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EDITORIAL

Gut microbiota in women: The secret of psychological and physical well-being

Giuseppe Marano, Gianandrea Traversi, Eleonora Gaetani, Antonio Gasbarrini, Marianna Mazza

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Giuseppe Marano, Marianna Mazza, Department of Neurosciences, Università Cattolica del Sacro Cuore, Rome 00168, Italy

Giuseppe Marano, Marianna Mazza, Unit of Psychiatry, Fondazione Policlinico Universitario Agostino Gemelli IRCCS, Rome 00168, Italy

Gianandrea Traversi, Unit of Medical Genetics, Department of Laboratory Medicine, Fatebenefratelli Isola Tiberina-Gemelli Isola, Rome 00186, Italy

Eleonora Gaetani, Department of Medical and Surgical Sciences, Università Cattolica del Sacro Cuore, Rome 00168, Italy

Eleonora Gaetani, Internal Medicine and Gastroenterology Unit, Fondazione Policlinico Universitario Agostino Gemelli IRCCS, Rome 00168, Italy

Antonio Gasbarrini, Medicina Interna e Gastroenterologia, CEMAD Digestive Disease Center, Università Cattolica del Sacro Cuore, Fondazione Policlinico Universitario A Gemelli IRCCS, Rome 00168, Italy

Corresponding author: Marianna Mazza, MD, PhD, Assistant Professor, Department of Neurosciences, Università Cattolica del Sacro Cuore, Largo A. Gemelli 8, Rome 00168, Italy. marianna.mazza@policlinicogemelli.it

Abstract

The gut microbiota works in unison with the host, promoting its health. In particular, it has been shown to exert protective, metabolic and structural functions. Recent evidence has revealed the influence of the gut microbiota on other organs such as the central nervous system, cardiovascular and the endocrine-metabolic systems and the digestive system. The study of the gut microbiota is outlining new and broader frontiers every day and holds enormous innovation potential for the medical and pharmaceutical fields. Prevention and treatment of specific women's diseases involves the need to deepen the function of the gut as a junction organ where certain positive bacteria can be very beneficial to health. The gut microbiota is unique and dynamic at the same time, subject to external factors that can change it, and is capable of modulating itself at different stages of a woman's life, playing an important role that arises from the intertwining of biological mechanisms between the microbiota and the female genital system. The gut microbiota could play a key role in personalized medicine.

Key Words: Gut microbiota; Women; Metabolites; Health promotion; Immune system; Gut-brain axis

Core Tip: The function of the gut microbiota on health is of primary importance, as it educates and controls the immune system, allows to metabolize and absorb nutrients correctly and protects from pathogens invasion. This paper focuses on the importance of the microbiota for women's physical and psychological well-being. The gut microbiota has a strategic role in crucial moments at every stage of a woman's life: From childhood to adolescence, from fertile age to pregnancy-partum, up to menopause. In the future, the study of the gut microbiota could be useful in the treatment of autoimmune and metabolic diseases and even in the fight against tumors, allowing the latest generation of oncological treatments, including immunotherapy, to be more effective.

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INTRODUCTION

Symbiotic microbes are present in several sites across the human body and contribute to the healthy physiology of our organism. The microbiota consists of all these microorganisms (bacteria, viruses, fungi and parasites) in a multicellular living organism that live in symbiosis with it without harming it. The assemblage of microbes and their respective genomes constitute our "microbiome" and contribute to shape and regulate many aspects of healthy bodily function. Gastrointestinal, skin, vaginal, and respiratory microbiomes are featured across those respective anatomical and functional sites[1].

In particular, the gastrointestinal tract is composed of different anatomical structures and is the theatre of complex biochemical processes, as well as parallel interactions with sensory, neurological, and endocrinological networks. Not surprisingly, the gut environment is characterized by a heterogeneous collection of distinct habitats along the rostral-caudal axis, which host the most abundant and diverse microbiota in the human body[2].

In recent years, a large body of studies on the human gut microbiota has increased impressively, deepening the awareness that the composition of the gut microbiota can greatly influence health status. Taking part in the digestive process, the gut microbiota plays a fundamental role in the synthesis of short-chain fatty acids, certain vitamins and essential amino acids, which contribute to the health of the body and the gut[3]. The gut microbiota has a strong influence on the immunoregulation, and on metabolic and cardiovascular health[4,5]. Furthermore, it can contribute to the correct functioning of the central nervous system and can even condition the response to drugs[3,6]. States of dysbiosis can adversely affect host health by promoting enrichment of pathogenic species, compromising the permeability of the intestinal barrier, and contributing to localized or generalized inflammatory states[7]. These conditions can lead to the onset of diseases such as cancer, inflammatory bowel disease, metabolic diseases, or even affect the health of other body districts *e.g.*, through gynaecological and dermatological diseases.

The gut microbiota is directly involved not only in the genesis of gastroenterological diseases, such as chronic inflammatory bowel disease, but also in the neurological and psychiatric fields due to the role of the so-called gut-brain axis[8]. The microbiota-gut-brain axis is a bidirectional communication system that connects the central nervous system with the gut microbiota. This axis describes a bidirectional interaction between the inside of the enteric environment (the intestinal epithelium, microbiota, enteric nervous system) and the outside (the central nervous system), connecting centers of the cognitive and emotional spheres, endocrine, and immunological activity. Growing scientific evidence indicates that the gut microbiota can modulate the functions of the central nervous system and *vice versa*[9]. The marked synergy and continuous exchange of information along this axis is possible because of the vast neurochemical pool available to the enteric nervous system, which innervates the gastrointestinal tract, comparable only to that of the central nervous system. Cells in both systems use the same chemical mediators[3]. Under stressful conditions, the autonomic nervous system can alter intestinal motility and blood flow, and lead to an excessive secretion of hormones and neurotransmitters such as adrenaline and cortisol[10]: All this translates into an alteration in the composition and functional activity of the gut microbiota (Figure 1).

Due to dysbiosis, injurious molecules released from the gut and mediators of the immune response (especially cytokines and interleukins) released during the subsequent chronic inflammatory process can damage and overcome the blood-brain barrier. As a result, brain areas that are critical for the control of emotions and behaviour, such as the limbic and frontal lobes, can be damaged[11]. The study conducted by Carloni *et al*[12] highlighted that alteration of the blood-brain barrier, induced by chronic intestinal inflammation, can be a cause of severe depressive and anxiety disorders.

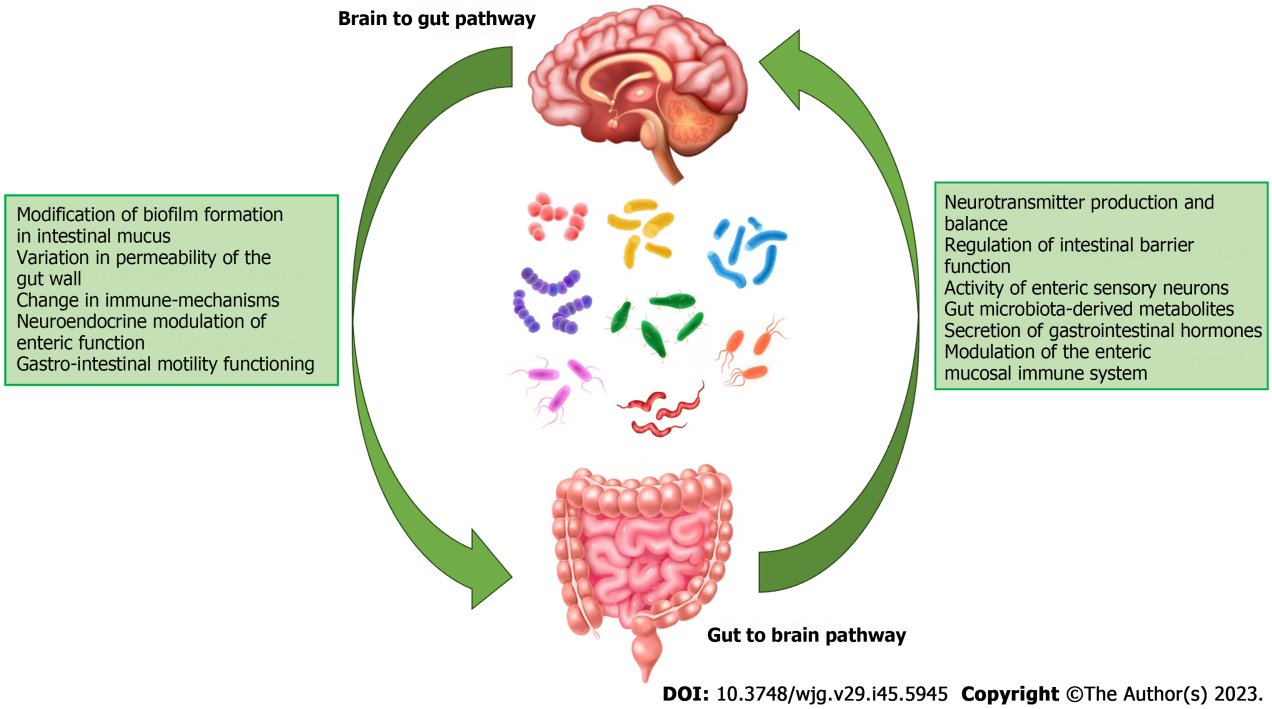

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Figure 1 Gut-brain axis interaction.

The emerging role of the gut microbiota in women

There is a complex controlled exchange of bacteria and other microorganisms from mother to child, even before birth. This dialogue prepares the newborn's immune system to face the outside world. Each individual acquires at birth his or her own personal gut microbiota, which will be an integral part of his or her defence mechanisms: An extremely heterogeneous set of bacteria, protozoa, fungi, and viruses that form the barrier between the external environment and the internal part of the organism (Figure 2). Its strength lies in diversity: A microbiota with high diversity can maintain certain functions and is usually a guarantee of a healthy immune system, while a low diversity microbiota can more easily undergo deficiencies and can cause impaired immune defences[13]. At birth, the digestive tract of newborns is completely sterile and is colonized immediately, starting from birth, by the microorganisms with which it comes into contact[13]. The gut microbiota of newborns delivered by caesarean section and/or those artificially breastfed appears profoundly different and takes longer to stabilize. In the first 4-36 mo of life, as a result of contact with parents, the external environment and food, the gut microbiota develops and changes rapidly.

In the first years of life, a real genetic "imprinting" of the gut microbiota takes place, which then determines the state of health in the rest of existence: There it is written whether or not there is predisposition toward certain diseases[14]. This is a crucial time in an individual's life, it occurs within the age of 4-6 years, and for this reason all factors that can alter it should be avoided[15].

At this age, any intervention on the bacterial flora takes on a very important meaning as it will leave an indelible mark on what will be the adult gut microbiota. From this individual basic nucleus, various changes continue to be observed at different stages of life or if particular pathological conditions are established.

Differences in gut microbiota between men and women

The factors that can influence the composition and balance of the gut microbiota in adulthood are the most diverse: Environmental conditions, stress levels, genetic predisposition, hormonal structure (such as pregnancy, menopause, premenstrual period), pharmacological therapies, eating habits, styles of life[16]. There are diversities between male and female gut microbiota[17] related to hormonal[18], autoimmune[19], ethnic[20] or purely physiological differences such as body mass index body mass index[21] and age[22]. Since microbes of the same species can produce different metabolites depending on the gender of the host and interact differently with sex hormones producing different effects [23] for better characterization it is important to take into account gender differences. These differences appear after puberty, suggesting that sexual hormones have a key role in influencing composition of gut microbiota[24]. The gut microbiota progressively develops along with the maturation of the immune and nervous system throughout the lifespan in men and women with resultant different microbial communities as well as immune and neuro-inflammatory pathways in adult males and females[24].

The embryo differentiates in a male direction due to the Y chromosome and the genes contained therein, which condition circulating testosterone levels. In mice, the microbiota also seems to follow the same paradigm. There is a basic female-type microbiota, which progressively differentiates into a male-type microbiota in parallel with the increase in circulating testosterone[25,26]. Boys and girls during childhood show significant differences in *Actinomycetota*, *Bacillota*, and *Bacteroidota* phyla amount with higher *Bacteroidota/Bacillota* ratio in boys compared to girls. In adult population men

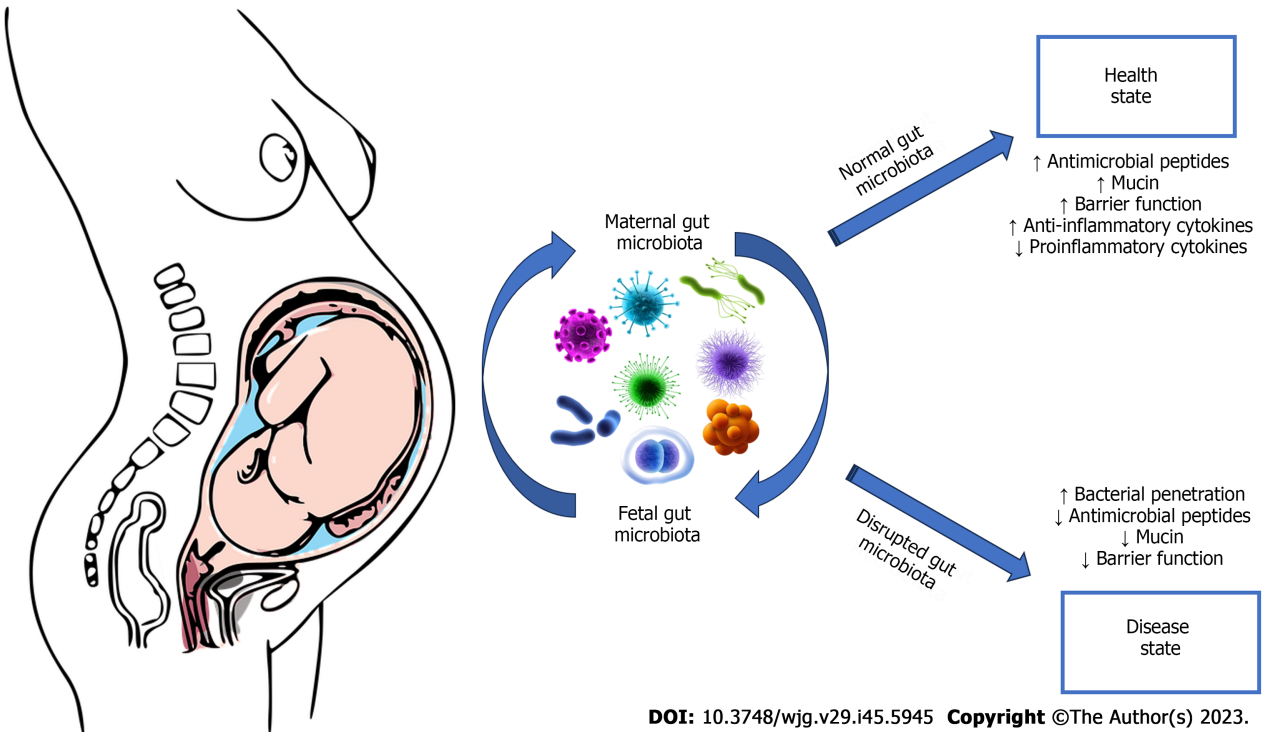

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Figure 2 Interplay between maternal and fetal microbiota.

and women maintain different amount of gut microbiota phyla (*Bacillota, Verrucomicrobia, Bacteroidota, Fusobacteria* and *Actinomycetota*)[27].

