787[1]
PSS score	22.06 (4.29)	22.42 (4.12)	21.93 (4.31)	0.6912[1]

[1]Between group comparison; one-way ANOVA.
[2]Between group comparison; Pearson chi-square test.
BMI: Body mass index; BP: Blood pressure; IBS-SSS: Irritable bowel syndrome-severity scoring system; APS-NRS: Abdominal pain severity-numeric rating scale; IBS-QoL: Irritable bowel syndrome-quality of life; PSS: Perceived stress scale; *L. plantarum*: *Lactiplantibacillus plantarum*.

0.001). No significant differences were observed between the dose groups in PSS. Further, a post-hoc analysis showed PSS response to be moderately but significantly correlated to IBS-SSS (r = 0.326, P < 0.001) and APS-NRS scores (r = 0.355, P < 0.001) over the intervention period.

Stool consistency

Figure 2B shows the percent of participants considered IBS-D stool consistency responders, based on a decrease of 50% or more in the number of days per week with at least one types 6-7 bowel movement, as compared to baseline[20]. On day 56, 62.5% and 88.4% of study participants met the criteria of IBS-D stool consistency responders in the *L. plantarum* 1B and *L. plantarum* 10B groups, respectively, as compared to 26.3% in the placebo group. Additionally, a greater number of responders were observed in the *L. plantarum* 10B group as compared to the *L. plantarum* 1B group (P < 0.001). A significant number of responders were also observed in the *L. plantarum* 1B (30.8%; P < 0.05 *vs* placebo) and *L. plantarum* 10B (48.4%; P < 0.001 *vs* placebo) groups after day 28.

Microbial profiling

There were no significant differences in alpha (Shannon) diversity or beta diversity between groups over the study period, suggesting no major shifts in microbiome composition. Figure 3 shows the proportional family (A) and genus (B) level abundance in participants receiving placebo, *L. plantarum* 1B, and *L. plantarum* 10B, respectively. The most abundant taxa at the phylum level, Firmicutes, Bacteroidetes, and Actinobacteria, did not differ significantly over time in the three groups. Similarly, the most abundant taxa at the family level, *Lachnospiraceae*, *Prevotellaceae*, *Ruminococcaceae*, and *Bifidobacteriaceae* were stable within group over the intervention period, with the exception of a significant reduction in *Ruminococcaceae* and *Bifidobacteriaceae* within the placebo group (LEfSe logarithmic LDA score < -3.0) that was not observed in the *L. plantarum* groups. Similarly, the placebo group demonstrated a significant reduction in *Bifidobacterium*, *Faecalibacterium,* and *F. prausnitzii* (LEfSe logarithmic LDA score < -3.0) over the study period that was not observed in the *L. plantarum* groups. LEfSe identified more taxa enriched in the higher dose *L. plantarum* group at the end of study visit as compared to placebo, including both *Lactiplantibacillus* and *L. plantarum* abundance (LEfSe logarithmic LDA score > 2.0).

Safety, compliance, and dietary profile

A total of 37 adverse events (AEs) were reported during the study, with seven reported during the run-in phase and 30 reported during the intervention phase. Of the 30 AEs reported post-randomization, 12, 13, and 5 were in the placebo, *L. plantarum* 1B, and *L. plantarum* 10B groups, respectively. A total of four AEs were suspected to be related to the study product with one AE (nausea and vomiting) in the *L. plantarum* 1B group and three AEs (heartburn and hyperacidity) in the placebo group. All AEs reported in the study were considered mild and resolved without complications. Vital signs, including systolic and diastolic blood pressure and pulse rate, were within clinically acceptable ranges over the intervention period with no significant differences between groups (Supplementary Table 2). Overall mean compliance over the study period was 98.7%, 98.4%, and 98.5% for the placebo, *L. plantarum* 1B, and *L. plantarum* 10B groups,

Table 2 Irritable bowel syndrome symptom severity total and domain-specific scores over the intervention period

	Placebo (*n* = 104)	*L. plantarum* 1B (*n* = 104)		*L. plantarum* 10B (*n* = 99)		
	mean (SD)	mean (SD)	*P vs* Placebo[1]	mean (SD)	*P vs* Placebo[1]	*P vs* 1B dose[2]
IBS-SSS total score						
Day 0	308.70 (70.91)	315.95 (62.07)		302.85 (61.06)		
AbsΔ (Day 28)	-35.68 (60.81)	-66.37 (72.27)	0.0098	-85.80 (89.53)	< 0.0001	0.0956
AbsΔ (Day 56)	-58.82 (74.75)	-128.45 (83.30)	< 0.0001	-156.77 (99.06)	< 0.0001	0.0298
Abdominal pain severity						
Day 0	67.05 (17.11)	68.80 (13.40)		67.07 (13.77)		
AbsΔ (Day 28)	-9.24 (19.67)	-15.45 (18.36)	0.0830	-20.76 (22.76)	< 0.0001	0.0736
AbsΔ (Day 56)	-14.38 (21.18)	-28.91 (20.36)	< 0.0001	-36.52 (24.39)	< 0.0001	0.0176
Abdominal pain duration						
Day 0	41.11 (14.13)	42.60 (14.55)		39.37 (13.75)		
AbsΔ (Day 28)	-5.35 (13.50)	-8.46 (12.05)	0.1975	-7.89 (12.62)	0.0484	0.7463
AbsΔ (Day 56)	-8.38 (13.75)	-15.29 (13.72)	0.0006	-19.68 (14.91)	< 0.0001	0.0315
Abdominal distension						
Day 0	65.08 (18.11)	66.31 (15.11)		63.07 (14.34)		
AbsΔ (Day 28)	-8.66 (18.19)	-14.69 (17.68)	0.0600	-18.55 (22.62)	< 0.0001	0.1849
AbsΔ (Day 56)	-14.00 (20.76)	-29.33 (21.64)	< 0.0001	-35.45 (24.00)	< 0.0001	0.0598
Bowel habits						
Day 0	68.32 (15.64)	69.35 (13.70)		66.72 (14.55)		
AbsΔ (Day 28)	-6.45 (12.02)	-14.36 (18.66)	0.0020	-19.52 (21.48)	< 0.0001	0.0714
AbsΔ (Day 56)	-11.19 (16.63)	-27.73 (19.54)	< 0.0001	-32.31 (25.12)	< 0.0001	0.1561
Effect on quality of life						
Day 0	67.13 (15.85)	68.90 (14.47)		66.62 (12.85)		
AbsΔ (Day 28)	-5.97 (12.03)	-13.40 (17.48)	0.0060	-19.08 (21.13)	< 0.0001	0.0395
AbsΔ (Day 56)	-10.86 (15.74)	-27.19 (19.19)	< 0.0001	-32.81 (23.66)	< 0.0001	0.0690

[1]ANCOVA using Dunnett's test adjustment with treatment as factor and baseline as covariate *vs* placebo.
[2]Two-sample *t*-test comparing dose groups.
IBS-SSS: Irritable bowel syndrome-severity scoring system; AbsΔ: Absolute change; *L. plantarum*: *Lactiplantibacillus plantarum*.

respectively, with no differences between groups ($P > 0.05$). Additionally, total caloric and macronutrient intake, including protein, carbohydrates, fat, and fiber, were stable over the intervention period with no significant differences between groups ($P > 0.05$) (Supplementary Table 3).

DISCUSSION

The present study was a randomized, double-blind, placebo-controlled trial to assess the efficacy of *L. plantarum* Lpla33 (DSM34428) in adults with IBS-D. The study is unique in its dose-ranging design in a well-powered study across 12 clinical sites. Enrolled participants were primarily 30 to 50 years of age, with a body mass index ranging from normal to borderline overweight and an IBS symptom score of at least moderate severity.

The study had a roughly equal allocation of females and males, which is in line with prevalence rates for the IBS-D subtype globally[24,25], as well as prior IBS studies in Asia[26-28], and in contrast with a significantly greater female prevalence among constipation-predominant (IBS-C) cohorts[2]. Within South Asia, chronic gut infections, gut microbial dysbiosis, altered intestinal permeability, and inflammation are suggested as contributing factors to IBS and IBS-D[26]. Dietary practices may also play a role, with a generally high consumption of short chain carbohydrates[29].