Gut microbiota during different stages of a woman's life

Puberty: In women, estrogens are important for differentiating the microbiota with health-promoting characteristics. The vaginal microbiota, whose population of microorganisms changes radically after puberty, is essential for the defence of the vagina throughout the fertile age, with a particular value for the pregnancy protection. After menopause, the impoverishment of the microbiota by sexual hormones deficiency leads to a depletion of many of its local, loco-regional and systemic beneficial health functions[24-26].

The estrobolome is capable of producing and metabolizing estrogen, which plays a key physiological role in maintaining women's health therefore modulating estrogen levels can have effects on weight, libido, and even mood, important dimensions for well-being of every person[28]. As regards estrogens in particular, intestinal microorganisms can modify their levels mainly through the activity of certain enzymes with which some bacteria are equipped, thus transforming estrogens into their active form capable of triggering physiological effects. An altered gut microbiota may play a leading role in the onset and development of reproductive system disorders with hormonal imbalances, such as polycystic ovary syndrome, endometriosis, and infertility[29]. Similarly, it has been suggested that microbiota imbalances with impairment of the gut's physiological "barrier" function and consequent translocation of microbes and/or microbial components have an active role in the development of vaginal and urinary tract infections[30,31].

Pregnancy: Pregnancy represents the period most clearly exemplifying the existing relationships between microbiota and hormone levels, where the modification of gut bacteria produces relevant and evident alterations ranging from a "low-grade" inflammatory condition typical of the pregnant state, to a reduced glucose tolerance, increased adiposity, among other transmissible as has been demonstrated in some experimental observations conducted on laboratory mice transplanted with the microbiota of women in the third trimester of pregnancy[30,31]. During pregnancy, the gut microbiota primarily defends the health of the mother, for example, by helping her not to develop gestational diabetes, and at the same time, it also preserves that of the child who will be exposed to the first "transfer" of microorganisms at the time of birth[32]. The vertical transmission of the microbiota from mother to fetus, which appears to begin during intrauterine life, contributes to the development of the child's gastrointestinal microbiota[33]. Going toward the third trimester, the relative abundance of some bacteria that are then transferred to the newborn in the peripartum (in the case of vaginal birth) increases: In fact, the child's gut microbiota consists of 22% bacteria derived from the mother's gut microbiota, particularly *Bifidobacteria*, *Bacteroides*, and *Escherichia coli*[31]. A more recent study showed important differences in the gut microbiota related to the sex of the individual and allowed the identification of microorganisms that remain in the female gut until fertile age and are then transmitted from mother to child during birth[34]. These strains are part of the genus *Bifidobacterium*, a group of microorganisms that has been much studied because they are associated with a positive impact on host health. Thus, knowing the composition of the microbiota during pregnancy can be a most useful tool both for preserving a woman's health status and for "working" to ensure that a healthy stock of microorganisms is inherited at the time of birth.

Menopause: In menopausal women it has been observed a depletion of *Bacillota* and a progressive reorganization of the gut microbiome (post-menopausal women tend to be more similar to age-matched men as to pre-menopausal women regarding *e.g.*, *Bacillota* to *Bacteroidota* ratio). This could suggest that testosterone may play a major role in shaping the gut microbiota, but more studies focusing on different ages with data comparing both male and female subjects are warranted in order to enlighten the distinct role of sexual hormones as direct or indirect choirmasters of human gut microbiota[27].

Gut microbiota and diseases in women

The fact that the gut microbiota differs between males and females can cause some sex-specific changes in immunity and it has been demonstrated that differences in male and female microbiota can drive chronic diseases, ranging from gastrointestinal inflammatory and metabolic conditions to neurological, cardiovascular, and respiratory illnesses[35]. Gut microbiota has a bidirectional relationship with inflammation and depending on its composition, it can inhibit or stimulate inflammatory pathways. Altered gut microbiota can contribute to subacute systemic inflammation reinforcing the disease state[36].

Potential role of the gut microbiota and its dysbiosis has been described in many diseases affecting women, such as polycystic ovarian syndrome, female cancers (breast, cervical, and ovarian cancer) and postmenopausal period illnesses such as menopausal obesity, Alzheimer's disease, and bone diseases[37]. The International Cancer Microbiome Consortium has suggested a key role of the gut microbiota in disease development, tumour protection, response to therapies, and control of side effects of cancer treatments[38]. Wang *et al*[39] found that the feces composition of women with benign pathology differed from that of women suffering from ovarian carcinoma where *Akkermansia* were low. The researchers showed that transferring gut microbes from ovarian carcinoma patients to mice with the same tumour increased their growth. Subsequent addition of *Akkermansia* bacteria to the microbiota transplant slowed tumour growth, probably due to increased T-cell activation. *Akkermansia* supplementation increased levels of short-chain fatty acids, which are associated with an improved ability of specific T lymphocytes to kill tumour cells.

The profile of the gut microbiota and its metabolites can serve as biomarkers for breast cancer. Some potentially pathogenic bacteria such as *Clostridium*, *Citrobacter*, and *Escherichia* may negatively influence the development and progression of breast cancer cells[40,41]. However, some bacterial species have shown a protective effect against this disease. *Faecalibacterium prausnitzii* and *Roseburia intestinalis* are producers of butyrate, this molecule has powerful anti-inflammatory and intestinal permeability-reducing activities, demonstrating a protective effect against the development of tumour cells[42,43].

The gut appears to be directly connected to the breast through an "axis", which may underlie a correlation between gut and breast microbiota dysfunction: The gut microbiota-mammary axis[44]. Altered composition of the various bacterial populations, no longer harmoniously represented within the microbiota, could affect not only local intestinal inflammation, but also systemic inflammation through "hyperactivation" of the immune system. In fact, several studies have shown the presence of dysbiosis in the breast microbiota of women with cancer, but not of healthy women[45]. Costantini *et al*[46] pointed out that the microbiota may predispose to neoplastic transformation. The clinical importance of this observation suggests that "working" on the microbiota to promote its rebalancing could result in a subsequent reduction in the risk of tumour transformation (Table 1).

The gut microbiota also actively and importantly influences the effectiveness of the response to immunotherapy. Several studies have observed that patients in whom immunotherapy is effective have a gut microbiome very rich in different species, whereas in patients who are resistant to this treatment the microbiota repertoire is more limited. Thus, the gut microbiota represents a first important modulation tool in regulating the anti-tumour immune response[47]. For example, *Bacteroidota* are biomarkers of response in melanoma patients: Their presence is associated with a possible reduction in response rates. In melanoma patients, the presence of three types of bacteria (*Bifidobacterium pseudocatenulatum*, *Roseburia* spp. and *Akkermansia muciniphila*) appears to be associated with better response to immunotherapy, but the link between microbiota and response to therapy involves different species in different patient groups [48]. On the other hand, *Faecalibacterium*, *Bifidobacterium* and *Oscillospiraceae* can improve the response to immune checkpoint inhibitors[49]. In addition, the gut microbiota may also play a role in the response to chemotherapy and in reducing the impact of treatment side effects, such as oral mucositis and inflammation[50,51].

CONCLUSION

Although there is a growing interest on influence of the gut microbiota on other organs, our knowledge on the role of gut microbiota in diseases is currently still limited. Some researchers have focused on the variation of the gut microbiome *via* diet and through supplementation with pre/pro/postbiotics in various female health issues. Interdisciplinary studies on the microbiota are achieving progress toward a better understanding of the molecular basis responsible for microorganism-host interaction and possible positive or negative implications on women's health. The modulation of the gut microbiome as a both preventative and therapeutic strategy needs to be accomplished. Theoretically, enriching the gut microbiota with "good" bacteria at the expense of "bad" bacteria, good health is promoted. However, there cannot be an ideal microbiota that is the same for everyone: Genes and individual characteristics play a determining role. Increasingly advanced techniques for analyzing the gut microbiota and its functions may also contribute to the development of precision medicine.

Table 1 Summary of gut microbiota in women

	Summary of gut microbiota
Healthy female[27]	Decreased: *Bacteroides* abundance with ↑diversity than in men
	Increased: *Lactobacillus*, *Bifidobacterium* and *Parabacteroides* than in men
Menstruation[29]	Decreased: *Bacteroidota*, *Butyricicoccus*, *Extibacter*, *Megasphaera*, *Parabacteroides*
Pregnancy[31-33]	Increased: *Actionbacteria*, *Proteobacteria*, *Akkermansia*, *Bifidobacterium*, *Bacillota*
	Decreased: Short chain fatty acids producers
Polycistic ovarian syndrome[36]	Increased: *Phocaeicola vulgatus*, *Bacillota*, *Streptococcus*, *Escherichia/Shigella*
	Decreased: *Tenericutes*, *Akkermansia*, *Oscillospiraceae*
Menopause[22]	Increased: *Bacillota*, *Roseburia*, *Lachnospira*, *Bacteroidota*
	Decreased: *Bilophila*, *Prevotella*, *Parabacteroides*
Breast cancer[40,41]	Increased: *Eubacteriales*, *Bacillus*, *Enterobacteriaceae*, *Staphylococcus*
Cervical cancer[37]	Increased: *Proteobacteria*, *Prevotella*, *Porphyromonas*, *Dialister*
	Decreased: *Bacteroides*, *Alistipes*, *Lachnospiraceae*
Ovarian cancer[39]	Increased: *Prevotella*, *Coriobacteriaceae*, *Bifidobacterium*

FOOTNOTES

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ORCID number: Giuseppe Marano 0000-0001-7058-4927; Antonio Gasbarrini 0000-0003-4863-6924; Marianna Mazza 0000-0002-3007-8162.

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ORIGINAL ARTICLE

Observational Study

Enduring association between irritable bowel syndrome and war trauma during the Nicaragua civil war period: A population-based study

Edgar M Peña-Galo, Daniel Wurzelmann, Javier Alcedo, Rodolfo Peña, Loreto Cortes, Douglas Morgan

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Edgar M Peña-Galo, Javier Alcedo, Department of Digestive Diseases, Miguel Servet University Hospital, Zaragoza 50009, Aragon, Spain

Edgar M Peña-Galo, Javier Alcedo, Aragon Health Research Institute, IIS Aragon, Zaragoza 50009, Aragon, Spain

Daniel Wurzelmann, Department of Mental Health, Carolina Partners, Durham, NC 27707, United States

Rodolfo Peña, Department of Data Analysis, CIDE (Research, Development and Epidemiology Center), Tegucigalpa 11101, Francisco Morazán, Honduras

Loreto Cortes, School of Medicine, National Autonomous University of Nicaragua, Leon 21000, Leon, Nicaragua

Douglas Morgan, Department of Gastroenterology and Hepatology, UAB University of Alabama Birmingham, Birmingham, AL 35233, United States

Douglas Morgan, School of Medcine, The University of North Carolina, Chapel Hill, NC 9500, United States

Corresponding author: Douglas Morgan, FACG, MD, Director, Professor, Department of Gastroenterology and Hepatology, UAB University of Alabama Birmingham, No. 373 Boshell Bldg, UAB, Birmingham, AL 35233, United States. drmorgan@uabmc.edu

Abstract

BACKGROUND

Psychosocial and physical trauma are known risk factors for irritable bowel syndrome (IBS), including in war veterans, whereas war exposure in civilians is unclear. Nicaragua experienced two wars, 1970-1990: The Sandinistas Revolution (1970s) and The Contra War (1980s). Our aim was to investigate the role of exposure to war trauma in the subsequent development of IBS in the context of an established health surveillance system (11000 households).

AIM

To investigate in a civilian population the relationship between exposure to war trauma and events and the subsequent development of IBS in the context of an

established public health and demographic surveillance system in western Nicaragua.

METHODS
We conducted a nested population-based, cross-sectional study focused on functional gastrointestinal disorders based on Rome II criteria. 1617 adults were randomly selected. The Spanish Rome II Modular Questionnaire and Harvard Trauma Questionnaire were validated in Nicaragua. War exposure was assessed with 10 measures of direct and indirect war trauma and post-war effects. Multiple exposures were defined by ≥ 3 measures.

RESULTS
The prevalence of IBS was 15.2% [Female (F) 17.1%, Male (M) 12.0%], war exposure 19.3% (F 9.3%, M 36.7%), and post-traumatic stress disorder (PTSD) 5.6% (F 6.4%, M 4.3%). Significant associations with IBS in the civilian population were observed (adjusted by gender, age, socioeconomic status, education): physical and psychological abuse [adjusted odds ratio (aOR): 2.25; 95% confidence interval: 1.1-4.5], witnessed execution (aOR: 2.4; 1.1-5.2), family member death (aOR: 2.2; 1.2-4.2), and multiple exposures (aOR: 2.7; 1.4-5.1). PTSD was independently associated with IBS (aOR: 2.6; 1.2-5.7).

CONCLUSION
An enduring association was observed in the Nicaragua civilian population between specific civil war-related events and subsequent IBS. Civilian populations in regions with extended armed conflict may warrant provider education and targeted interventions for patients.

Key Words: Irritable bowel syndrome; Functional gastrointestinal disorders; War trauma; Civil war; Post-traumatic stress disorder; Central America

Core Tip: What is known: Psychosocial and physical trauma are known risk factors for irritable bowel syndrome (IBS), including in war veterans. What is new: An enduring association was observed in the Nicaragua civilian population between specific civil war-related events and subsequent IBS. Civilian populations in regions with extended armed conflict may warrant provider education and targeted interventions for patients.

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INTRODUCTION

Irritable bowel syndrome (IBS) is an important functional gastrointestinal disorder (FGID) affecting 1.1% to 35.5% of the general population. In North America, Europe, Australia and New Zealand the prevalence is estimated to be 8.1% [95% confidence interval (95%CI): 7.0-8.3][1]. Recent data developed in North America and the United Kingdom estimated an IBS prevalence with Rome IV criteria in 4.6% (95%CI: 4.1%-5.2%)[2]. In Latin America, the prevalence may be 2-3 times higher (17.5%, 95%CI: 16.9%- 18.2%)[1]. Furthermore, in central America the IBS studies are scarce, and not reported prevalence of IBS according to subtype[3]. The pathophysiology of IBS is a complex bio-psychosocial disease model. Variation in disease epidemiology may be explained by the heterogeneity of the biology, cultural and socioeconomic context, evolution of diagnostic criteria, and the microbiome, including parasite and viral infections[1,4-6] IBS is associated with multiple comorbidities, including somatic pain syndromes, other gastrointestinal disorders and psychiatric disorders, which affect patient quality of life (QOL) and health care utilization[1,7]. IBS and war veterans most studies of FGIDs and war trauma have focused on professional soldiers. Studies in deployed United States veterans identified a positive association between IBS, life stressors and gastroenteritis[8-10]. An analysis from the millennium cohort study, antecedent infectious gastroenteritis, female gender, life stressors, and anxiety are significantly associated with IBS[8]. This suggests that FGIDs may develop following war exposure, and may exert significant long term adverse effects. In addition, the risk of IBS in women veterans is nearly three times higher [odds ratio (OR): 2.82, 95%CI: 2.06-3.85) in those with posttraumatic stress disorder (PTSD)[11]. A recent meta-analysis based on 8 studies found PSTD to be a significant risk factor for IBS (OR: 2.80, 95%CI: 2.06-3.54)[12]. In Gulf War veterans, a case-control study found a positive association with IBS with chronic multi-symptom illness condition (OR: 11.57, 95%CI: 3.66-36.58)[9,10]. Similarly, Tuteja *et al*[10], founded a long-term persistence of FGIDs up to 16 years post-deployment, and a positive association with psychological disorders, extra-intestinal symptoms, and lower of QOL. The Central America Four region (CA-4), Nicaragua, Honduras, El Salvador, and Guatemala, are interconnected by geography, history, language, and development indices

[13]. In 2006, the CA-4 opened borders, similar to the European Union, and has been in transition toward a union of many aspects of their infrastructure, which has had implications for the health systems. The development indices in the CA-4 rank among the lowest in the Western Hemisphere: Multidimensional poverty approaches 20%, and nearly half (48%) of the CA-4 population lives in rural areas[13]. Civil wars dominated the region from 1970-1996, centered in Nicaragua, El Salvador and Guatemala. Importantly, the CA-4 countries account for a large number of the recent immigrant population to the United States, which makes the region unique among global low/middle income countries (LMICs) from a United States perspective[13]. A number equivalent to one quarter of the population of El Salvador lives in the United States, and most are foreign born. From the United States viewpoint, research and prevention initiatives in the CA-4 may be informative for United States. Hispanic populations, particularly for those from the region (> 5 million)[13]. The Nicaragua Civil War period, 1970-1990 Nicaragua suffered through two civil wars in 1970s and 1980s: The Popular Sandinista Revolution in the 1970s, with peak combat intensity in 1975-1979, and the counter-revolution "Contra War" in the 1980s. The Sandinista Revolution was an intense conflict throughout the country, with the greatest intensity in the western region in the cities of León and Chinandega. The civilian population of Leon lived with armed confrontations and aerial bombing[14]. The Contra War developed after the Sandinista victory, in the context of regional conflicts in Central America and the Cold War. It is estimated that 50000 people were killed in each of the two conflicts, and tens of thousands more were exposed to war events and trauma[14,15]. Based upon the enduring oral history of the civil wars in the Leon region, and the emerging bio-psychosocial model of IBS, we hypothesized a potential association between civil conflict and IBS. Our aim was to investigate in a civilian population the relationship between exposure to war trauma and events and the subsequent development of IBS in the context of an established public health and demographic surveillance system in western Nicaragua.

MATERIALS AND METHODS

Study design

We conducted a nested population-based, cross-sectional study of IBS and the FGIDs with the Rome II criteria in western Nicaragua, utilizing the León Health and Demographic Surveillance System (León-HDSS). The Centre of Epidemiology and Health Research, within the National Autonomous University of Nicaragua-León, oversees the León-HDSS, established in 1993. The municipality of León has an area of 820 km^2, including rural and urban populations[16]. The Leó n-HDSS encompasses 10994 households with 54647 individuals, distributed in 208 randomly-distributed geographical clusters, which encompass approximately 22% of the population of the municipality of León[16]. The current study is nested within a comprehensive study of the population prevalence of the FGIDs, based upon Rome 2 criteria, with final enrollment of 1617 persons randomly selected from urban and rural areas (the sample size was based upon an estimated IBS prevalence of 11%, alpha 5%, and 5% losses to follow-up within the León-HDSS). Thereafter, individuals born before 1975 were selected to meet the criteria of having been at least 5 years of age during the peak war period of 1975-1979 in the Leon region (n = 1012).

Variables and instruments

Socioeconomic status was measured by a validated poverty index based on the United Nations unsatisfied basic needs index (UBN)[16,17]. This is a scale from 0 to 4 (0-1 non-poor, 2-3 poor, and 4 extremely poor) that is based on four indicators: Household condition, water and sanitation services, school enrolment, and number of dependents in the household. This scale has been adapted and validated to specific conditions in Nicaragua[16]. Educational level was categorized into four levels: No schooling or illiterate, primary school, secondary school and university/professional formation. The Rome II Modular Questionnaire (R2MQ) was used to identify IBS cases, which were confirmed by physician interview[18,19]. To validate the R2MQ instrument in Nicaragua, we performed an independent selection of 400 individuals (not included in the study sample) for dual R2MQ assessments by trained field coordinators with an interval of 15 d (± 1 d) between the first and second interview. Alternate field coordinators conducted the second R2MQ assessment. The study subjects were randomly selected per the León-HDSS standing protocol. Three hundred and eighty subjects completed the two R2MQ assessments. Case status was confirmed thereafter *via* interviews by trained physicians, who were blinded to the survey results. We documented an R2MQ accuracy of 88% and repeatability of 84% (data not shown). The assessment of war trauma included direct exposures, indirect exposures, and post-war effects. The direct exposures refer to those experienced by the individual and included forced conscription, service in combat, personal trauma and disability, witnessed executions, and physical or psychological abuse. Indirect exposures included experiences of family members, and death of a family member. The post-war effects were focused on unemployment, loss of personal property, and rejection by family members. Unemployment includes difficulty finding employment in the immediate post-war period, and extended unemployment. The Harvard Trauma Questionnaire (HTQ) was used as a proxy to measure PTSD. The war trauma instrument was developed by Mollica *et al*[20], and includes a section covering 30 symptoms likely to be present after traumatic experiences, scored from 1 (not at all) to 4 (extremely). The trauma symptoms section of the HTQ is derived from the Diagnostic and Statistical Manual IV criteria for PTSD, with some additional questions specific to refugee trauma. The HTQ was validated in victims after the 1974 earthquake in Nicaragua, achieved a sensitivity of 95% and specificity of 77% with a cut-off 50/51 out of a maximum score 120[21].

Statstical methods and ethics

The variable distribution by groups [gender and strata age (29-39, 40-49 and ≥ 50)] was analysed with Mantel-Haenszel chi square test for categorical variables. The prevalence of IBS across the exposure variables (gender, age, socioeconomic

Table 1 Demographic, irritable bowel syndrome and war exposure summary

Variable	Total	Gender		*P* value[1]	Age group			*P* value[1]
	N (%)	Men, *n* (%)	Women, *n* (%)		29-39, *n* (%)	40-49, *n* (%)	50-65, *n* (%)	
Poverty				0.175				0.010[2]
Non-poor	657 (64.9)	229 (62.2)	428 (66.5)		227 (59.6)	224 (65.9)	206 (70.8)	
Poor/extremely poor	355 (35.1)	139 (37.8)	216 (33.5)		154 (40.4)	116 (34.1)	85 (29.1)	
Educational level				0.933				< 0.001[2]
Primary school	489 (48.3)	175	314 (48.8)		117 (30.7)	163 (47.8)	209 (71.8)	
High school	372 (36.8)	(47.6) 137	235 (36.5)		183 (48.0)	124 (36.5)	65 (22.3)	
Professional	151 (14.9)	(37.2) 56 (15.2)	95 (14.7)		81 (21.2)	53 (15.6)	17 (5.8)	
IBS				0.029[2]				0.593
No	858 (84.8)	324 (88.0)	534 (82.9)		320 (84.0)	286 (84.1)	252 (86.6)	
Yes	154 (15.2)	44 (12.0)	110 (17.1)		61 (16.0)	54 (15.9)	39 (13.4)	
PTSD				0.180				0.537
No	955 (94.4)	352 (95.7)	603 (93.6)		362 (95.0)	317 (93.2)	276 (94.8)	
Yes	57 (5.6)	16 (4.3)	41 (6.4)		19 (5.0)	23 (6.8)	15 (5.2)	
War				< 0.001[2]				0.022[2]
No	817 (80.7)	233 (63.3)	584 (90.7)		321 (84.3)	259 (76.2)	237 (81.4)	
Yes	195 (19.3)	135 (36.7)	60 (9.3)		60 (15.7)	81 (23.8)	54 (18.6)	
PTSD + War				0.422				0.095
No	987 (97.0)	357 (97.0)	630 (97.8)		376 (98.7)	327 (96.2)	284 (97.6)	
Yes	25 (3.0)	11 (3.0)	14 (2.2)		5 (1.3)	13 (3.8)	7 (2.4)	
Dose Effect				< 0.001[2]				0.199
0-2 exposures	945 (93.4)	308 (83.7)	637 (98.9)		358 (94.0)	311 (91.5)	276 (94.8)	
≥ 3 exposures	67 (6.6)	60 (16.3)	7 (1.1)		23 (6.0)	29 (8.5)	15 (5.2)	
Total	1012 (100.0)	368 (100.0)	644 (100.0)		381 (100.0)	340 (100.0)	291 (100.0)	

[1]Mantel-Haenszel chi square test.
[2]*P* value < 0.05.
IBS: Irritable bowel syndrome; PTSD: Post-traumatic stress disorder.

status, educational level, war exposure, and PTSD). The multiple logistic regression model was adjusted for age, gender, education, and poverty level. In the analysis, age [age of ≥ 45 years old (29-44 or 45-65)], socioeconomic status [non-poor (0 to 1) *vs* poor (2 to 4)], and educational level (elementary primary school or less educational level *vs* secondary school or more) were dichotomized. The model was used to examine the relationship between IBS and war events and exposures (direct and indirect exposures, and postwar effects). A distinct analysis was conducted for PTSD with and without war exposure and the association with IBS. The 95%CI and *P* value < 0.05 is used for the reported values. All analyses were done using IBM SPSS Statistics for Mac, Version 20.0 (IBM Corp., Armonk, NY, United States). This study was approved by the Institutional Review Boards of the University of North Carolina, Chapel Hill (#02-MED-461) and UNAN-Leon. All Good Clinical Practice standards were followed, including informed consent with each subject, participant confidentiality and anonymized data. There were no conflicts of interest with respect to industry or government.

RESULTS

A total of 1617 individuals were randomly selected and consented from the León-HDSS for household interviews, of whom 1012 met age inclusion criteria (see Methods). Nearly two-thirds (n = 644), were women in Table 1. The mean age was 43.8 years old (range 29-65). The distribution of poverty and educational level by gender was similar, with p values of 0.175 and 0.933, respectively. Men were exposed to a greater number of war events than women (36.7% *vs* 9.3%, *P* <

Table 2 Irritable bowel syndrome prevalence by Rome II criteria among 1012 study participants

Variables	IBS		*P* value[1]
	N (%)	95%CI	
Gender			0.029[2]
Male	44 (12.0)	9.0–15.7	
Female	110 (17.1)	14.4–20.2	
Age group			0.593
29-39	61 (16.0)	12.7-20.0	
40-49	54 (15.9)	12.4-20.1	
≥ 50	39 (13.4)	9.9-17.8	
Socioeconomic status[3]			0.298
Non-poor	97 (14.8)	12.3-17.7	
Poor/Extremely poor	57 (16.1)	12.6-20.24	
Educational level			0.701
Primary school/Lower	70 (14.3)	11.5-17.7	
High school	61 (16.4)	13.0-20.5	
University/Professional	23 (15.2)	10.4-21.8	
War			0.237
No	119 (14.6)	12.3-17.1	
Yes	35 (17.9)	13.2-23.9	

[1]Mantel-Haenszel chi square test.
[2]*P* value < 0.05.
[3]Based on United Nations Poverty Index, adapted to Nicaraguan population.
95%CI: 95% confidence interval; IBS: Irritable bowel syndrome.

0.001), and men were more likely than women to have three or more war exposures (16.3% and 1.1%, $P < 0.001$). Participants ages 40-49 were more likely to have war experiences (23.8%, $P = 0.022$) compared with the 29-39 age group (15.7%) and 50-65 years old group (18.6%).

IBS prevalence and associations

The overall prevalence of IBS was 15.2% (95%CI: 13.1-17.6), and significantly higher among women (17.1% *vs* 12.0%, $P = 0.029$). No significant differences in IBS prevalence were noted by age, education, or poverty level. The IBS prevalence was greater among individuals with war exposure, but not statistically significant (17.9% and 14.6%, respectively, $P = 0.237$) in Table 2. Three specific war exposures were significantly associated with over two times the future risk of IBS in the logistic regression model adjusted for age, gender, education, and poverty level in Table 3. These included physical or psychological abuse [adjusted OR (aOR): 2.25, 1.1-4.5], a witnessed execution (aOR: 2.4, 1.1-5.2), and the death of a family member (aOR: 2.2, 1.2-4.2). In addition, a dose effect was noted, as participants with ≥3 war exposures also had over two times the IBS risk (aOR: 2.7, 1.4-5.1), with a prevalence of 23.9%. The remaining war event exposures were not associated with IBS, including direct combat involvement, physical injury or disability, a wounded family member, and post-war events (loss of personal property, rejection by family and unemployment in table.

PTSD relationchip to war exposure and IBS

The overall prevalence of PTSD was 5.6%, and without differences observed by gender or age (Table 1). PTSD specifically caused by war exposure was 3.0% overall, and similar in men and women (3.0 *vs* 2.2, $P = 0.422$) and among the age strata (1.3% *vs* 3.8% *vs* 2.4%, $P = 0.095$) in Table 1. Notably, 42.1% (95%CI: 30.2-55.0) and 56% (95%CI: 37.1-73.3) of subjects who fulfilled the criteria for IBS had PTSD and war-associated PTSD, respectively, in Table 2. Adjusted for age, gender, education, and poverty level, a strong relationship was observed between IBS and war-associated PTSD (aOR: 3.3, 1.1-10.0) in Table 3.

Table 3 Association between Irritable bowel syndrome and specific war exposures

Condition	IBS, *n* (%)	Non-IBS, *n* (%)	Unadjusted[1], OR (95%CI)	Adjusted[2], OR (95%CI)
Direct Exposures				
War experience	35 (22.7)	160 (18.6)	1.28 (0.84-1.94)	1.30 (0.81-2.09)
Direct combat involvement	13 (8.4)	77 (9.0)	0.93 (0.50-1.72)	0.61 (0.27-1.38)
Abuse, physical or psychological[3]	17 (11.0)	47 (5.5)	2.19 (1.19-3.83)	2.25 (1.12-4.49)
Witnessed execution[3]	17 (11.0)	51 (5.9)	1.96 (1.10-3.50)	2.41 (1.11-5.23)
Physical injury or disability	3 (1.9)	8 (0.9)	2.11 (0.55-8.04)	1.29 (0.27-6.02)
Indirect Exposures				
Death of a family member[3]	20 (13.0)	68 (7.9)	1.73 (1.01-2.94)	2.21 (1.17-4.17)
Family member wounded	9 (5.8)	42 (4.9)	1.20 (0.57-2.53)	0.80 (0.33-1.93)
Post-war effects				
Loss of personal property	3 (1.9)	19 (2.2)	0.87 (0.25-3.00)	0.86 (0.24-3.08)
Rejection by family	5 (3.2)	16 (1.9)	2.58 (0.88-7.54)	2.57 (0.82-8.07)
Unemployment	9 (5.8)	36 (4.2)	1.41 (0.66-3.00)	1.58 (0.69-3.65)
Dose effect				
≥ 3 exposures[3]	16 (10.4)	67 (7.8)	1.83 (1.01-3.30)	2.67 (1.39-5.13)
PTSD				
PTSD[3]	24 (15.6)	33 (3.8)	4.61 (2.64-8.05)	2.63 (1.21-5.73)
PTSD, war associated[3]	14 (9.1)	11 (1.3)	7.70 (3.42-17.3)	3.31 (1.09-10.0)

[1]Crude odds ratio.
[2]Multiple logistic regression analysis adjusted by gender, age, poverty and educational level.
[3]Significant association with IBS.
95%CI: 95% confidence interval; IBS: Irritable bowel syndrome; PTSD: Post-traumatic stress disorder.