In the present study, *L. plantarum* Lpla33, at daily doses of 1×10^9 or 1×10^{10} CFU, reduced IBS-SSS by more than twice the magnitude of the placebo group after 56 d, thus achieving the primary outcome while surpassing a clinically

Table 3 Abdominal pain severity, quality of life, and perceived stress scores over the intervention period

	Placebo (*n* = 104)	*L. plantarum* 1B (*n* = 104)		*L. plantarum* 10B (*n* = 99)		
	mean (SD)	mean (SD)	*P vs* Placebo[1]	mean (SD)	*P vs* Placebo[1]	*P vs* 1B dose[2]
APS-NRS score						
Day 0	7.14 (0.69)	7.14 (0.77)		7.17 (0.86)		
AbsΔ (Day 28)	-0.61 (1.12)	-0.87 (1.06)	0.1356	-0.92 (0.90)	0.0733	0.7089
AbsΔ (Day 56)	-0.94 (1.36)	-1.83 (1.38)	< 0.0001	-2.39 (1.47)	< 0.0001	0.0057
IBS-QoL total score						
Day 0	36.19 (17.97)	37.83 (17.25)		37.54 (17.02)		
AbsΔ (Day 28)	4.20 (14.19)	8.82 (17.38)	0.0208	17.65 (19.47)	< 0.0001	0.0009
AbsΔ (Day 56)	4.90 (14.29)	17.47 (19.54)	< 0.0001	28.78 (23.64)	< 0.0001	0.0003
PSS score						
Day 0	22.06 (4.29)	22.42 (4.12)		21.93 (4.31)		
AbsΔ (Day 28)	-0.12 (3.92)	-2.38 (4.54)	< 0.0001	-2.76 (4.69)	< 0.0001	0.5690
AbsΔ (Day 56)	-0.46 (4.14)	-4.33 (6.51)	< 0.0001	-5.59 (6.43)	< 0.0001	0.1708

[1]ANCOVA using Dunnett's test adjustment with treatment as factor and baseline as covariate *vs* placebo.
[2]Two-sample *t*-test comparing dose groups.
APS-NRS: Abdominal pain severity-numeric rating scale; IBS-QoL: Irritable bowel syndrome-quality of life; PSS: Perceived stress scale; AbsΔ: Absolute change; *L. plantarum*: *Lactiplantibacillus plantarum*.

meaningful response[18]. Further, the higher dose *L. plantarum* group demonstrated a greater effect size than the lower dose *L. plantarum* group. Previously, an *L. acidophilus* strain, administered at daily doses of 1×10^9 and 1×10^{10} CFU for 12 wk, exhibited a treatment effect in a post-hoc analysis of IBS participants with elevated abdominal pain levels[30]. However, no dose-response was observed. Similarly, a meta-analysis reported a similar improvement in IBS symptoms when comparing probiotic regimens greater than or less than 1×10^{10} CFU daily[30]. In contrast, a *L. gasseri* strain resulted in a reduction in abdominal pain severity *vs* placebo at a daily dose of 1×10^{10} CFU, but had no effect in groups receiving 2×10^8 or 2×10^9 CFU daily[31]. Another meta-analysis looked at 14 different probiotic products over a total of 45 intervention arms, of which 36%, 51%, and 11% used a daily dose of 10^6-10^9, 10^{10}, and 10^{11} CFU, respectively[14]. Of these, a dose response was noted for a 4-strain probiotic combination at 10^{10} CFU daily[32,33], as well as a *B. infantis* strain, which demonstrated efficacy at a dose of 10^8 CFU daily but not at doses of 10^6 or 10^{10} CFU daily[34].

Both *L. plantarum* dose groups displayed a significant number of IBS-SSS responders using either 95-point[18] or 50-point[17] reduction thresholds, which have independently been correlated with clinically relevant responses. Further, the high responder rates across participants with heterogeneous baseline symptoms[35] may also be applicable to the general healthy population experiencing GI discomfort with less frequency or severity[36].

Abdominal pain severity, assessed *via* the APS-NRS, was also significantly reduced with *L. plantarum* supplementation, with the higher dose group achieving a clinically relevant reduction of over 30%[20]. It should be noted that the placebo response of approximately 20% in the current study was less than those in prior IBS-D studies, which ranged from 32% to 44%[37-39]. This may have been due, in part, to the placebo run-in period as well as geographic, cultural, or dietary differences. Additionally, the higher-dose *L. plantarum* group exhibited a mean increase in IBS related QoL of 28.8 points, above the clinically meaningful threshold of 10 points[40]. This is of note given the lower reported QoL in IBS-D as compared to IBS-C[41], in part due to unpredictable defecation habits that limit daily activity. In addition, perceived stress in the *L. plantarum* groups was reduced compared to placebo. Of note, moderate but significant correlations were observed between reduced IBS symptomology, abdominal pain, and stress levels, with effects possibly linked to a more normalized gut-brain signaling or barrier function.

Rome Foundation reports have supported the concept that the intestinal microbiota is perturbed in IBS[42,43], and both incidence and symptom severity have been inversely associated with microbiome diversity[44,45]. Further, reports have linked IBS pathogenesis to dysbiosis of the microbiota[9,46], which may in turn impact pathogen binding and mucosal barrier integrity. However, there remain no uniform characteristics of an IBS-related gut microbiota[47]. The current study demonstrated a significant reduction in type 6 or 7 stool form *via* stool consistency responder analysis[20], including a dose effect among the *L. plantarum* groups. In contrast, no significant shifts in fecal microbial diversity were observed, in line with systematic reviews of probiotic studies showing no effects on alpha diversity, richness, or evenness, as well as composition[48]. *Lactiplantibacillus* and *L. plantarum* abundances were enriched at the end of study visit in the higher dose group as compared to placebo. Further, *Ruminococcaceae, Bifidobacterium,* and *F. prausnitzii* significantly decreased over the study period in the placebo group. Multi-omics studies have implicated *F. prausnitzii*, *Ruminococcus* spp., and *Bifidobacterium* in IBS treatment response[47], but it is unclear if this played a role here. Overall, the most abundant taxa did not significantly change over time. However, the site of action of *L. plantarum* Lpla33 is postulated to

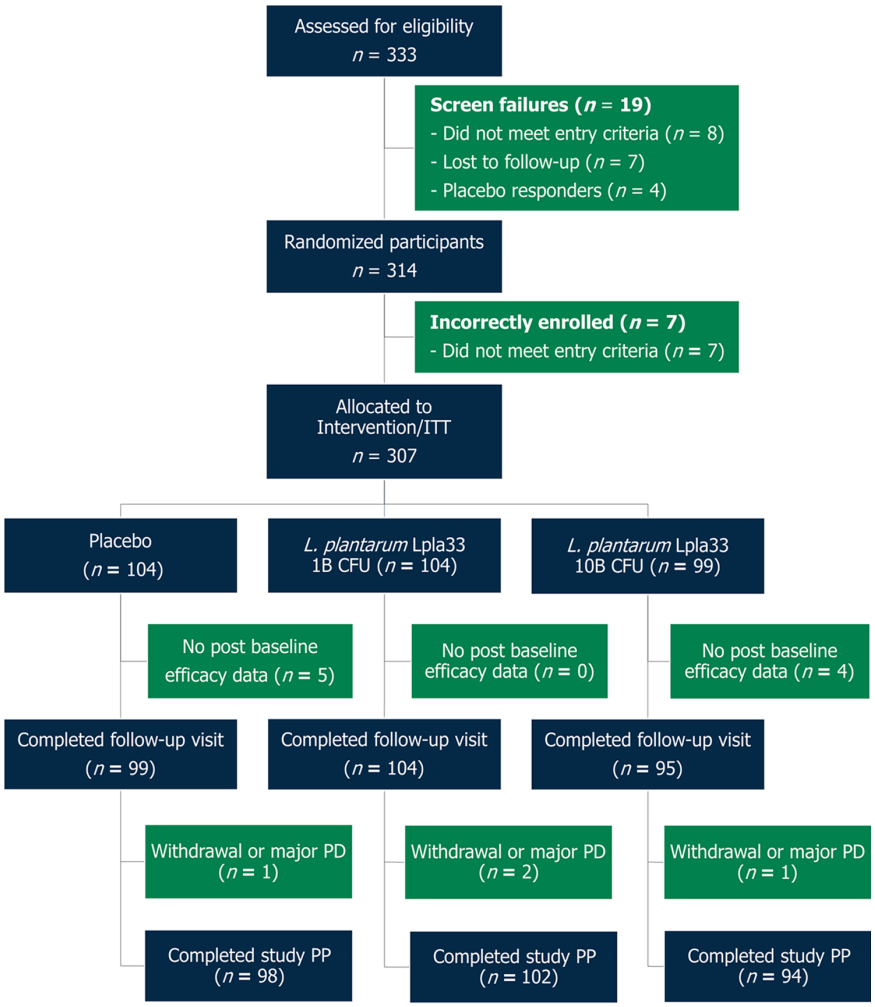

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Figure 1 Participant flow chart. ITT: Intention-to-treat; PD: Protocol deviation; PP: Per protocol; *L. plantarum*: *Lactiplantibacillus plantarum*.