DISCUSSION

Our population-based study in the civilian population of Nicaragua demonstrates an association of direct and indirect exposure to war events with the subsequent development of IBS. IBS was associated with specific war exposures, including physical or psychological abuse, a witnessed execution, and the death of a family member. A 'dose effect' is also observed, with an increased risk of IBS among those with ≥ 3 types of war trauma. Riddle *et al*[8] describe an increase association in relation with the numbers of life stressors and female gender. Notably, the majority of studies to date have evaluated the association between deployed veterans and risk of gastrointestinal symptoms or IBS, and from the first Gulf War in particular[9,22-24]. The prevalence of IBS was 14.2% (95%CI: 12.1-16.5) in the general population (without war trauma exposures), which is similar to estimations in Latin America 17.5% (95%CI: 16.9-18.2), some of which hvw used different Rome instruments[1]. The IBS prevalence in females (17.1%; 95%CI: 14.4-20.2) was greater than males (12.0%; 95%CI: 9.0-15.7), also consistent with Latin America data[1,25]. We observed a lower IBS prevalence in subjects ≥ 50 years old, similar to the findings by Lovell and Ford[26] in a review of 14 studies (OR: 0.75; 95%CI: 0.62-0.92). The IBS prevalence was not significantly different among levels of poverty. In this regard, Lovell and Ford[26] noted an insufficient number of studies (n = 4) of the IBS association with socioeconomic status, and insufficient for assessment of heterogeneity. We found that the abuse of civilians in a civil armed conflict setting, physical or psychological, was independently associated with IBS. The association of abuse and IBS is a consistent finding in patients[27-29]. Koloski *et al*[29] reported likelihood abuse (physical, emotional and/or verbal) during adulthood in subjects with symptoms of IBS in contrast with controls. Kanuri *et al*[28] demonstrated a higher prevalence of abuse in the IBS population in comparison with the non-FGID population. Specific forms of abuse, including physical, emotional and sexual, were higher in IBS groups. Abuse experiences have lasting effects on the mental and gastrointestinal health of individuals, thereby establishing a relationship between these experiences and IBS during the adulthood[27-29]. In the Nicaraguan civilian population, IBS was associated with PTSD, likely linked to prior war events exposure (42.1%, 95%CI: 30.2%-55.0%, P value < 0.001). The increased risk of gastrointestinal syndromes among men and women is a consistent finding with warzone exposure[23,30]. Increased rates of IBS among deployed United States veterans[9,22,24]. In a population-based military survey in China, appreciable differences in the prevalence of IBS were noted between aircrew and ground personnel (5.72% *vs* 3.70%), P < 0.05)[31]. In one of the major cohort studies among United States military personnel from all service branch, Riddle *et al*[8], identified a strong association between IBS and PTSD. Maguen *et al*[32] conducted a

retrospective, cross-sectional analysis of > 600000 Iraq and Afghanistan War veterans, finding that IBS was more likely among both males and females among those with PTSD. A meta-analysis with 648375 subjects reported a pooled IBS risk OR of 2.80 (95%CI: 2.06-3.54; $P < 0.001$) with PTSD[12]. Our finding demonstrated similar association in the logistic regression model. In 2016, the Institute of Medicine confirmed that there was sufficient evidence demonstrating an association between deployment to the Gulf War and gastrointestinal symptoms consistent with FGIDs such as IBS or functional dyspepsia[22]. Conversely, a United Kingdom study on the Iraq War estimated that the prevalence of IBS was relatively low on return from Iraq, although it was significant higher during deployment[33]. Tuteja *et al*[10], demonstrated the long-term persistence of IBS with higher scores for all psychological disorders under study (depression, anxiety, somatization, and global symptoms index) in deployed Gulf War veterans. Our results in the Nicaragua civil conflict setting suggest a long-term risk of IBS and its association with PTSD and specific war exposures. Likewise, Riddle *et al*[8] describe an increase association in relation with the numbers of life stressors and female gender. Our study has several strengths including the random selection of a civilian population from an established health and demographic surveillance system, thereby minimizing selection bias. The variables under study were well defined and used instruments validated in the Nicaraguan population. Our study evaluated a general civilian population and therefore may generalize to civilians in prior or current war zones. The study focused on the period over 15 years after the ending of Contra-Revolution war, and consistent with studies suggesting longstanding risk for the development of clinical IBS [10,34]. Several study limitations are noted. The cross-sectional design does not confirm causality between war exposure and IBS. Rome II criteria were used, which have different IBS sensitivity and specificity in comparison with Rome III and Rome IV criteria, but notably, each IBS case was verified by a physician interview in our protocol. Our study was conducted several years after war exposure and potentially subject to recall bias, although arguably these recollections are vivid. Lastly, the impact of gastrointestinal infections in this study population in the tropical environment was not evaluated[6].

CONCLUSION

The prevalence of IBS in the LMIC setting of in Central America is significant and consistent with studies in Latin America. An enduring association in the civilian population of IBS with prior exposure to specific war-related events is observed, in a region of extended civil conflict. Our findings have important implications for healthcare programs for providers and civilian populations in the post-war period[35].

ARTICLE HIGHLIGHTS

Research background

Post-traumatic stress disorder (PTSD) in veterans, and the association with irritable bowel syndrome (IBS) is well described, but the impact on civilian population is poor described.

Research motivation

According to United Nations Refugee Agency 108.4 million of people were forced to flee their homes to escape conflicts in 2023. During this exodus the people are exposed to suffer different PTSD. The Nicaraguan population between 1970 and 1988 was part of this worldwide phenomena.

Research objectives

To determinate the association between PTSD and IBS in civilians exposed to war.

Research methods

A nested cross-sectional study was design. A population data set was used to develop a random selection. Different instruments were validated to collect data. The instruments were focused on IBS, PTSD and poverty. Logistic regression model was developed to respond to our aim.

Research results

Positive association between IBS and PTSD by war exposure in civilians was obtained.

Research conclusions

PTSD and IBS symptoms are persistent over the time. The association between then is positive.

Research perspectives

Other populations in the world could be affected by IBS as a result of PTSD originated in different stressful conflicts. Interventions in primary health care could be implemented to improve the gut and mental health.

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FOOTNOTES

Author contributions: Morgan D and Peña R designed the research; Peña-Galo EM, Peña R, and Cortes L contributed with data acquisition; Peña-Galo EM, Wurzelmann D, Peña R, Alcedo J, and Morgan D analyzed and interpreted of data; Morgan D, and Peña EM wrote manuscript; Peña-Galo EM, Peña R, Alcedo J, Wurzelmann D, Cortes L, and Morgan D performed a critical review.

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Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

Conflict-of-interest statement: There were no conflicts of interest with respect to industry or government.

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ORCID number: Edgar M Peña-Galo 0000-0003-2858-3079; Javier Alcedo 0000-0001-6522-6682; Rodolfo Peña 0009-0008-8496-8452; Douglas Morgan 0000-0002-1136-0963.

Corresponding Author's Membership in Professional Societies: American College of Gastroenterology; American Gastroenterological Association; American Society for Gastrointestinal Endoscopy.

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ORIGINAL ARTICLE

Observational Study

Association of low muscle strength with metabolic dysfunction-associated fatty liver disease: A nationwide study

Gyu Bae Lee, Youn Huh, Sang Hyun Lee, Byoungduck Han, Yang-Hyun Kim, Do-Hoon Kim, Seon Mee Kim, Youn Seon Choi, Kyung Hwan Cho, Ga Eun Nam

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Gyu Bae Lee, Byoungduck Han, Yang-Hyun Kim, Do-Hoon Kim, Seon Mee Kim, Youn Seon Choi, Kyung Hwan Cho, Ga Eun Nam, Department of Family Medicine, Korea University College of Medicine, Seoul 02841, South Korea

Youn Huh, Department of Family Medicine, Uijeongbu Eulji Medical Center, EULJI University, Daejeon 11759, South Korea

Sang Hyun Lee, School of Electrical Engineering, Korea University, Seoul 02841, South Korea

Corresponding author: Ga Eun Nam, MD, PhD, Associate Professor, Department of Family Medicine, Korea University College of Medicine, 73 Goryeodae-ro, Seongbuk-gu, Seoul 02841, South Korea. silver79@korea.ac.kr

Abstract

BACKGROUND

There is limited evidence regarding the association between muscle strength and metabolic dysfunction-associated fatty liver disease (MAFLD).

AIM

To investigate the association between muscle strength and MAFLD in the general population in Korea.

METHODS

This nationwide representative cross-sectional study included 31649 individuals aged ≥ 19 years who participated in the Korea National Health and Nutrition Examination Survey between 2015 and 2018. Odds ratios (ORs) and 95% confidence intervals (95%CIs) for MAFLD according to sex-specific quartiles of muscle strength, defined by relative handgrip strength, were calculated using multivariable logistic regression analysis. Additionally, multivariable logistic regression analysis was used to assess the association between muscle strength and probable liver fibrosis in patients with MAFLD.

RESULTS

Of all the participants, 29.3% had MAFLD. The prevalence of MAFLD was significantly higher in the lower muscle strength quartile groups for all participants, sexes, and age groups ($P < 0.001$). A 1.92-fold (OR = 1.92, 95%CI: 1.70–2.16) and 3.12-fold (OR = 3.12, 95%CI: 2.64–3.69) higher risk of MAFLD was

observed in the lowest quartile (Q1) group than in the other groups (Q2–Q4) and the highest quartile (Q4) group, respectively. The ORs of MAFLD were significantly increased in the lower muscle strength quartile groups in a dose-dependent manner (*P* for trend < 0.001). These associations persisted in both sexes. An inverse association between muscle strength and the risk of MAFLD was observed in all subgroups according to age, obesity, and diabetes mellitus. In patients with MAFLD, the odds of severe liver fibrosis were higher in Q1 (OR = 1.83, 95%CI: 1.25–2.69) than in other groups (Q2–Q4).

CONCLUSION

Among Korean adults, low muscle strength was associated with an increased risk of MAFLD and liver fibrosis in patients with MAFLD.

Key Words: Muscle strength; Handgrip strength; Metabolic dysfunction-associated fatty liver disease; Liver fibrosis; Korea National Health and Nutrition Examination Survey

Core Tip: Limited evidence exists regarding the association between muscle strength and metabolic dysfunction-associated fatty liver disease (MAFLD). This nationwide cross-sectional study analyzed 17349 individuals in the general community who participated in the Korea National Health and Nutrition Examination Survey and measured their grip strength between 2015 and 2018. Among the participants, 29.3% had MAFLD. The prevalence of MAFLD was significantly higher in the lower muscle strength quartiles. The odds ratios of MAFLD were significantly increased in the lower muscle strength quartile groups in a dose-response manner. Among Korean adults, low muscle strength was associated with an increased risk of MAFLD and liver fibrosis in patients with MAFLD.

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INTRODUCTION

The prevalence of non-alcoholic fatty liver disease (NAFLD) has surged alongside the obesity pandemic, making it a significant global public health issue, particularly within the Asian population. Over the last three decades, the overall burden of NAFLD has grown exponentially[1-3]. NAFLD has significant clinical implications because it causes liver cirrhosis and liver cancer, which are major causes of liver-related mortality[1]. In response to the broader multi-system nature of NAFLD and the rising prevalence of metabolic disorders, a recent introduction has been made regarding a new definition: metabolic dysfunction-associated fatty liver disease (MAFLD)[2]. While reports suggest that the prevalence of MAFLD in Asian countries ranges from 10%–30%, it exhibits a discernible upward trajectory[2]. Notably, a 23-year follow-up United States study revealed that MAFLD was associated with increased all-cause mortality; furthermore, advanced fibrosis in MAFLD had a higher all-cause mortality risk than that in NAFLD[4]. Given the clinical significance and the imperative to alleviate the disease burden associated with MAFLD, a thorough analysis of risk factors is essential.

Previous studies have suggested an association between sarcopenia and NAFLD. Specifically, muscle mass or strength has shown an inverse relationship with NAFLD[5-7]. Furthermore, it is worth noting that reduced muscle strength has shown a significant association with liver fibrosis in individuals afflicted with NAFLD[8]. Muscle strength, a marker for cardiometabolic fitness, is inversely associated with morbidity[9,10], encompassing conditions such as metabolic syndrome and mortality, particularly within the context of NAFLD[11-14]. Thus, we hypothesized that sarcopenia, particularly muscle strength, is associated with the risk of MAFLD and advanced fibrosis in MAFLD.

Although the body of evidence is evolving, it is worth noting that only a few cross-sectional studies have shown that sarcopenia, measured using dual-energy X-ray absorptiometry and mid-upper arm circumference, was associated with liver fibrosis in patients with MAFLD[15,16]. Limited evidence exists on the association between muscle strength and the prevalence of MAFLD and liver fibrosis in MAFLD in the general population[17]. In light of the public health burden of MAFLD in Asia, we investigated the association between muscle strength and MAFLD using a Korean nationally representative database.

MATERIALS AND METHODS

Survey description and study participants

This study used data from the Korea National Health and Nutrition Examination Survey (KNHANES). The Ministry of Health and Welfare and the Korea Disease Control and Prevention Agency jointly conduct the KNHANES to calculate national health statistics, which is the fundamental database for healthcare policymaking in South Korea. This annual examination recruits an average of 10000 participants in South Korea and consists of 11 different variables, including blood tests and physical examinations, such as measuring body weight, height, handgrip strength, and blood pressure (BP). The KNHANES ensures the quality of the data entered into the database through data collection by well-trained staff and quality control of procedures by internal and external professionals. Details of the KNHANES database have been covered in depth in the past[18].

Because the KNHANES database includes pediatric and adolescent participants, only Korean citizens aged ≥ 19 years who participated in the KNHANES during 2015-2018 were initially included in the analysis. Among the 31649 individuals who participated in the KNHANES between 2015 and 2018, we excluded individuals aged < 19 years (n = 6315), heavy drinkers who consumed ≥ 210 g of alcohol per week for men and ≥ 140 g per week for women (n = 2712) based on self-reported questionnaires, those who tested positive serological markers for hepatitis B or C virus (n = 976), those diagnosed with liver cirrhosis or hepatocellular carcinoma (n = 84), and those with missing data (n = 4213). Ultimately, the data from 17349 individuals were included in the analysis. All participants provided written informed consent for data collection.

Assessment of muscle strength

Muscle strength was assessed using relative handgrip strength, which has been utilized in prior studies as an indicator of muscle strength[8,19]. To measure handgrip strength, the participants were instructed to squeeze a handgrip dynamometer (Digital grip strength dynamometer, T.K.K 5401, Takei Scientific Instruments Co., Ltd., Tokyo, Japan) for at least 3 S using their dominant arm with the elbow extended and the participant in a standing position. Handgrip strength was measured thrice with a 1-min interval for rest between each measurement. Muscle strength was defined as the mean handgrip strength (kg) divided by the body mass index (BMI, kg/m^2). As the present study aimed to understand the association between muscle strength and MAFLD, the study participants were divided into sex-specific quartile groups of muscle strength, with Q1 and Q4 being the lowest and highest quartiles, respectively. The cutoff values for the quartiles were 1.30, 1.53, and 1.77 in men, and 0.76, 0.94, and 1.11 in women, respectively.

Definition of MAFLD and liver fibrosis

NAFLD was defined using a validated fatty liver prediction model called the hepatic steatosis index (HSI)[20]. HSI was defined as 8 × alanine aminotransferase (ALT)/aspartate aminotransferase (AST) + body mass index (BMI, + 2, if diabetic; +2, if female). HSI > 36 was defined as NAFLD[20]. Previous studies have reported that HSI could predict NAFLD with high sensitivity and specificity in the Korean population[20,21].

MAFLD was defined as NAFLD (HSI > 36) with the presence of at least one of the following metabolic risk factors[2]: (1) Overweight or obesity (BMI ≥ 23 kg/m^2) based on Asian standards[22]; (2) Type 2 diabetes [physician diagnosis, fasting serum glucose ≥ 126 mg/dL, or glycated hemoglobin (HbA1c) ≥ 6.5%]; and (3) Normal BMI (< 23 kg/m^2) with two or more of the following metabolic risk factors[23]: (1) Waist circumference (WC) ≥ 90 cm and ≥ 80 cm for men and women; (2) BP ≥130/85 mmHg or being administered anti-hypertensive medication(s); (3) Triglyceride level ≥ 150 mg/dL or being administered lipid-lowering medication(s); (4) High-density lipoprotein cholesterol (HDL-C) < 40 mg/dL for men and < 50 mg/dL for women, or being administered lipid-lowering medication(s); (5) Diagnosis of prediabetes state, defined as fasting serum glucose of 100-125 mg/dL or HbA1c of 5.7%–6.4%; and (6) Serum high-sensitivity C-reactive protein (hs-CRP) level > 2 mg/L.

To evaluate advanced liver fibrosis in patients with MAFLD, we used the following prediction equation: Fibrosis-4 (FIB-4) score = age (years) × AST (IU/L)/[platelet (10^9/L)] × [ALT (IU/L)]$^{1/2}$. The risk of advanced fibrosis in MAFLD was classified as either 1.3 ≤ FIB-4 score < 2.67 (intermediate risk) or FIB-4 ≥ 2.67 (high risk)[24].

Measurements and covariates

Participants' sociodemographic information and data on health behaviors were assessed using a self-report questionnaire. Smoking status was classified based on whether the participant was a current smoker. Based on the modified version of the International Physical Activity Questionnaire[25,26], regular physical activity was defined as: (1) Moderate-intensity physical activity for ≥ 150 min/wk; (2) High-intensity physical activity for ≥ 75 min/week; or (3) A combination of moderate- and high-intensity physical activity per week, where 1 min of high-intensity physical activity is equivalent to 2 min of moderate-intensity physical activity, with the collective minutes satisfying either one of the above criteria. Household income was segmented into quartiles, and educational attainment was assessed based on whether participants had completed more than 12 years of education (or high school graduate).

The physical examination was conducted by certified staff. Height, body weight, and WC were measured, and BMI was defined as the weight in kilograms divided by the square of height in meters. Using a standard sphygmomanometer, three BP measurements were conducted at 5-min intervals, and the mean values of the second and third BP measurements were recorded. Blood samples were drawn after fasting for ≥ 8 h, and the serum concentrations of AST, ALT, total cholesterol, HDL-C, low-density lipoprotein cholesterol (LDL-C), triglycerides, fasting glucose, HbA1c, hs-CRP, hepatitis B surface antigen, hepatitis C virus antibody, and platelet count were assessed.

Hypertension was defined as either a medical diagnosis by a physician or a systolic/diastolic BP reading of ≥ 140/90 mmHg[27]. Diabetes mellitus (DM) was defined as having a physician's diagnosis, a fasting glucose level of ≥ 126 mg/dL, or an HbA1c level of ≥ 6.5%[28]. Dyslipidemia was characterized by either a physician's diagnosis or a total cholesterol level of ≥ 240 mg/dL[29]. Obesity was defined as BMI ≥ 25 kg/m^2[30].

Statistical analyses

Continuous variables were summarized as mean ± SE and categorical variables as percentages and were compared using analysis of variance and the Rao–Scott chi-square test, respectively. We performed multivariable logistic regression analysis to evaluate the association between muscle strength and the risk of MAFLD and calculated the odds ratios (ORs) and 95% confidence intervals (95%CIs). Model 1 was not adjusted, and Model 2 was adjusted for age, sex, income, education, smoking status, and physical activity. In Model 3, adjustments were made for ALT, obesity, hypertension, DM, dyslipidemia, and hs-CRP levels, in addition to the confounders in Model 2. The adjusted variables were selected from the statistically significant variables in Table 1, the clinical factors that were expected to be associated with muscle strength and MAFLD, and based on the results of the preliminary logistic regression analysis between baseline variables and MAFLD (Supplementary Table 1) and a literature search. The association between muscle strength and the risk of MAFLD was also evaluated in subgroups stratified by sex, age, obesity, and DM. Multivariable logistic regression analysis was used to assess the association between muscle strength and probable liver fibrosis in patients with MAFLD. Statistical analyses, including pairwise comparison, were performed using IBM SPSS Statistics ver. 22.0 (IBM Corp., Armonk, NY, United States). Complex sample procedures were performed based on the survey design. Statistical significance was set at $P < 0.05$. The statistical methods used in this study were reviewed by Dr. Youn Huh from Uijeongbu Eulji Medical Center.

RESULTS

Characteristics of study participants

Among a total of 31649 potentially eligible individuals, 17349 individuals were included and analyzed in the study. The characteristics of the participants according to the muscle strength quartiles are presented in Table 1. Individuals with weaker muscle strength were older and had lower socioeconomic status. Those with weaker muscle strength were less likely to be current smokers and engaged in regular physical activities (P for trend < 0.001). The mean values of cardiometabolic parameters, such as BMI, WC, BPs, AST, ALT, total cholesterol, LDL-C, triglycerides, fasting glucose, HbA1c, and hs-CRP, tended to be higher in the lower muscle strength quartile groups (P for trend < 0.001). The proportion of those with obesity, hypertension, DM, and dyslipidemia also increased as muscle strength decreased (P for trend < 0.001).

Prevalence of MAFLD according to muscle strength

Among all participants, including within various sex and age groups, the prevalence of MAFLD was significantly higher in the lower muscle strength quartile groups ($P < 0.001$ in Figure 1). Additionally, regardless of sex and age group, the prevalence was higher in the lowest quartile (Q1) group of muscle strength than in the remaining quartile (Q2–Q4) groups ($P < 0.001$ in Figure 1B and C).

Association between muscle strength and the risk of MAFLD

Among all participants, the Q1 group had higher odds of MAFLD than the other groups (Q2–Q4) (OR = 1.92, 95%CI: 1.70–2.16) (Model 3, Table 2). Compared with the Q4 group, the ORs of MAFLD significantly increased in the Q3 (OR = 1.41, 95%CI: 1.22–1.65), Q2 (OR = 2.19, 95%CI: 1.85–2.58), and Q1 (OR = 3.12, 95%CI: 2.64–3.69) groups. The ORs were higher in the lower muscle strength quartile groups in a dose-dependent manner (P for trend < 0.001). Among both men and women, higher odds of MAFLD were observed in the Q1 group than in the Q2–Q4 (OR = 2.05, 95%CI: 1.70–2.47 in men and OR = 1.76, 95%CI: 1.47–2.10 in women) and Q4 groups (OR = 2.88, 95%CI: 2.20–3.78 in men and OR = 3.09, 95%CI: 2.43–3.92 in women). The ORs of MAFLD tended to be higher in the lower muscle strength quartile groups for both sexes (P for trend < 0.001). Furthermore, the receiver operating characteristic analysis to assess the relationship between muscle strength and prevalence of MAFLD revealed that the area under the curve for the entire participant group, as well as for men and women separately, were 0.764, 0.701, and 0.740, respectively (all $P < 0.001$, Supplementary Figure 1).

Subgroup analyses on the association between muscle strength and MAFLD

As indicated in Table 3, a noteworthy inverse relationship between muscle strength and the risk of MAFLD remained consistent across all subgroups. Importantly, no significant interactions were observed based on sex, age, obesity, or DM status in the association between muscle strength and MAFLD.

Association between muscle strength and probable liver fibrosis assessed by FIB-4 in MAFLD

After adjusting for all confounding factors, muscle strength was not significantly associated with the intermediate risk for advanced liver fibrosis (defined as 1.3 ≤ FIB-4 < 2.67) in patients with MAFLD (Table 4). However, the lowest muscle strength group (Q1) had a higher odds of a high risk of advanced fibrosis (defined by FIB-4 ≥ 2.67) than the Q2–Q4 group (OR = 1.83, 95%CI: 1.25–2.69).

Table 1 Characteristics of study participants according to the quartiles of muscle strength

	Q1	Q2	Q3	Q4	*P* for trend
N (unweighted)	4305	4347	4397	4300	
Age (yr)	55.9 ± 0.4	49.2 ± 0.4	44.6 ± 0.3	39.9 ± 0.2	< 0.001
Sex (men)	45.2 (1.0)	45.9 (0.9)	46.2 (0.9)	48.0 (0.9)	0.172
Current smoker	13.6 (0.7)	16.6 (0.7)	17.7 (0.7)	20.3 (0.8)	< 0.001
Physical activity	38.5 (1.0)	46.4 (1.0)	51.1 (0.9)	53.1 (0.9)	< 0.001
Income (lowest quartile)	28.8 (1.0)	16.5 (0.7)	10.7 (0.6)	8.0 (0.5)	< 0.001
Education (≤ 12 yr)	45.4 (1.2)	28.1 (0.9)	16.9 (0.7)	8.5 (0.5)	< 0.001
BMI (kg/m^2)	26.0 ± 0.1	24.7 ± 0.1	23.5 ± 0.1	21.8 ± 0.1	< 0.001
Waist circumference (cm)	88.0 ± 0.2	84.0 ± 0.2	80.5 ± 0.2	76.3 ± 0.2	< 0.001
Handgrip strength (kg)	21.8 ± 0.2	27.8 ± 0.2	31.2 ± 0.2	35.6 ± 0.2	< 0.001
Muscle strength (handgrip strength/body mass index)	0.8 ± 0.01	1.1 ± 0.01	1.3 ± 0.01	1.6 ± 0.01	< 0.001
Systolic BP (mmHg)	122.7 ± 0.4	118.2 ± 0.3	115.2 ± 0.3	112.1 ± 0.3	< 0.001
Diastolic BP (mmHg)	75.3 ± 0.2	75.8 ± 0.2	75.2 ± 0.2	74.2 ± 0.2	< 0.001
AST (IU/L)	24.5 ± 0.2	23.1 ± 0.2	21.5 ± 0.2	20.2 ± 0.1	< 0.001
ALT (IU/L)	26.0 ± 0.5	24.2 ± 0.4	21.0 ± 0.3	18.4 ± 0.2	< 0.001
Total cholesterol (mg/dL)	191.4 ± 0.7	194.3 ± 0.7	194.1 ± 0.6	188.8 ± 0.6	< 0.001
HDL-C (mg/dL)	47.4 ± 0.2	49.9 ± 0.2	51.6 ± 0.2	54.0 ± 0.2	< 0.001
LDL-C (mg/dL)	115.0 ± 0.7	116.9 ± 0.6	116.5 ± 0.6	112.3 ± 0.5	< 0.001
Triglycerides (mg/dL)	145.6 ± 2.0	137.5 ± 2.0	130.1 ± 1.9	112.5 ± 1.6	< 0.001
Fasting glucose (mg/dL)	106.1 ± 0.5	100.8 ± 0.4	97.6 ± 0.4	93.8 ± 0.3	< 0.001
HbA1c (%)	5.9 ± 0.02	5.7 ± 0.01	5.6 ± 0.01	5.4 ± 0.01	< 0.001
hs-CRP (mg/L)	1.7 ± 0.04	1.2 ± 0.04	1.1 ± 0.03	0.8 ± 0.03	< 0.001
Obesity	56.7 (0.9)	44.0 (1.0)	28.7 (0.8)	11.6 (0.6)	< 0.001
Hypertension	45.4 (1.0)	30.5 (0.8)	21.5 (0.7)	12.4 (0.6)	< 0.001
Diabetes mellitus	22.5 (0.7)	13.1 (0.6)	7.8 (0.4)	3.9 (0.3)	< 0.001
Dyslipidemia	31.8 (0.9)	28.1 (0.9)	21.2 (0.7)	14.1 (0.6)	< 0.001

Data are presented as mean ± SE or percentage (SE). Q: Quartile; BMI: Body mass index; BP: Blood pressure; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; HbA1c: Glycated hemoglobin; hs-CRP: High-sensitivity C-reactive protein.

DISCUSSION

In this large-scale nationwide study, we found that the prevalence of MAFLD was greater among individuals with lower muscle strength, which was associated with a higher risk of MAFLD after adjusting for potential confounding variables. The lowest muscle strength quartile group had 3.12-fold, 2.88-fold, and 3.09-fold higher odds of MAFLD than the highest quartile group in all participants, men, and women, respectively. These associations persisted in the subgroups stratified by age, obesity, and DM. Furthermore, in patients with MAFLD, the lowest muscle strength quartile group had 1.83-fold increased odds of high risk of advanced liver fibrosis compared to the other groups.

To the best of our knowledge, no prior studies have examined the association between muscle strength and the risk of MAFLD. However, few studies have examined the association between muscle strength and NAFLD[31]. One cross-sectional study, utilizing data from the KNHANES database, found an association between low muscle strength and NAFLD[8]. In our study, we have effectively demonstrated a significant association between muscle strength and MAFLD. Importantly, our findings suggest that low muscle strength may be a modifiable risk factor for MAFLD. This study also suggests an association between low muscle strength and a high probability of advanced liver fibrosis in patients with MAFLD. Our findings are in line with previous studies reporting an association between low muscle strength and advanced liver fibrosis[8,32]. Although the association did not persist when each muscle strength group was analyzed, the current study demonstrated that low muscle strength might be associated with advanced liver fibrosis in

Table 2 Odds ratios (95% confidence intervals) of metabolic dysfunction-associated fatty liver disease according to the categories of muscle strength

Muscle strength	Model 1	Model 2	Model 3
Total			
Q1	3.63 (3.34–3.95)	3.24 (2.94–3.58)	1.92 (1.70–2.16)
Q2–Q4	1 (reference)	1 (reference)	1 (reference)
P value			
Q1	9.12 (7.98–10.41)	8.61 (7.41–10.01)	3.12 (2.64–3.69)
Q2	4.92 (4.29–5.64)	4.75 (4.10–5.50)	2.19 (1.85–2.58)
Q3	2.48 (2.17–2.84)	2.42 (2.10–2.78)	1.41 (1.22–1.65)
Q4	1 (reference)	1 (reference)	1 (reference)
P for trend	< 0.001	< 0.001	< 0.001
Men			
Q1	3.51 (3.02–4.09)	3.31 (2.80–3.90)	2.05 (1.70–2.47)
Q2–Q4	1 (reference)	1 (reference)	1 (reference)
P value	< 0.001	< 0.001	
Q1	7.30 (5.67–9.39)	7.20 (5.52–9.38)	2.88 (2.20–3.78)
Q2	3.68 (2.83–4.78)	3.68 (2.82–4.80)	1.75 (1.32–2.31)
Q3	2.02 (1.54–2.66)	2.03 (1.55–2.68)	1.23 (0.93–1.63)
Q4	1 (reference)	1 (reference)	1 (reference)
P for trend	< 0.001	< 0.001	< 0.001
Women			
Q1	4.20 (3.74–4.71)	3.17 (2.79–3.60)	1.76 (1.47–2.10)
Q2–Q4	1 (reference)	1 (reference)	1 (reference)
P value	< 0.001	< 0.001	< 0.001
Q1	11.76 (9.87–14.02)	9.14 (7.56–11.05)	3.09 (2.43–3.92)
Q2	6.09 (5.15–7.20)	5.30 (4.47–6.30)	2.52 (2.05–3.10)
Q3	2.76 (2.34–3.26)	2.59 (2.20–3.06)	1.52 (1.25–1.85)
Q4	1 (reference)	1 (reference)	1 (reference)
P for trend	< 0.001	< 0.001	< 0.001

Odds ratios (95% confidence intervals) were calculated using multivariable logistic regression analysis; Model 1 = not adjusted; Model 2 = adjusted for age, sex, income, education, smoking status, and physical activity; Model 3 = Model 2 + alanine aminotransferase, obesity, hypertension, diabetes mellitus, dyslipidemia, and high-sensitivity C-reactive protein. Q: Quartile.

patients with MAFLD.