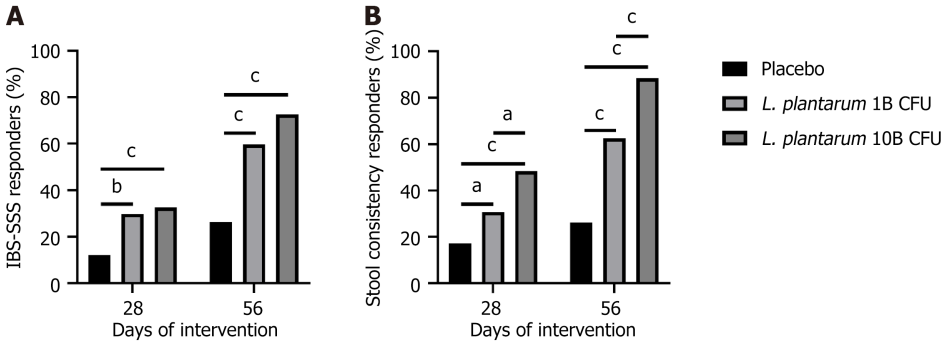

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Figure 2 Responder profile over the intervention period. A: Percentage of irritable bowel syndrome-severity scoring system (IBS-SSS) responders, as defined by a decrease of 95 points or more in the IBS-SSS total score from baseline, in participants receiving placebo or *Lactiplantibacillus plantarum* (*L. plantarum*) (1B or 10B CFU) capsules; B: Percentage of stool consistency responders, as defined by a decrease of at least 50% in the number of days per week with at least one stool that has a consistency of type 6 or 7 compared with baseline, in participants receiving placebo or *L. plantarum* (1B or 10B CFU) capsules. Between group comparison *via* Pearson chi-square test. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. *L. plantarum*: *Lactiplantibacillus plantarum*.

be proximal to the colon, and fecal samples may not accurately reflect the small intestinal profile or corresponding changes in mucosal inflammation or epithelial function[6].

L. plantarum is a well-documented species, with several prior studies demonstrating efficacy in managing IBS symptomology, albeit with strain specific differences[49-53]. Recent studies with *L. plantarum* strains have also reported improved QoL and defecation frequency in IBS-D[50,52]. In rodent models, *L. plantarum* strains have been shown to

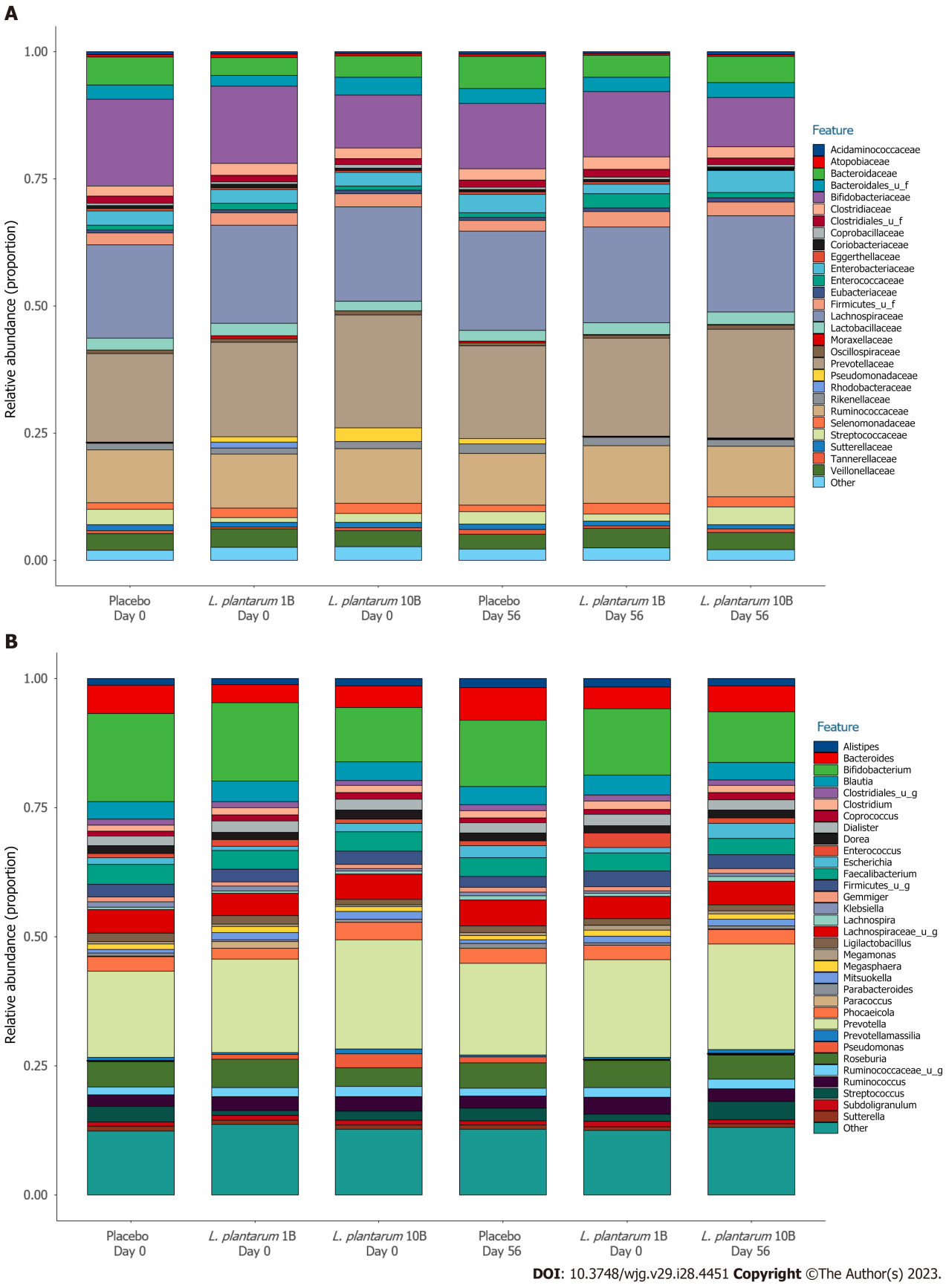

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Figure 3 Fecal microbial profile over the intervention period. A: Proportional family level abundance in participants receiving placebo or *Lactiplantibacillus plantarum* (*L. plantarum*) (1B or 10B CFU) capsules; B: Proportional genus level abundance in participants receiving placebo or *L. plantarum* (1B or 10B CFU) capsules.

relieve diarrhea through inflammation modulation and increased SCFAs[54], as well as upregulate the expression of brain-derived neurotrophic factor, serotonin transporter, and intestinal serotonin levels[55]. SCFAs play a critical role in gut homeostasis *via* mucus production, protection from inflammation, and immunomodulation[56]. Further, SCFA-producing microorganisms can also affect neurotransmitter levels and therefore gut-brain signaling, as well as activate and directly act on intestinal vagal terminals[57]. Additionally, SCFAs, particularly butyrate, may help promote intestinal barrier function[58], a feature of several *L. plantarum* strains[59]. Bile acid (BA) metabolism may also be implicated, as *L. plantarum* Lpla33 possesses significant bile salt hydrolase activity. Of note, diarrhea and visceral hypersensitivity have been associated with decreased 7α-dehydroxylation of primary BAs to secondary BAs[60]. Additionally, the ability of *L. plantarum* Lpla33 to modulate intestinal barrier function, inhibit key pathogens, and moderate inflammatory markers may have played a role in the observed effects.

Limitations of the study include the absence of biomarkers assessing immunity, inflammation, and intestinal barrier function, in part due to the large enrollment across several sites. Additionally, the study assessed fecal samples but not proximal sites of interest or corresponding changes in mucosal profile, inflammation, or epithelial function. Further, the study did not incorporate metabolite profiles in serum, feces, or urine to assess changes in immune or inflammation-related pathways[47]. Additionally, while macronutrient intake was shown to be stable over the study period, the association of diet with the microbial community or metabolite profiles would have been of interest to explore in more detail. Going forward, multi-omics studies should play an important role in further understanding therapeutic mechanisms of probiotic and diet-based interventions in IBS-D. Nevertheless, the study was well-powered to evaluate its primary outcome, incorporated 12 clinical sites and multiple doses, and integrated IBS trial design considerations[61].

CONCLUSION

The present randomized controlled trial demonstrates that *L. plantarum* Lpla33 (DSM34428) at a dose of 1×10^9 and 1×10^{10} CFU/day was well tolerated and met the primary outcome (reduction in IBS-SSS total score at day 56) in both dose groups compared to placebo. The study also met the first secondary outcome (reduction in IBS-SSS score at day 28) as well as outcomes related to abdominal pain severity, stool normalization, QoL, and perceived stress, when compared to placebo over the intervention period. Lastly, a *L. plantarum* Lpla33 dose response was observed in several key outcomes in females and males with IBS-D.

ARTICLE HIGHLIGHTS

Research background

Irritable bowel syndrome (IBS) is a disorder of gut-brain interaction characterized by abdominal pain in association with altered bowel habits and further classified by the predominant stool pattern. Global prevalence is high, with diarrhea predominant subtype (IBS-D) considered the most common. IBS-D has a significant impact on quality of life, and clinical management remains challenging due to the variety of symptoms to address.