The mechanisms underlying the association between low muscle strength and MAFLD have not yet been fully elucidated. However, potential pathways may include insulin resistance due to hepatic steatosis[33] and physical inactivity and disuse, leading to decreased muscle function[34]. Metabolically inactive muscles undergo disuse atrophy, in which sarcomeres are catabolized and capillaries decrease in number[35]. This risk is especially pronounced in individuals with metabolic syndrome and a sedentary lifestyle. Weight gain leads to adipocyte dysfunction and increased local inflammation, eventually resulting in insulin resistance. Disturbances in fat storage in adipocytes increase the release of fatty acids. Excess fatty acids produce strain in the hepatic mitochondria and subsequently result in the production of reactive oxygen species and mitochondrial damage. Excess fatty acids also increase stress on the endoplasmic reticulum, contributing to mitochondrial dysfunction and cellular death[33]. In particular, reduced muscle strength may be linked to mitochondrial dysfunction in both skeletal muscles and the liver, as suggested by previous studies[36-41]. Data accumulated thus far have indicated that mitochondrial dysfunction may play a role in the development of insulin resistance, and MAFLD and mitochondrial dysfunction may additionally contribute to low muscle strength[42-46]. In relation to hepatic fibrosis, stellate cells are thought to be involved in inflammatory and fibrotic changes in fatty liver disease in response to damage-associated molecular patterns from dying hepatocytes, free

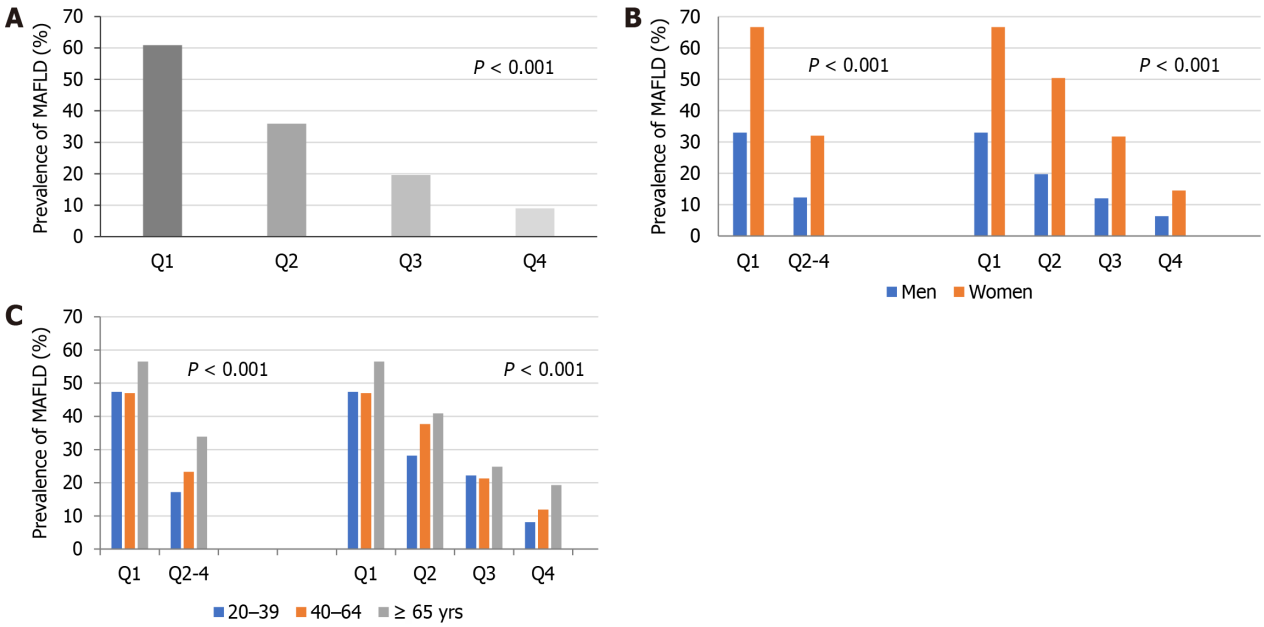

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Figure 1 Prevalence of metabolic dysfunction-associated fatty liver disease by muscle strength. A: The prevalence of metabolic dysfunction-associated fatty liver disease (MAFLD) in the total participants; B: the prevalence in men and women; C: the prevalence in different age groups. The prevalence of MAFLD was higher in the lower muscle strength quartile groups in total participants and age and sex groups. The prevalence was higher in the lowest muscle strength quartile (Q1) group than in the remaining groups (Q2–Q4) in both sexes and all age groups. A dose-response relationship between lower muscle strength quartile and MAFLD was also observed. Q: Quartile; MAFLD: Metabolic dysfunction-associated fatty liver disease.

cholesterol, toll-like receptors, and oxidative stress[33,47].

Sarcopenia is a muscle disease of both young and old and increases the risks of falls, fractures, and mortality[34]. Studies have revealed that muscle strength, rather than muscle mass, is a predictor of mortality[48-50]; thus, the guideline from the European Working Group on Sarcopenia in Older People highlighted low muscle strength as the main characteristic of sarcopenia[34]. Low muscle strength alone is adequate for making a clinical diagnosis of probable sarcopenia. Subsequently, diagnostic assessments for potential underlying causes and appropriate interventions can be initiated[34,51]. Additionally, the guideline recommended handgrip strength as a proxy for whole-body strength[34]. Overall, these findings suggest that muscle strength is a crucial parameter in detecting sarcopenia[52]. Accordingly, we used muscle strength, defined by handgrip strength, as the primary independent variable in this study.

MAFLD increases the risk of liver fibrosis, hepatocellular carcinoma, and cardiovascular diseases; hence, controlling metabolic disorders is essential in the management of MAFLD[2]. Treatment objectives encompass the reversal of hepatic steatosis, steatohepatitis, and hepatic fibrosis, as well as the reduction of cardiovascular risk associated with MAFLD. The mainstay of treatment is the modification of metabolic risk factors and lifestyle[2]. Weight loss can reduce liver steatosis and reverse steatohepatitis or fibrosis[53]. Generally, low-carbohydrate, low-fat, and Mediterranean-type dietary plans with moderate-intensity exercise for 30 min/d for ≥ 5 d/wk or ≥ 150 min/wk or vigorous-intensity exercise for ≥ 20 min/d for ≥ 3 d/wk are recommended[2,54]. The guidelines endorse the inclusion of both aerobic and resistance exercises[2]. In light of the observed association between low muscle strength and a higher prevalence of MAFLD in this study, it is imperative to underscore the importance of resistance training during patient education.

While this study has provided valuable insights, it is important to acknowledge that it also had several limitations. First, the findings of this study are not generalizable to other ethnic groups since only Koreans were included in the analysis. Second, although liver biopsy is the gold standard for diagnosis, the present study used HSI to diagnose NAFLD and MAFLD. However, the diagnostic reliability of the HSI has been validated in previous studies[20,21]. Third, causal relationships could not be fully determined owing to the cross-sectional design of the study. Future prospective longitudinal studies are needed to confirm the role of muscle strength in MAFLD. Fourth, the lack of data on medications for chronic diseases and those potentially affecting liver steatosis is a limitation. Thus, we defined hypertension, DM, and dyslipidemia using a combination of prior physician diagnoses and laboratory blood tests. Fifth, handgrip strength was used to diagnose probable sarcopenia in the present study. The prevalence of probable sarcopenia can vary depending on the diagnostic methods, especially in liver steatosis[55]. Nonetheless, handgrip strength is easy to incorporate into clinical settings and is also a well-studied parameter of sarcopenia in many studies. Despite these shortcomings, the use of KNHANES enabled us to study important exposures and muscle strength in a large representative Korean population and make adjustments for a variety of potential confounders. Furthermore, we were able to successfully assess the new definition of MAFLD and study its prevalence and association with muscle strength, which extends beyond previous studies where only NAFLD was considered.

Table 3 Subgroup analysis for the association between muscle strength and metabolic dysfunction-associated fatty liver disease

	Muscle strength	OR (95%CI)	*P* for interaction
Sex			0.126
Men	Q2–Q4	1 (reference)	
	Q1	2.05 (1.70–2.47)	
Women	Q2–Q4	1 (reference)	
	Q1	1.76 (1.47–2.10)	
Age			0.057
< 65 yr	Q2–Q4	1 (reference)	
	Q1	1.97 (1.70–2.29)	
≥ 65 yr	Q2–Q4	1 (reference)	
	Q1	1.74 (1.44–2.09)	
Obesity			0.264
No	Q2–Q4	1 (reference)	
	Q1	1.63 (1.36–1.96)	
Yes	Q2–Q4	1 (reference)	
	Q1	2.09 (1.75–2.48)	
Diabetes mellitus			0.622
No	Q2–Q4	1 (reference)	
	Q1	1.97 (1.72–2.26)	
Yes	Q2–Q4	1 (reference)	
	Q1	1.71 (1.33–2.22)	

Odds ratios (95% confidence intervals) were calculated using multivariable logistic regression analysis after adjusting for age, sex, income, education, smoking status, physical activity, alanine aminotransferase, obesity, hypertension, diabetes mellitus, dyslipidemia, and high-sensitivity C-reactive protein; Stratified variables (sex, age, obesity, and diabetes mellitus) were omitted from the adjusted variables during the respective subgroup analyses. OR: Odds ratio; 95%CI: 95% confidence interval; Q: Quartile.

Table 4 Association between muscle strength and probable liver fibrosis assessed using fibrosis-4 among patients with metabolic dysfunction-associated fatty liver disease

Muscle strength	OR (95%CI)		
	Model 1	Model 2	Model 3
1.3 ≤ FIB-4 < 2.67			
Q1	2.67 (2.35–3.02)	0.96 (0.82–1.12)	1.03 (0.87–1.21)
Q2–Q4	1 (reference)	1 (reference)	1 (reference)
P value	< 0.001	0.588	0.773
Q1	4.09 (3.50–4.79)	0.89 (0.72–1.09)	1.01 (0.81–1.26)
Q2	2.31 (2.00–2.66)	0.85 (0.71–1.01)	0.94 (0.78–1.13)
Q3	1.67 (1.43–1.94)	0.97 (0.81–1.16)	1.04 (0.86–1.25)
Q4	1 (reference)	1 (reference)	1 (reference)
P for trend	< 0.001	0.117	0.809
FIB-4 ≥ 2.67			
Q1	4.71 (3.38–6.56)	1.72 (1.19–2.50)	1.83 (1.25–2.69)
Q2–Q4	1 (reference)	1 (reference)	1 (reference)

P value	< 0.001	0.004	0.002
Q1	8.13 (4.74–13.95)	1.68 (0.89–3.17)	1.85 (0.92–3.74)
Q2	2.25 (1.22–4.15)	0.78 (0.41–1.47)	0.82 (0.41–1.66)
Q3	2.20 (1.18–4.13)	1.26 (0.65–2.46)	1.31 (0.65–2.65)
Q4	1 (reference)	1 (reference)	1 (reference)
P for trend	< 0.001	0.113	0.073

Odds ratios (95% confidence intervals) were calculated using multivariable logistic regression analysis; Model 1 = not adjusted; Model 2 = adjusted for age, sex, income, education, smoking status, and physical activity; Model 3 = Model 2 + alanine aminotransferase, obesity, hypertension, diabetes mellitus, dyslipidemia, and high-sensitivity C-reactive protein. OR: Odds ratio; 95%CI: 95% confidence interval; FIB-4: Fibrosis-4; Q: Quartile.

CONCLUSION

In this nationwide study of the Korean adult population, low muscle strength was associated with a dose-dependent higher risk of MAFLD in all participants and subgroups. Low muscle strength is associated with a high probability of liver fibrosis in patients with MAFLD. The identification and management of low muscle strength may play a crucial role in preventing MAFLD and liver fibrosis. Nonetheless, additional research is necessary to validate this association.

ARTICLE HIGHLIGHTS

Research background

More evidence is needed regarding the association between muscle strength and metabolic dysfunction-associated fatty liver disease (MAFLD) and only a few cross-sectional studies have shown that sarcopenia was associated with liver fibrosis in patients with MAFLD. In response to the increasing public health burden of MAFLD in Asia, we investigated the association between muscle strength and MAFLD using a Korean nationally representative database.

Research motivation

A recent introduction has been made regarding a new definition: MAFLD. Importantly, MAFLD is associated with increased all-cause mortality and advanced fibrosis in MAFLD had a higher all-cause mortality risk than that in non-alcoholic fatty liver disease. However, the link between muscle strength and MAFLD is not well studied.

Research objectives

We aimed to investigate the association between muscle strength and MAFLD in the general population in Korea. Additionally, we sought to study the risk of liver fibrosis in patients with MAFLD according to muscle strength.

Research methods

This study used data from the Korea National Health and Nutrition Examination Survey. Muscle strength was assessed using relative handgrip strength and the participants were categorized into muscle strength quartiles. We performed multivariable logistic regression analysis to evaluate the association between muscle strength and the risk of MAFLD and calculated the odds ratios and 95% confidence intervals.

Research results

Twenty-nine point three per cent of the participants had MAFLD. The lowest quartile was significantly associated with higher prevalence of MAFLD for all participants, sexes, and age groups. In patients with MAFLD, the odds of severe liver fibrosis were higher in Q1 than in other groups (Q2–Q4). However, causality should be investigated in future studies.

Research conclusions

The nationwide study of the Korean adult population revealed that low muscle strength was associated with a dose-dependent higher risk of MAFLD in all participants and subgroups. Additionally, low muscle strength is associated with a high probability of liver fibrosis in patients with MAFLD. The identification and management of low muscle strength may play a crucial role in preventing MAFLD and liver fibrosis.

Research perspectives

Prospective cohort or randomized controlled trials are needed to confirm the relationship between muscle strength and MAFLD. Future studies should focus on whether physical activity can prevent or reverse MAFLD and liver fibrosis in patients with MAFLD.

FOOTNOTES

Author contributions: Nam GE designed the study; Nam GE and Huh Y were responsible for developing the methodology; Nam GE, Huh Y, Lee GB, and Lee SH participated in the formal analysis and investigation; Lee GB wrote the original draft; Lee GB, Han B, Kim YH, Kim DH, Kim SM, Choi YS, Cho KH, and Nam GE participated in the review and editing; Nam GE acquired funding.