Research motivation

A recent meta-analysis showed probiotics to be safe and superior to placebo for alleviating global IBS-D symptoms. However, the certainty of evidence is low, due in part to significant heterogeneity between studies. There is therefore a need for well-powered randomized controlled trials on promising probiotic candidates for IBS-D.

Research objectives

To assess the efficacy of a probiotic candidate strain, *Lactiplantibacillus plantarum* (*L. plantarum*) Lpla33 (DSM34428), in adults with IBS-D. The primary outcome was the change in the IBS severity scoring system (IBS-SSS) total score after 8 wk. Additional outcomes included the change in abdominal pain severity, IBS-related quality of life, stool and microbial profile, and perceived stress.

Research methods

Adults meeting Rome IV diagnostic criteria for IBS-D were recruited from 12 gastroenterology specialized centers across India. In this randomized, double-blind, placebo-controlled, multi-center, parallel-arm, and dose-ranging study, a total of 307 adults meeting the inclusion criteria were allocated (1:1:1) to receive placebo or *L. plantarum* Lpla33 at one of two doses [1×10^9 colony-forming units (CFU)/d (1B) or 1×10^{10} CFU/d (10B)] over 8 wk.

Research results

The primary outcome, IBS-SSS total score, was significantly reduced after 8 wk in participants receiving *L. plantarum* compared to placebo ($P < 0.001$), with a dose-ranging effect when comparing the two *L. plantarum* groups ($P < 0.05$). In total, 59.6% and 72.6% of participants in the *L. plantarum* 1B and *L. plantarum* 10B groups, respectively, were considered significant responders based on a 95-point reduction threshold, as compared to 26.3% in the placebo group ($P < 0.001$). Additionally, 62.5% and 88.4% of participants administered *L. plantarum* 1B and 10B, respectively, demonstrated a significant reduction in diarrheal stool form as compared to 26.3% in the placebo group ($P < 0.001$).

Research conclusions

L. plantarum Lpla33 is well tolerated and demonstrates dose-ranging efficacy in alleviating IBS symptom severity with a corresponding normalization of bowel habits in adults with IBS-D.

Research perspectives

Future research should incorporate multi-omics analyses and associated biomarkers to better understand the mechanisms of action involved.

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FOOTNOTES

Author contributions: Martoni CJ, Damholt A, and Leyer GL conceived, designed, and planned the study; Srivastava S directed the study, data analysis, and reporting; Martoni CJ prepared the original manuscript; Srivastava S, Damholt A, and Leyer GL reviewed and edited the manuscript; and all authors have read and approved the final version.

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META-ANALYSIS

One in four patients with gastrointestinal bleeding develops shock or hemodynamic instability: A systematic review and meta-analysis

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Abstract

BACKGROUND

Hemodynamic instability and shock are associated with untoward outcomes in gastrointestinal bleeding. However, there are no studies in the existing literature on the proportion of patients who developed these outcomes after gastrointestinal bleeding.

AIM

To determine the pooled event rates in the available literature and specify them based on the bleeding source.

METHODS

The protocol was registered on PROSPERO in advance (CRD42021283258). A systematic search was performed in three databases (PubMed, EMBASE, and CENTRAL) on 14th October 2021. Pooled proportions with 95%CI were calculated with a random-effects model. A subgroup analysis was carried out based on the time of assessment (on admission or during hospital stay). Heterogeneity was assessed by Higgins and Thompson's I^2 statistics. The Joanna Briggs Institute

Prevalence Critical Appraisal Tool was used for the risk of bias assessment. The Reference Citation Analysis (https://www.referencecitationanalysis.com/) tool was applied to obtain the latest highlight articles.

RESULTS

We identified 11589 records, of which 220 studies were eligible for data extraction. The overall proportion of shock and hemodynamic instability in general gastrointestinal bleeding patients was 0.25 (95%CI: 0.17-0.36, I^2 = 100%). In non-variceal bleeding, the proportion was 0.22 (95%CI: 0.14-0.31, I^2 = 100%), whereas it was 0.25 (95%CI: 0.19-0.32, I^2 = 100%) in variceal bleeding. The proportion of patients with colonic diverticular bleeding who developed shock or hemodynamic instability was 0.12 (95%CI: 0.06-0.22, I^2 = 90%). The risk of bias was low, and heterogeneity was high in all analyses.

CONCLUSION

One in five, one in four, and one in eight patients develops shock or hemodynamic instability on admission or during hospitalization in the case of non-variceal, variceal, and colonic diverticular bleeding, respectively.

Key Words: Gastrointestinal bleeding; Hemodynamic instability; Shock; Meta-analysis; Statistics; Review

Core Tip: Gastrointestinal bleeding is one of the most common gastrointestinal emergencies with estimated mortality up to 10%. It is associated with significant morbidity, additional burden, and health care costs. It is documented that hemodynamic instability and shock are highly associated with untoward outcomes; they lead to a higher mortality rate, rebleeding risk, prehospital transfusion, and sedation complications. Our study provides clear evidence that hemodynamic instability and shock are common presentations and complications in gastrointestinal bleeding and gives insight into some possible predictor factors.

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INTRODUCTION

The annual incidence of gastrointestinal bleeding (GIB) is 100 per 100000 population, and it is one of the most common gastroenterological emergencies with an estimated mortality rate in the range of 2%-10%, primarily due to complications related to the admission state and individual patient factors[1-3]. It is associated with significant morbidity, additional burden, and health care costs[4,5]. The mortality rate of upper GIB has not considerably decreased over the past decades, despite the improvement in the diagnosis and endoscopic treatment[6]. We contemplate that pre-endoscopic assessment and post-endoscopic care may contribute effectively to better outcomes.

Several studies showed that hemodynamic instability (HI) and shock in GIB are highly associated with untoward outcomes; they can lead to higher mortality rates, prehospital transfusion, rebleeding risk, and endoscopic sedation might be complicated with unfavorable hemodynamics if the patient presents with massive bleeding[7-9]. Furthermore, the hospital mortality rate of bleeding with shock can be 10 times higher than without shock[10].

Early intensive resuscitation of HI decreases complications in patients with upper GIB[11]. However, there are not enough details in the guidelines regarding the management of hemodynamically unstable patients; there are still some uncertainties about the optimal fluid rate and the ideal type of fluid to be used in treating those patients[12-15].

At the time of our systematic search, there were no published systematic reviews assessing the proportion of hemodynamically unstable and shocked patients in GIB. There are large variations in the proportions of these outcomes. Some studies in variceal and non-variceal bleeding resulted in proportions of 10% or lower[16-19], whereas others exceeded 60%[20-22]. Therefore, we aimed to highlight the importance of recognizing those patients by quantifying the pooled event rates based on the bleeding source. Additionally, we did a subgroup analysis based on the assessment time of these outcomes (on admission or during hospital stay).

MATERIALS AND METHODS

Our systematic review and meta-analysis was conducted following the recommendation of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 guideline[23]. The recommendations of the Cochrane

Handbook were also followed[24]. The study protocol was registered on PROSPERO (CRD42021283258), and we fully adhered to it[25]. In addition, we applied the Reference Citation Analysis (RCA) tool, which is based on artificial intelligence technology. This tool allowed us to access a comprehensive database of citations across multiple disciplines, aiding us in identifying the most recent and significant articles for our research.

Eligibility criteria

We applied the CoCoPop (condition, context, and population) framework to establish the eligibility criteria[26]; the condition was hemodynamic instability and/or shock, gastrointestinal bleeding as a context, and our population was adult patients. All definitions of hemodynamic instability and shock were accepted.

Randomized Controlled Trials (RCTs), cohorts, and case-control studies were included. Cross-sectional studies were included only if the hemodynamic parameters were assessed on admission. We included studies only if the primary cause of hospital admission was gastrointestinal bleeding and excluded articles that assessed our investigated outcomes after specific interventions. Articles that could not be found were sought for retrieval by contacting the journals and the authors. In the case of studies with overlapping populations, we kept the ones with larger sample sizes.

Information sources

Our systematic search was conducted in three main databases: MEDLINE (*via* PubMed), EMBASE, and Cochrane Central Register of Controlled Trials (CENTRAL) from the inception to 14th October 2021. No language or other restrictions were applied.

Search strategy

Our search key contained two main concepts: All types of bleeding sources and hemodynamic instability or shock. For the detailed search strategy, see Supplementary Table 1.

Screening and selection

Following the systematic search, the yielded articles were imported into a reference management program (EndNote 20.1). Duplicate articles were eliminated automatically and manually with overlapping publication years, authors, and titles. The screening and selection were performed by two independent reviewers (Obeidat M and Tari E) first by title and abstract, and then by full text (considering the eligibility criteria). Cohen's kappa coefficient (κ) was calculated at both levels of selection to measure the inter-reviewer reliability. In case of any disagreement, a consensus was reached after a discussion with the corresponding author (Erőss B).

Data extraction

The relevant data from the eligible studies were extracted independently by two authors (Obeidat M and Rancz A). Disagreements were resolved by involving the corresponding author (Erőss B). All data were manually collected and introduced into an Excel spreadsheet (Office 365, Microsoft, Redmond, WA, United States) for analysis. The following data were extracted: First author, the year of publication, Digital Object Identifier, geographical location, study period and design, number of centers, basic demographics, source of bleeding, the total number of GIB patients and those who developed HI or shock, definitions of the investigated outcomes, and the time of detection (on admission or during hospital stay).

Risk of bias assessment and quality of evidence

Two independent authors (Obeidat M and Tari E) performed the risk of bias assessment using the 'Joanna Briggs Institute Prevalence Critical Appraisal Tool'[26]. A third reviewer resolved potential disagreements (Rancz A). The tool contains nine items regarding the target population and study settings. Each item was rated as 'yes', 'no', 'unclear', or 'not applicable' according to information provided in each study, with a maximum score of nine points. The higher the score, the lower the risk of bias.

We followed the Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach[27] to evaluate the quality of evidence of our results, and the GRADEpro tool (software) was used. Study design, risk of bias, inconsistency, indirectness, and imprecision were the determinant factors.

Statistical synthesis

The statistical analysis of the data was conducted by the R programming language using the meta package. We used forest plots to summarize the findings of the studies and show the pooled result. Pooled event rates were calculated with 95%CIs. The random-effect model was anticipated as applied in all analyses as considerable between-study heterogeneity. The random intercept logistic regression model method was used for pooling method as recommended by Schwarzer *et al*[28]. To estimate the heterogeneity variance measure τ^2, the maximum likelihood method was used. For the outcomes where the study number was at least five, a Hartung-Knapp adjustment was used[29,30]. Below five studies, we applied the adjustment if it was more conservative than without the adjustment. Statistical heterogeneity was assessed by Higgins and Thompson's I^2[31].

Egger's test with the Peter's modification and funnel plots were applied to report and visualize publication bias if at least 10 studies were involved in the analysis[32]; $P < 0.1$ indicates potential publication bias. We also performed an influential sensitivity analysis with leave-one-out method to evaluate whether a single study could have a marked influence on the overall proportional rate or heterogeneity.

A subgroup analysis was carried out based on the time of assessment (on admission or during hospitalization) of HI or shock. Studies where there were no data about the time when the patients were assessed, were considered (during hospitalization). We used a fixed-effects "plural" model. We assumed that subgroups had different τ^2 values as we anticipated differences in the between-study heterogeneity in the subgroups, although a common τ^2 assumption was used for practical reasons if the subgroup size was maximum five. To assess the difference between the subgroups, a Cochrane Q test was used between subgroups[33]. We did not calculate the overall effect and heterogeneity for subgroups where less than three studies were included. We calculated the prediction intervals for our outcomes to assess the probability that future studies would have the same result in a similar setting[34]. The statistical methods of this study were reviewed by Veres DS who is a verified biostatistician from the Centre for Translational Medicine, Semmelweis University.

RESULTS

Search and selection

Altogether, 11589 studies were identified by our search key through three main databases, 8129 in EMBASE, 3134 in Medline (*via* PubMed), and 326 in CENTRAL. Of them, 9192 records remained for title and abstract selection after duplicate removal. A total of 601 studies were sought for full-text selection, out of which 164 records were not found. We managed to retrieve 29, but 135 records were still inaccessible. In total, 466 studies were assessed for full-text eligibility, of which 246 were excluded (Supplementary Table 2). Eleven studies were removed for overlapping populations (Supplementary Table 3). Details of search and selection are illustrated in the PRISMA 2020 flow chart (Figure 1).

Basic characteristics of included studies

Most of the included studies were cohort studies. We also included 28 RCTs, 6 case-control, and 4 cross-sectional studies. Eighty records were from Asia, 66 from Europe, 25 from North America, and 13 from Africa. In total, more than six million patients were included in the analysis. However, the study with the largest sample size included 6411838 patients with different bleeding sources from a 12-year national analysis in the United States[10]. The main characteristics of the enrolled studies are detailed in Supplementary Table 4.

Hemodynamic instability and shock in general gastrointestinal bleeding sources

We included all studies with unspecified bleeding sources[8,10,35-50]. HI was assessed on admission and during hospital stay with pooled event rates of 0.29 (95%CI: 0.12-0.56, I^2 = 87%) and 0.34 (95%CI: 0.11-0.68, I^2 = 93%), respectively. Shock on admission was 0.27 (95%CI: 0.08-0.60, I^2 = 92%), whereas during hospital stay it was 0.15 (95%CI: 0.05-0.36, I^2 = 99%). One in four patients with GIB developed HI or shock; 0.25 (95%CI: 0.17-0.36, I^2 = 100%) (Figure 2).

Hemodynamic instability and shock in non-variceal upper GIB

In the case of non-variceal bleeding, more than three million patients were included in the analysis from 25 studies[10,17,20,21,51-71]. The proportion of hemodynamically unstable patients on admission was 0.21 (95%CI: 0.12-0.36, I^2 = 97%). Two studies assessed HI during hospitalization, Hwang *et al*[58] and Kwon *et al*[60] where the event rate was 0.10 (95%CI: 0.08-0.11) and 0.57 (95%CI: 0.42-0.70), respectively. Moreover, shock on admission was the highest at 0.36 (95%CI: 0.21-0.53, I^2 = 98%), with a noticeable difference from those who developed shock during hospitalization with a rate of 0.07 (95%CI: 0.02-0.18, I^2 = 100%). Altogether, 0.22 (95%CI: 0.14-0.31, I^2 = 100%) of non-variceal bleeders developed shock or HI on admission or during the hospital stay (Figure 3).

Hemodynamic instability and shock in variceal upper GIB

In total, 34 studies were included in this analysis[10,16,20,21,61,65,70,72-98]. The rate of patients with variceal bleeding who presented with HI on admission was 0.38 (95%CI: 0.12-0.73, I^2 = 98%). Two studies assessed HI during hospitalization, Farooqi and Farooqi[96] and Choi *et al*[75] where the event rate was 0.21 (95%CI: 0.14-0.29) and 0.52 (95%CI: 0.40-0.63), respectively. The shock rate on admission was 0.26 (95%CI: 0.18-0.36, I^2 = 100%), whereas it was 0.18 (95%CI: 0.10-0.30, I^2 = 99%) during the hospital stay. In total, one in four patients with variceal bleeding developed shock or HI at presentation or during hospital stay 0.25 (95%CI: 0.19-0.32, I^2 = 100%) (Figure 4).

Hemodynamic instability and shock in peptic ulcer bleeding

Peptic ulcer bleeding (PUB) was the most reported source of bleeding among the included studies. Sixty-seven studies were involved in the subgroups. On admission, 0.22 (95%CI: 0.09-0.44, I^2 = 96%) of the patients were hemodynamically unstable, whereas during the hospital stay, it was 0.41 (95%CI: 0.12-0.78, I^2 = 89%). The rate of shock on admission was 0.25 (95%CI: 0.19-0.32, I^2 = 98%), whereas 0.24 (95%CI: 0.17-0.33, I^2 = 97%) developed shock during hospitalization. As an overall effect, one in four PUB patients was affected by HI or shock on admission or during hospital stay; 0.25 (95%CI: 0.21-0.30, I^2 = 98%) (Supplementary Figure 1).

Hemodynamic instability and shock in upper GIB

The studies included in this plot contain various upper GIB sources. All the studies that reported HI were assessed on admission, with a rate of 0.33 (95%CI: 0.21-0.48, I^2 = 97%). Seventeen studies were included in the shock on admission subgroup with a rate of 0.15 (95%CI: 0.09-0.25, I^2 = 99%), whereas 18 studies evaluated shock during hospitalization with a rate of 0.20 (95%CI: 0.12-0.32, I^2 = 100%). In total, one in five patients with upper GIB developed shock or HI; 0.20