Institutional review board statement: The institutional review board of the Korea University Guro Hospital in Seoul, Republic of Korea approved the study protocol (No. 2022GR0322).

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Country/Territory of origin: South Korea

ORCID number: Gyu Bae Lee 0000-0002-1650-1278; Youn Huh 0000-0001-8899-9637; Sang Hyun Lee 0000-0001-5385-2061; Byoungduck Han 0000-0003-2830-1174; Yang-Hyun Kim 0000-0003-3548-8758; Do-Hoon Kim 0000-0001-7421-4501; Seon Mee Kim 0000-0002-1957-2928; Youn Seon Choi 0000-0003-2406-5848; Kyung Hwan Cho 0000-0003-2521-3064; Ga Eun Nam 0000-0002-6739-9904.

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Thioridazine reverses trastuzumab resistance in gastric cancer by inhibiting S-phase kinase associated protein 2-mediated aerobic glycolysis

Zheng-Yan Yang, Yi-Wei Zhao, Jing-Rui Xue, Ran Guo, Zhi Zhao, Han-Di Liu, Zhi-Guang Ren, Ming Shi

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Zheng-Yan Yang, Ran Guo, Han-Di Liu, Zhi-Guang Ren, Department of Pathology, School of Basic Medical Sciences, Henan University, Kaifeng 475004, Henan Province, China

Yi-Wei Zhao, Jing-Rui Xue, Joint National Laboratory for Antibody Drug Engineering, Henan University, Kaifeng 475004, Henan Province, China

Zhi Zhao, Department of Pathology, Henan University-affiliated Zhengzhou Yihe Hospital, Zhengzhou 450000, Henan Province, China

Zhi-Guang Ren, Key Laboratory of Clinical Resources Translation, The First Affiliated Hospital, Henan University, Kaifeng 475004, Henan Province, China

Ming Shi, Center of Clinical Oncology, The Affiliated Hospital of Xuzhou Medical University, Xuzhou 221002, Jiangsu Province, China

Ming Shi, Jiangsu Center for the Collaboration and Innovation of Cancer Biotherapy, Xuzhou Medical University, Xuzhou 221004, Jiangsu Province, China

Corresponding author: Zhi-Guang Ren, Doctor, Associate Professor, Department of Pathology, School of Basic Medical Science, Henan University, No. 1 Jinming Street, Kaifeng 475004, Henan Province, China. renzhiguang66@outlook.com

Abstract

BACKGROUND

Trastuzumab constitutes the fundamental component of initial therapy for patients with advanced human epidermal growth factor receptor 2 (HER-2)-positive gastric cancer (GC). However, the efficacy of this treatment is hindered by substantial challenges associated with both primary and acquired drug resistance. While S-phase kinase associated protein 2 (Skp2) overexpression has been implicated in the malignant progression of GC, its role in regulating trastuzumab resistance in this context remains uncertain. Despite the numerous studies investigating Skp2 inhibitors among small molecule compounds and natural products, there has been a lack of successful commercialization of drugs specifically targeting Skp2.

AIM

To discover a Skp2 blocker among currently available medications and develop a therapeutic strategy for HER2-positive GC patients who have experienced progression following trastuzumab-based treatment.

METHODS

Skp2 exogenous overexpression plasmids and small interfering RNA vectors were utilized to investigate the correlation between Skp2 expression and trastuzumab resistance in GC cells. Q-PCR, western blot, and immunohistochemical analyses were conducted to evaluate the regulatory effect of thioridazine on Skp2 expression. A cell counting kit-8 assay, flow cytometry, a amplex red glucose/glucose oxidase assay kit, and a lactate assay kit were utilized to measure the proliferation, apoptosis, and glycolytic activity of GC cells in vitro. A xenograft model established with human GC in nude mice was used to assess thioridazine's effectiveness *in vivo*.

RESULTS

The expression of Skp2 exhibited a negative correlation with the sensitivity of HER2-positive GC cells to trastuzumab. Thioridazine demonstrated the ability to directly bind to Skp2, resulting in a reduction in Skp2 expression at both the transcriptional and translational levels. Moreover, thioridazine effectively inhibited cell proliferation, exhibited antiapoptotic properties, and decreased the glucose uptake rate and lactate production by suppressing Skp2/protein kinase B/mammalian target of rapamycin/glucose transporter type 1 signaling pathways. The combination of thioridazine with either trastuzumab or lapatinib exhibited a more pronounced anticancer effect in vivo, surpassing the efficacy of either monotherapy.

CONCLUSION

Thioridazine demonstrates promising outcomes in preclinical GC models and offers a novel therapeutic approach for addressing trastuzumab resistance, particularly when used in conjunction with lapatinib. This compound has potential benefits for patients with Skp2-proficient tumors.

Key Words: Gastric cancer; Trastuzumab resistance; Thioridazine; S-phase kinase associated protein 2; Glycolysis

Core Tip: S-phase kinase-interacting protein 2 (Skp2) has been shown to be a reliable prognostic indicator of unfavorable outcomes for gastric cancer (GC). However, no agents specifically targeting Skp2 have been successfully developed. In this study, we proved that thioridazine restores the sensitivity of GC cells to trastuzumab both *in vivo* and *in vitro* by inhibiting Skp2-mediated glycolysis. Furthermore, the combination of thioridazine and lapatinib exhibits enhanced inhibitory effects compared with either monotherapy on the growth and survival of trastuzumab-resistant GC cells. Overall, this study suggests the potential of a thioridazine-based therapy to overcome trastuzumab resistance in human epidermal growth factor receptor 2-positive GC by targeting Skp2.

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INTRODUCTION

Gastric cancer (GC) is the fifth most common cancer worldwide, with more than 1 million new cases diagnosed in 2020, and it is the fourth leading cause of cancer-related death[1]. Approximately 7.3%-20.2% of GCs are positive for human epidermal growth factor (EGF) receptor 2 (HER2) /neu, c-ERBB2[2]. Positive expression of HER2 was proven to be associated with many tumorigenic processes and poor prognosis in patients with GC[3,4]. Trastuzumab is an effective anti-HER2 therapeutic agent that showed a survival benefit in the ToGA trial[5]. Trastuzumab in combination with chemotherapy was previously the first-line treatment for HER2-positive metastatic GC. However, due to primary or acquired drug resistance, only 12.8% of patients with HER2-positive GC respond to trastuzumab[6,7]. To overcome trastuzumab resistance, many new agents and combination therapies, such as pertuzumab, margetuximab, lapatinib, tucatinib, trastuzumab emtansine, and pembrolizumab, have emerged. However, the application of most of these drugs in the treatment of trastuzumab-resistant HER-2-positive GC is still in the investigative stage. Thus, the development of new drugs or combination therapies to increase trastuzumab sensitivity is a critical need.

Cancer cells exhibit high levels of glucose uptake and glycolysis, which allow the production of high levels of ATP to facilitate cell proliferation and survival, a phenomenon called the "Warburg effect"[8]. It has been reported that the GATA6 binding protein 6 protein contributes to resistance to trastuzumab in GC by regulating metabolic reprogramming, including reprogramming toward glycolysis[9]. According to Liu *et al*[10], MACC1 activates the phosphatidylin-

ositol-3-kinase (PI3K)/protein kinase B (AKT) signaling pathway to promote the Warburg effect, and downregulation of MACC1 reverses trastuzumab resistance in GC cells. Wang *et al*[11] and colleagues found that combination treatment with metformin and trastuzumab in a circadian pattern resensitized GC cells to trastuzumab, partially by disrupting the BMAL1–CLOCK–PER1–HK2 axis, thus controlling fluctuations in glycolysis. Therefore, glycolysis inhibitors could be used appropriately to overcome trastuzumab resistance in GC. However, historically, glycolysis inhibitors have not been widely applied clinically due to their obvious side effects[12].

S-phase kinase associated protein 2 (Skp2) is a constituent of the F-box protein family and functions as a substrate recognition component within the Skp2-SCF complex, which plays a crucial role in the regulation of ubiquitination, cell cycle progression, cell proliferation, and apoptosis[13]. Extensive evidence has demonstrated that Skp2 acts as an oncogene[14], exhibiting elevated expression levels in breast cancer[15], GC[16], prostate cancer[17], and various other malignant tumors, thereby exhibiting a strong association with poor outcomes in affected individuals. Recent studies have shown that Skp2 regulates glycolysis, trastuzumab sensitivity, and tumorigenesis in breast cancer[18]. However, whether Skp2 regulates trastuzumab sensitivity in GC is unknown.

Several investigations have been conducted on small structure-based inhibitors of Skp2. For instance, the compounds SZL-P1-41[19], SKPin C1[20,21], and DT204[22] were identified as Skp2 inhibitors that could suppress tumor growth. However, treatment with these chemical inhibitors is accompanied by adverse effects. Several natural compounds, such as diosmetin[23], safranal[24], dioscin[25], gartanin[26], betulinic acid[27], linichlorin A[28], and gentian violet[29], have been identified to function as potential antitumor agents through Skp2 inhibition. However, these studies are still in the preliminary stages of preclinical development.

This study proposes that the antipsychotic drug thioridazine can specifically decrease the expression of Skp2, thereby increasing the responsiveness of HER-2-positive GC cells to trastuzumab through the attenuation of glycolysis.

MATERIALS AND METHODS

Cell lines and reagents

The human GC cell lines HGC-27, SGC-7901, MGC-803, MKN-45, and NCI-N87 were purchased from the American Type Culture Collection (Manassas, United States). HGC-27, NCI-N87, and MGC-803 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS); Biological Industries, Israel). SGC-7901 and MKN-45 cells were grown in RPMI 1640 medium containing 10% FBS. Trastuzumab-resistant HGC-27 and SGC-7901 cells were established by culturing cells with increasing concentrations of trastuzumab (Roche, Switzerland) over half a year and were designated HGC-27-R and SGC-7901-R cells, respectively. All cells were cultured in incubators at 37 ℃ with 5% CO_2. Thioridazine and lapatinib were obtained from Selleck Chemicals (United States).

RT-PCR analysis

Quantitative real-time reverse transcription polymerase chain reaction analysis using a Real-Time PCR Detection System (Agilent Technologies) was performed to validate the effect of thioridazine on SKP2 gene expression. The sequences of the primers used were as follows: *5′-ATGCCCCAATCTTGTCCATCT-3′* and *5′-CACCGACTGAGTGATAGGTGT-3′* for SKP2; *5′-GTGGGGCGCCCCAGGCACCA-3′* and *5′-CTTCCTTAATGTCACGCACGATTTC-3′* for β-actin.

Construction of the exogenous overexpression plasmids and SKP2 RNA interference vectors

The pcDNA3.1-3 × Flag-C plasmid carrying the Skp2 coding sequence was constructed. The sense primer sequence was *5'-CCGGAATTCCGGAGGATGCACAGGAAGCACCTCCAGGAG-3'*, and the antisense primer sequence was *5'-CCGCTC-GAGTAGACAACTGGGCTTTTGCAGTGT-3'*. The two recombinant plasmids confirmed to contain the correct sequence were named OX-SKP2-1 and OX-SKP2-2. Small interfering RNA (siRNA) for SKP2 and the negative control (NC) oligonucleotide sequence were synthesized by Sangon Biotech (Guangdong, China). The siRNA duplexes were transfected into HGC27-R cells using Lipofectamine 2000 (Invitrogen, United States).

CCK-8 assay

A cell counting kit-8 (CCK-8), Japan, was used to assess cell proliferation. In brief, cell suspensions (5 × 10^3 cells/well) were seeded in 96-well plates in triplicate, and the plates were incubated for 48 h. Each well was filled with 10 μL of CCK-8 assay solution and incubated for 4 h. A microplate reader was used to measure the optical density at 450 nm.

Western blot

Immunoblotting was performed using antibodies against the following proteins: Poly ADP-ribose polymerase (PARP); 9352, glucose transporter type 1 (Glut1); 73015, Skp2 (2652), p-signal transducer and activator of transcription 3 (9134p), p-AKT (4060), p-mammalian target of rapamycin (mTOR) (5336), and GAPDH (5174) (all obtained from Cell Signaling Technology, United States).

Molecular docking

The 3D structure of thioridazine was obtained from the PubChem Substance database (https://www.ncbi.nlm.nih.gov/) by minimizing structural energy using the ChemBioDraw 3D module. The crystal structure of Skp2 was retrieved from the RCSB Protein Data Bank (PDB ID: 1fs2) and subsequently modified (dehydration and hydrogenation) using AutoDockTools 1.5.6 21 before being exported in pdbqt format. Following definition of the grid on the active site of the

receptor protein, the docking procedure was executed using AutoDock Vina 1.1.2, and the output score was displayed in kcal/mol. PyMOL 2.3.0 and BIOVIA Discovery Studio were utilized in this process.

Apoptosis assays

For apoptosis assays, the following steps were performed according to the instructions of the annexin V-FITC Apoptosis Detection Kit (Solarbio, China). Cells in each sample were washed, suspended in 100 μL of 1 × binding buffer and stained with 5 μL of FITC-labeled annexin V and 5 μL of PI for 5 min. Apoptotic cells were detected by a flow cytometer (BD, United States) at wavelengths of 488 nm and 630 nm.

Cellular thermal shift assay

HGC-27-R cells were exposed to dimethyl sulfoxide or thioridazine for 24 h, collected, washed with PBS containing protease inhibitors, aliquoted into PCR tubes, and heated in a thermal cycler (Bio-Rad, T100) at the indicated temperature for 3 min to denature proteins. The cells were then resuspended in NP40 buffer, subjected to three freeze-thaw cycles with liquid nitrogen, and centrifuged at 20000 × g for 20 min at 4 ℃. The supernatant was boiled in loading buffer for western blotting.

Glycolysis assay

Cells were seeded in 12-well plates at 5 × 10^5 cells/well. After the cells were treated with different reagents for 48 h, the supernatant was collected. An amplex red glucose/glucose oxidase assay kit (Molecular Probes, Carlsbad, CA, United States) was used for glucose uptake measurements. A lactate assay kit (BioVision, Mountain View, CA, United States) was used to detect the production of lactate in the medium.

Animal studies

The animal procedures were approved by the Henan University Institutional Experimental Animal Care and Use Committee (ID: HUSOM2022-439). All experiments were designed and conducted in accordance with the Animal Research: Reporting of In Vivo Experiments guidelines; the United Kingdom Animals (Scientific Procedures) Act 1986 and associated guidelines; and the European Union (EU) Directive 2010/63/EU for animal experiments. Five-week-old male BALB/c athymic nude mice (weighing 16-18 g, SPF grade) were purchased from Peking Vital River Laboratory Animal Technology Company. Prior to use, all cages, bedding, and drinking water were sterilized. The cages, feed, and drinking water were replaced biweekly. The breeding environment adhered to the following specifications: temperature range from 20 to 26 ℃, humidity range from 40% to 70%, and light cycle consisting of 12 h of illumination followed by 12 h of darkness (lights activated from 8 am to 8 pm). A total of 3 × 10^6 HGC27-R or 1 × 10^6 SGC7901-R cells were injected subcutaneously (s.c.) into each mouse. The mice were randomly grouped into four groups with five mice in each group. The mice received either vehicle control, thioridazine (25 mg/kg), trastuzumab (5 mg/kg), lapatinib (70 mg/kg), and thioridazine (25 mg/kg) plus trastuzumab (5 mg/kg) or lapatinib (70 mg/kg) by intraperitoneal injection daily. After two weeks of drug administration, the mice were sacrificed, and tumor weights were determined.