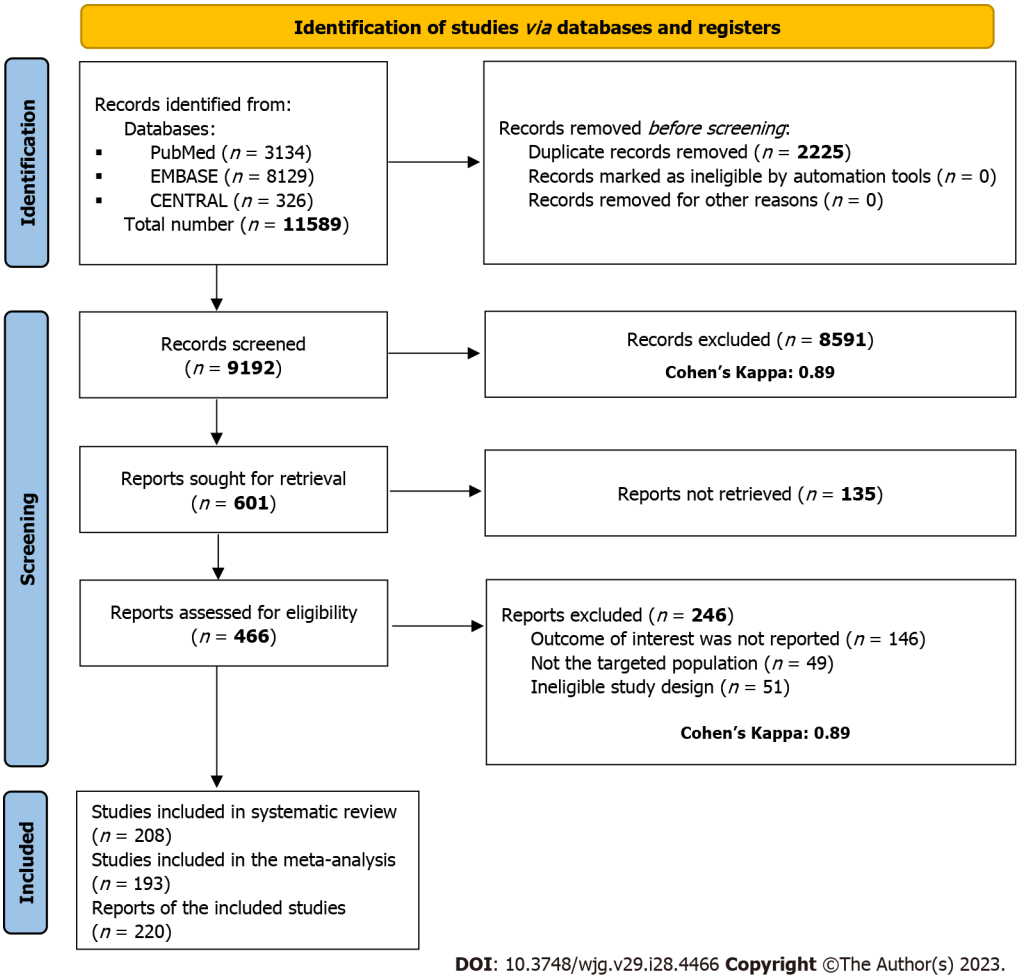

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Figure 1 PRISMA 2020 flow chart of the screening and selection process of the studies.

(95%CI: 0.15-0.27, I^2 = 100%) (Supplementary Figure 2).

Hemodynamic instability and shock in lower GIB

In total, 17 studies were included in this analysis[10,48,99-113]. Thirteen studies evaluated HI in lower GIB population: Three studies on admission with a rate of 0.14 (95%CI: 0.01-0.81, I^2 = 83%), and 10 studies during hospitalization with a rate of 0.49 (95%CI: 0.27-0.71, I^2 = 94%). Two studies assessed shock on admission, Oakland *et al*[109] and Li *et al*[105] where the pooled event rates were 0.02 (95%CI: 0.02-0.03) and 0.03 (95%CI: 0.03-0.03), respectively. Another two studies assessed shock during hospital stay. In the study by Siddiqui *et al*[10] the shock rate was 0.02 (95%CI: 0.02-0.02). The study by Lv and Gu[106], which involved patients with life-threatening bleeding, resulted in the highest pooled event rate of shock with a rate of 0.68 (95%CI: 0.50-0.82). In total, of the general lower GIB population, 0.27 (95%CI: 0.13-0-49, I^2 = 100%) developed shock or HI (Figure 5).

Hemodynamic instability and shock in colonic diverticular bleeding

All studies assessed the investigated outcomes on admission only. Six studies evaluated shock in colonic diverticular bleeding (CDB) with a rate of 0.12 (95%CI: 0.05-0.26, I^2 = 91%). Only two studies reported HI, that of Gilshtein *et al*[114] reported a rate of 0.05 (95%CI: 0.02-0.11), and Ichiba *et al*[115] a rate of 0.21 (95%CI: 0.17-0.26). As an overall effect, the proportion of shock and HI in CDB was 0.12 (95%CI: 0.06-0.22, I^2 = 90%) (Supplementary Figure 3).

Risk of bias assessment

Most of the studies received a score of 6 or higher, indicating a moderate to low risk of bias. Only 10 studies were rated with a score less than six. The sample size was not adequate in 33 studies. The results of the risk of bias assessment are presented in Supplementary Table 5.

Heterogeneity and publication bias

Serious heterogeneity (with more than 80%) was observed in all our analyses. The large number of included studies with heterogeneous populations regarding age and sex could explain this. The definitions of HI and shock in the studies were not the same resulting in considerable heterogeneity, too.

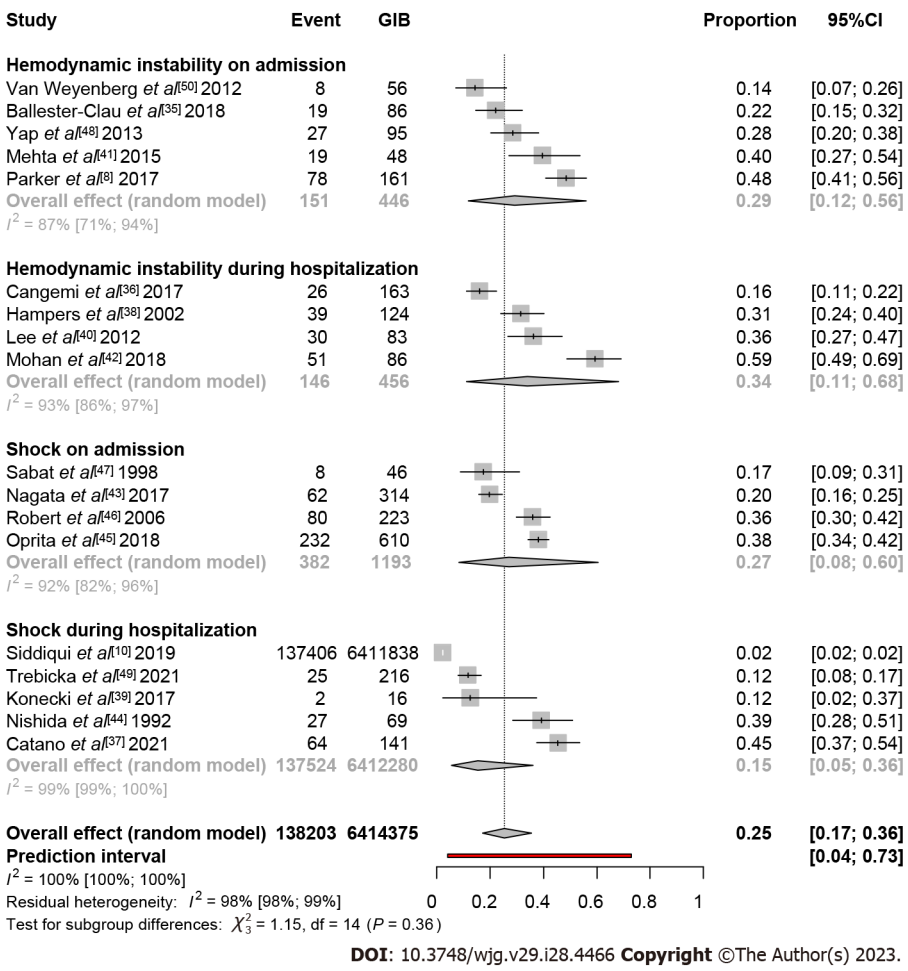

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Figure 2 Forest plot demonstrating the proportion rates for hemodynamic instability and shock in general gastrointestinal bleeding sources. GIB: Gastrointestinal bleeding.

All of our meta-analytical calculations that included 10 or more studies were investigated for publication bias. CDB was an exception where only eight studies were included. We found potential publication bias in all of our analyses except for non-variceal bleeding based on Egger's test. This result could be explained by the very large heterogeneity of the study estimates. Additionally, a highly influential large study by Siddiqui *et al*[10] led to a false positive result for Egger's test.

Leave-one-out sensitivity analysis showed some variability for some potential outliers. The proportion of our outcomes changed from 0.25 (95%CI: 017-0.36, I^2 = 100%) to 0.29 (95%CI: 0.22-0.37, I^2 = 90%) if Siddiqui *et al*[10] study was eliminated from the GIB analysis. This study did not only include a large sample size compared to other studies but also used the National Inpatient Sample database using International Classification of Diseases (ICD-9) codes to analyze patient data, which might have failed to identify some affected patients. Results of Egger's test, funnel plots, and leave-one-out analysis are found in Supplementary Figures 4-16.

Certainty of evidence

Based on the results and the careful evaluation of the evidence level, the certainty levels were low or very low for each outcome. The very high heterogeneity in almost all analyses was the main reason for that. In addition, all the included studies were considered observational studies, which contributes to the low level of evidence. (Supplementary Tables 6-12).

DISCUSSION

Our study found that HI and shock are common complications of GIB. Either shock or HI affects one in every four patients; even the lowest proportion, one in eight colonic diverticular bleeders, is still a significant portion of patients.

Variceal bleeding resulted in the highest HI on admission, with a rate of (38%) among various bleeding sources. In contrast, the highest HI rates during hospitalization were observed in PUB (41%) and LGIB (49%). The rate of shock on admission was generally the highest among different non-variceal bleeding sources (36%), whereas PUB specifically led to the highest rate of shock during hospitalization (24%).

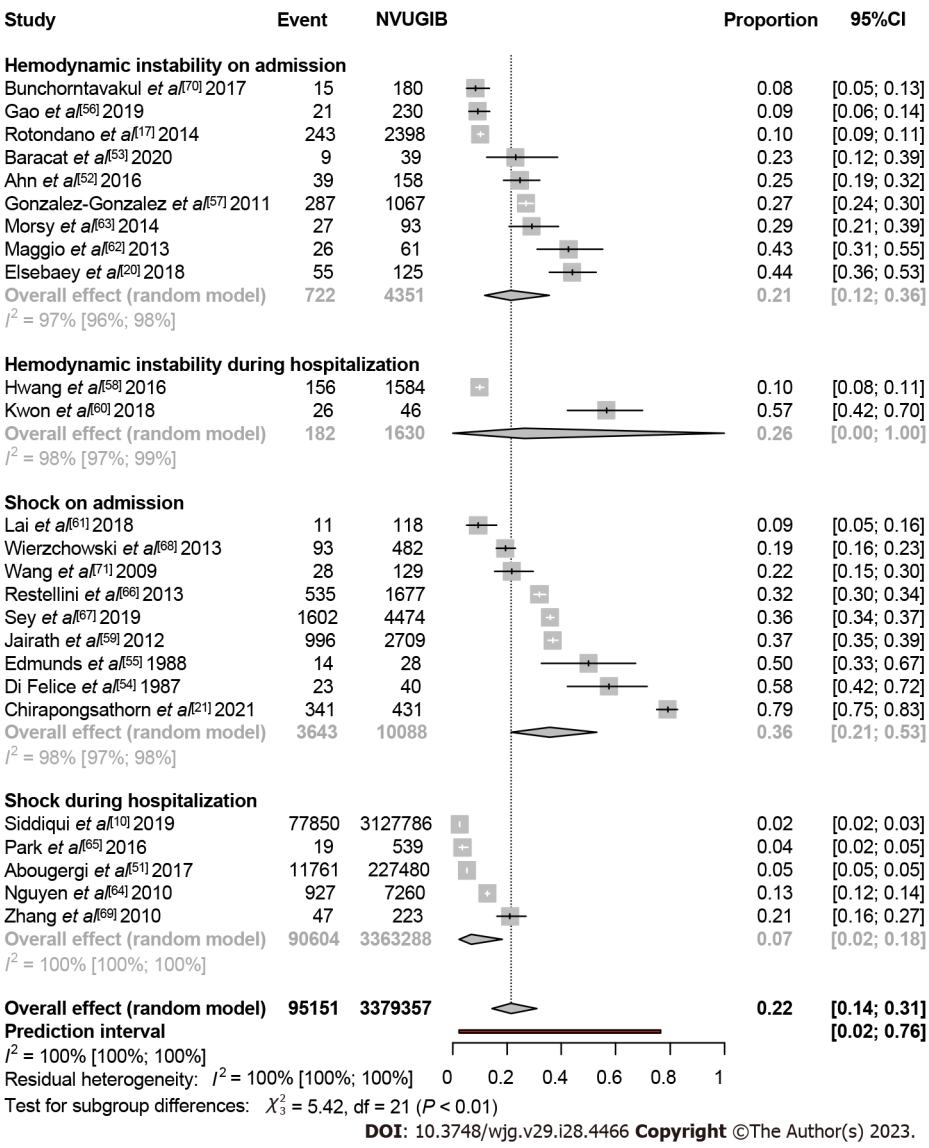

Figure 3 Forest plot demonstrating the proportion rates for hemodynamic instability and shock in non-variceal bleeding. NVUGIB: Non-variceal upper gastrointestinal bleeding.

Our results about unspecified GIB sources, non-variceal, and PUB showed higher rates of HI during hospitalization than on admission and higher rates of shock on admission than during hospitalization. In contrast, variceal bleeding showed higher rates of HI and shock on admission than during hospitalization. Lower GIB, on the other hand, showed higher rates of these outcomes during hospitalization than on admission.

Blood loss leads to HI characterized by a decrease in systolic blood pressure (BP) and an increase in heart rate (HR). Eventually, it can lead to a more severe state of shock, which is caused by a rapid reduction of intravascular blood volume resulting in decreasing hemoglobin levels, thereby decreasing the oxygen delivery capacity of the heart. HI is not just a sign; it is the starting point of a chain of events leading to hypoxemia and hypoperfusion. If it is not appropriately treated as soon as possible, it will lead to multiple organ failures. Therefore, health care providers must emphasize continuous monitoring and efficient stabilization for those patients[11].

Serious heterogeneity was observed in all our analyses. The reason for this lies in the large number of included articles. The population had different geographical locations, ethnicities, several comorbidities, age ranges, and access to different qualities of health care systems. Thus, there was even a variation in the definitions; most of the included studies defined HI as a decrease of systolic BP < 100 mmHg and/or an increase in HR > 100 bpm[6]. However, some definitions included syncope, orthostatic changes[115], or signs of organ hypoperfusion[52]. All these factors contributed noticeably, resulting in a very serious heterogeneity. All definitions of HI and shock can be found in Supplementary Tables 13 and 14, respectively.

Possible predictors were observed that resulted in higher rates of our investigated outcomes. We observed some outliers in different sources of bleeding; in variceal bleeding, intensive care unit admission[79,82,97], elderly population [20], and severe uncontrolled bleeding[75] were possible predictors for higher rates of shock and HI. In non-variceal bleeding, elderly patients > 60 years[20] and those who underwent embolization[60] accounted for the highest rate of HI on admission and during hospitalization, respectively. As for upper GIB in general, the study by Chirapongsathorn *et al*

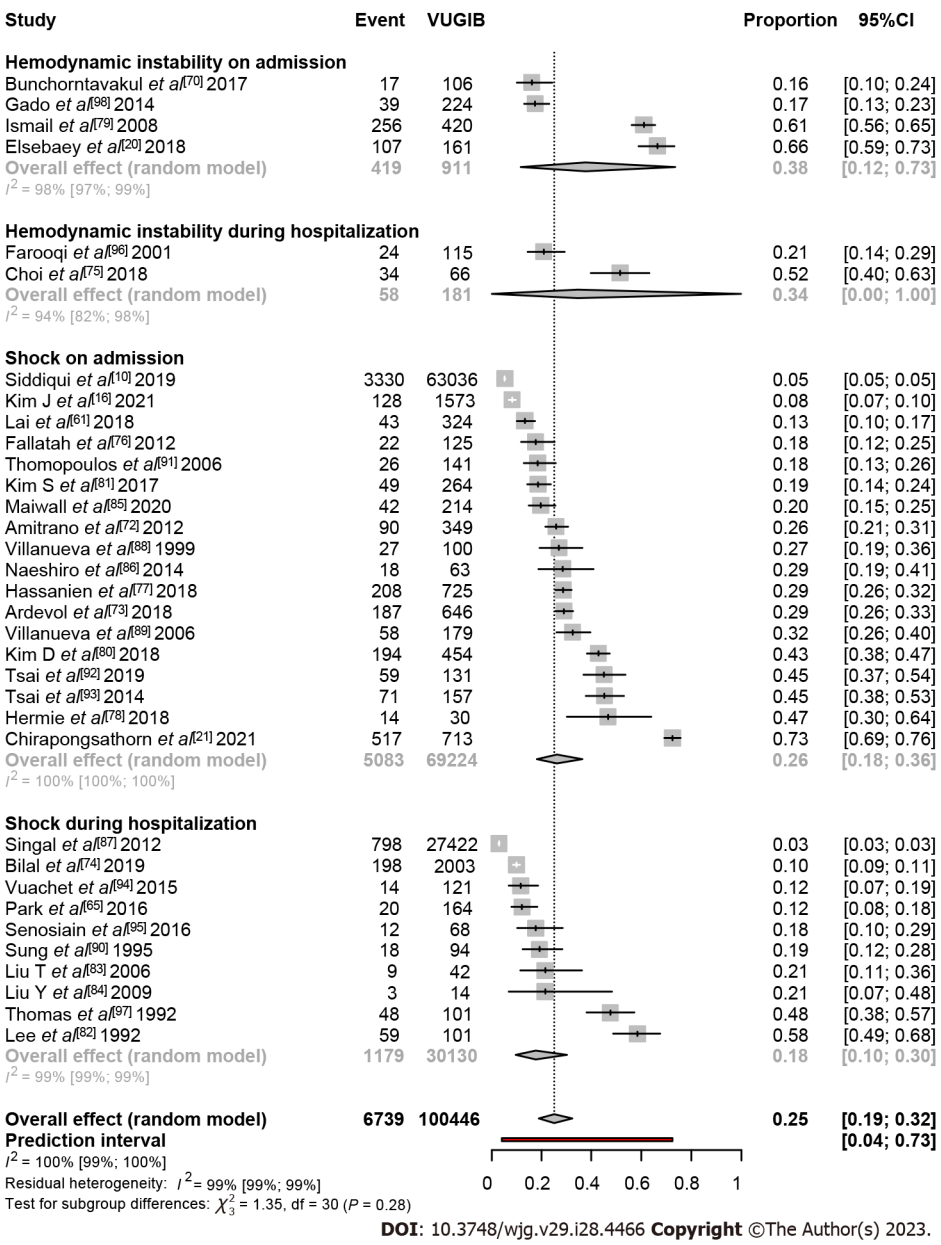

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Figure 4 Forest plot demonstrating the proportion rates for hemodynamic instability and shock in variceal bleeding. VUGIB: Variceal upper gastrointestinal bleeding.

[21] included variceal and non-variceal bleeders, where they defined shock as mean arterial pressure lower than 50 mmHg, which results in a very high rate of shock (75%).

Lower GIB is three times less common than upper GIB and has not been the focus of much attention yet. Mortality rises to 20%-40% in the case of massive lower GIB complicated by unstable hemodynamics[116]. Super-selective patients who underwent arterial embolization[104], angiography[112], or were diagnosed with acute severe bleeding[103] showed higher rates of the investigated outcomes.

Strengths and Limitations

This is the first comprehensive overview to assess the proportion of patients affected by HI and shock in GIB and specify it according to the bleeding source. Our study included many studies with an extensive sample size. Additionally, subgroup analysis, which was based on the time of assessment, whether on admission or during hospital stay, provided a more precise overview. This study also gives an insight into some of the possible predictors that result in higher rates of our investigated outcomes.

Considering the limitations of this work, the definitions of HI and shock were different among the included studies or even missing. Different characteristics of the included population led to high heterogeneity in almost all analyses. The presence of low certainty of evidence in some domains is another limitation.

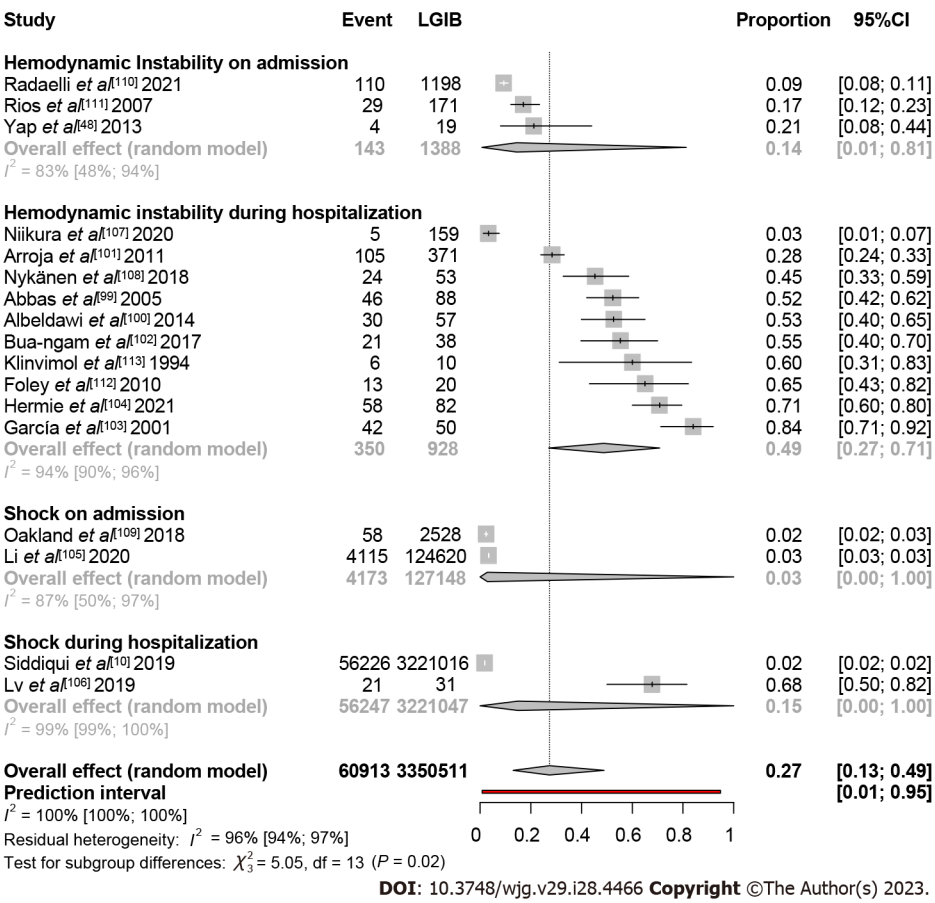

Figure 5 Forest plot demonstrating the proportion rates for hemodynamic instability and shock in lower gastrointestinal bleeding sources. LGIB: Lower gastrointestinal bleeding.

Implications for practice and research

Based on our results, we suggest standardizing the definition of HI and shock, and establishing a protocol to proactively screen and monitor the affected patients in routine management. Physicians involved in the treatment of the affected patients should focus more on early and rapid correction of hemodynamics because it significantly decreases mortality [11]. Therefore, a careful pre-endoscopic assessment and strong adherence to risk stratification scores need to be highlighted. Furthermore, cautious care and continuous monitoring of the affected patients should be emphasized, especially for high-risk patients.

CONCLUSION

Our study has provided clear evidence that hemodynamic instability and shock are common presentations and complications of GIB. On the basis of our findings, a high majority of patients are affected; one in five, one in four and one in eight patients develops shock or hemodynamic instability on admission or during the hospital stay in the case of non-variceal, variceal, and colonic diverticular bleeding, respectively. Patients need a more proactive treatment strategy and require continuous monitoring to prevent untoward outcomes.

ARTICLE HIGHLIGHTS

Research background

Hemodynamic instability (HI) and shock are associated with unfavorable outcomes in gastrointestinal bleeding (GIB). Understanding the proportion of these outcomes is essential for several reasons. Firstly, it provides valuable insight into the severity and potential risks associated with the condition. Knowing the proportion of patients who develop shock or HI helps healthcare providers anticipate the need for immediate interventions and allocate appropriate resources accordingly.

Research motivation

At the time of our systematic search, there was no data in the current literature describing these proportions in GIB based on the bleeding source. Additionally, monitoring changes in these patients over time can serve as an indicator of the effectiveness of medical interventions and guide future treatment strategies to improve patient outcomes.

Research objectives

Our aim is to quantify the pooled event rates of HI and shock in GIB. This will help in risk stratification and determining the overall severity of the condition. By understanding how frequently these outcomes occur, healthcare providers can identify high-risk patients who require immediate and intensive management.

Research methods

We conducted a systematic review with meta-analysis to determine the proportions of HI and shock in different GIB sources. The R programming language, using the meta package, was employed to perform statistical analysis on the data. Forest plots were utilized to summarize the study findings and present the results. Pooled event rates with 95%CIs, were computed to provide a measure of the overall outcomes.

Research results

The overall proportion of HI and shock was found to be 25% across all sources of GIB, 22% in non-variceal bleeding, 25% in variceal bleeding, and 12% in colonic diverticular bleeding. However, our findings also revealed a high degree of heterogeneity, highlighting the significance of our study. This heterogeneity suggests a lack of consensus in the guidelines in this field, as evidenced by the varied definitions of our included outcomes.

Research conclusions

Our study provides compelling evidence that HI and shock are frequently observed complications and presentations in GIB. One in four patients with GIB develops shock or HI on admission or during the hospital stay.

Research perspectives

Given our findings, we recommend the establishment of a standardized definition for HI and shock in GIB. Additionally, implementing a protocol for proactive screening and continuous monitoring of affected patients should be considered as part of routine management. Emphasizing a thorough pre-endoscopic assessment and strict adherence to risk stratification scores is crucial. Furthermore, rigorous care and attentive monitoring should be emphasized, particularly for high-risk patients.

FOOTNOTES

Author contributions: Obeidat M contributed to conceptualization, investigation, project administration, visualization, validation, writing – original draft; Teutsch B contributed to conceptualization, methodology, project administration, validation, writing – review & editing; Rancz A contributed to conceptualization, investigation, writing – review & editing; Tari E: conceptualization, investigation, writing – review & editing; Márta K contributed to conceptualization, writing – review & editing; Veres DS contributed to conceptualization, formal analysis, software, writing – review & editing; Hosszúfalusi N contributed to conceptualization, writing – review & editing; Mihály E contributed to conceptualization, writing – review & editing; Hegyi P contributed to conceptualization, writing – review & editing; Erőss B contributed to conceptualization, supervision, validation, writing – review & editing; All authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript.

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