Statistical analysis

Data were expressed as mean ± SD. Comparisons between two groups were performed using a *t* test. One-way ANOVA with the Bonferroni correction was used to analyze differences among three or more groups. Statistical significance was defined as a value of $P < 0.05$.

RESULTS

Upregulation of Skp2 expression promotes trastuzumab resistance in HER2-positive GC cells

To ascertain the correlation between Skp2 expression and trastuzumab sensitivity in GC, Skp2 expression was analyzed by immunoblotting in a panel of human GC cell lines with HER2-positive status. Subsequently, the HGC27 and SGC7901 cell lines, exhibiting Skp2 overexpression, were selected for further examination (Figure 1A). Following chronic treatment with 10 μg/mL trastuzumab, two cell lines, HGC27-R and SGC7901-R, were identified as being more resistant to trastuzumab than their parental counterparts. Notably, the Skp2 level was higher in HGC27-R and SGC7901-R cells than in the corresponding parental HGC27 and SGC7901 cells (Figure 1B and C). HGC27 cells with exogenous Skp2 overexpression were employed to investigate the potential decrease in antiproliferative activity associated with upregulated Skp2 expression. The results indicated a decline in trastuzumab activity in isogenic stable Skp2 transfectants (HGC27-OX-SKP2-2 cells) (Figure 1D and E). Additionally, transfection of the SKP2-targeted siRNA effectively downregulated Skp2 expression and significantly enhanced trastuzumab activity in HGC27-R cells (Figure 1F and G). These findings suggest a significant relationship between Skp2 expression and trastuzumab insensitivity, highlighting the importance of Skp2 as a potential therapeutic target in GC.

Thioridazine decreases Skp2 expression in GC

A docking analysis conducted with the drug repurposing compound library revealed that thioridazine exhibits favorable binding potential with Skp2. Thioridazine exhibits a high binding potential (affinity score: -7.0 kcal/mol) for the active pocket of Skp2, as shown in Figure 2A. Specifically, thioridazine engages in van der Waals, pi-alkyl and alkyl interactions with the branched-chain amino acids Leu130, Lys131, Pro132 and Ile205 in Skp2, resulting in a robust interaction between

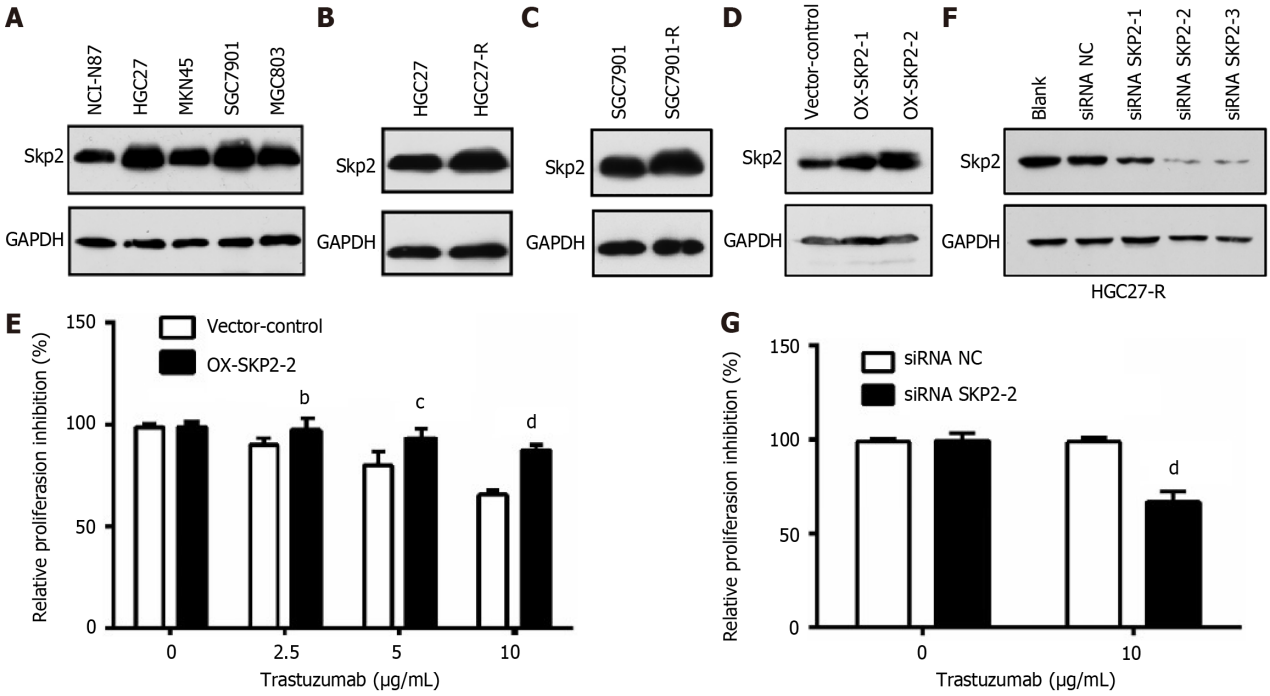

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Figure 1 Elevated S-phase kinase associated protein 2 expression contributes to trastuzumab resistance in human epidermal growth factor receptor 2-positive gastric cancer cells. A: Western blotting was used to measure the S-phase kinase associated protein 2 (Skp2) expression level in human gastric cancer (GC) cell lines; B and C: Skp2 expression in HGC27 and HGC27-R cells and SGC7901 and SGC7901-R cells was analyzed by Western blotting; D: Skp2 expression levels in HGC27 cells transfected with empty vector (pcDNA3.1), OX-SKP2-1 or OX-SKP2-2 (pcDNA3.1/SKP2) were determined by western blotting; E: HGC27 cells stably transfected with empty vector or OX-SKP2-2 were cultured with or without trastuzumab (0, 2.5, 5, and 10 μg/mL) for 48 h. Proliferation activity was analyzed by cell counting kit-8 (CCK-8) assays; F: HGC27-R cells were transfected with or without small interfering RNA-negative control (siRNA-NC) or siRNA-SKP2 for 72 h. Skp2 expression was measured by western blotting; G: HGC27 cells transfected with siRNA-NC or siRNA-SKP2-2 were cultured with or without 10 μg/mL trastuzumab. CCK-8 assays were used to analyze proliferation activity. The experiments were performed in triplicate. $^{b}P < 0.01$; $^{c}P < 0.001$; $^{d}P < 0.0001$. Skp2: S-phase kinase associated protein 2; siRNA: Small interfering RNA; NC: Negative control.

the ligand and the Skp2 protein (Figure 2B and C). Cellular thermal shift assay was performed to evaluate the binding affinity of thioridazine for the Skp2 protein. Administration of thioridazine increased the thermal stability of Skp2 but not of GAPDH (Figure 2D), implying a direct binding interaction between thioridazine and the Skp2 protein.

To explore the effect of thioridazine on Skp2 expression, HGC27-R and SGC7901-R cells were treated with varying concentrations of thioridazine (0, 1.25, 2.5, and 5 μM). Western blot analysis revealed a notable decrease in the expression level of Skp2 protein after treatment with thioridazine (Figure 2E and F). MG132 reversed the decrease in Skp2 protein expression induced by thioridazine, thereby indicating that thioridazine may increase the degradation of Skp2 *via* the ubiquitin–proteasome pathway (Figure 2G). Additionally, the mRNA expression level of SKP2 exhibited a dose-dependent decline as the concentration of thioridazine increased, as shown in Figure 2H and I. It has been established that Skp2 plays a role in governing the phosphorylation and activation of Akt in response to ErbB receptor signaling[18], and we found that the phosphorylation of Akt, which is stimulated by EGF and facilitated by Skp2, was abolished after thioridazine treatment (Figure 2J). In a similar vein, our observations also revealed that Skp2 expression and Akt phosphorylation induced by heregulin was effectively inhibited through the administration of thioridazine (Figure 2K). These results suggest that thioridazine can impede the Skp2-mediated Akt phosphorylation and activation induced by ligands of the ErbB2 family.

Thioridazine increases GC cell sensitivity to trastuzumab in vitro and in vivo

Furthermore, we verified that cotreatment with thioridazine and trastuzumab resulted in a further decrease in Skp2 expression (Figure 3A and B). We next assessed the effect of thioridazine on the proliferation and survival of HGC27-R and SGC7901-R cells. As shown in Figure 3C and D, thioridazine significantly decreased the viability and restored the trastuzumab sensitivity of HGC27-R and SGC7901-R cells. To determine whether thioridazine promotes apoptosis in trastuzumab-resistant cells, we treated HGC27-R and SGC7901-R cells with thioridazine and trastuzumab alone and in combination. PARP is a substrate of caspases. PARP splicing is a key indicator of apoptosis. As shown in Figure 3E and F, trastuzumab induced increased expression of Skp2 but not splicing of PARP. In contrast, combined treatment with thioridazine and trastuzumab increased PARP splicing. The rate of early apoptotic HGC27-R cells treated with thioridazine was 2-fold that of HGC27-R cells treated with trastuzumab, and the difference even increased to 4-fold thioridazine was combined with trastuzumab (Figure 3G and H). These data suggest that thioridazine and trastuzumab synergistically suppress the proliferation and decrease the survival of GC cells.

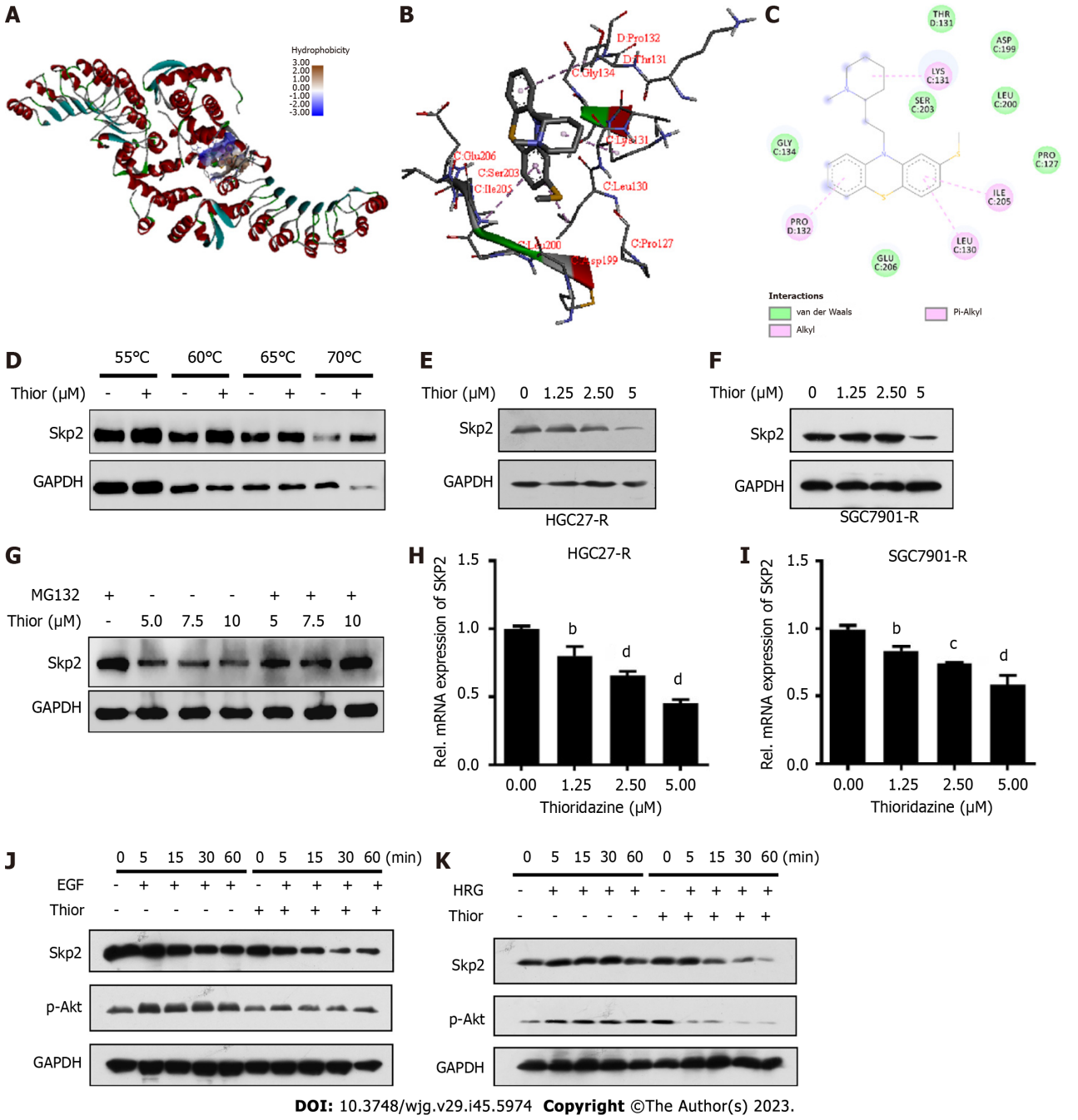

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Figure 2 Thioridazine can inhibit S-phase kinase associated protein 2 expression and protein kinase B activation triggered by ErbB signaling. A: The interaction between the ligand thioridazine and the active pocket of S-phase kinase associated protein 2 (Skp2) (1FS2) was examined; B: 3D structural schematic showing the binding of thioridazine to Skp2; C: Dihedral angle diagram showing the mode of interaction between thioridazine and Skp2; D: HGC-27-R cells were exposed for 24 h to dimethyl sulfoxide or 30 μM thioridazine and subjected to the Cellular Thermal Shift Assay; E and F: HGC27-R and SGC7901-R cells were treated with different concentrations of thioridazine for 24 h. Western blotting was used to analyze Skp2 expression; G: HGC27 cells were treated with the indicated concentrations for 42 h and were then treated with 5 μM MG132 for 6 h. The protein expression level of Skp2 was analyzed by western blotting; H and I: HGC27-R and SGC7901-R cells were treated with various concentrations of thioridazine for 24 h. The mRNA expression level of SKP2 was analyzed by Q-PCR; J and K: HGC27-R cells were pretreated with or without thioridazine (5 μM) for 2 h, followed by stimulation with epidermal growth factor (100 μg/mL) or heregulin (50 μg/mL). At the indicated times, Skp2 and p-protein kinase B protein levels were assessed. $^{b}P < 0.01$; $^{c}P < 0.001$; $^{d}P < 0.0001$. Thior: Thioridazine; Skp2: S-phase kinase associated protein 2; EGF: Epidermal growth factor; HRG: Heregulin.

To investigate the efficacy of trastuzumab and thioridazine in vivo, we treated mice bearing HGC27-R xenografts with trastuzumab and thioridazine alone or in combination. As monotherapies, trastuzumab and thioridazine showed a limited effect on tumor growth, whereas combined administration of trastuzumab and thioridazine resulted in greater reductions in tumor volume and tumor weight (Figure 3I and J). However, lower expression of Skp2 was found in xenograft tissues of the combined administration group (Figure 3K). These data demonstrate that thioridazine enhances the antitumor activity of trastuzumab *in vitro* as well as *in vivo*.

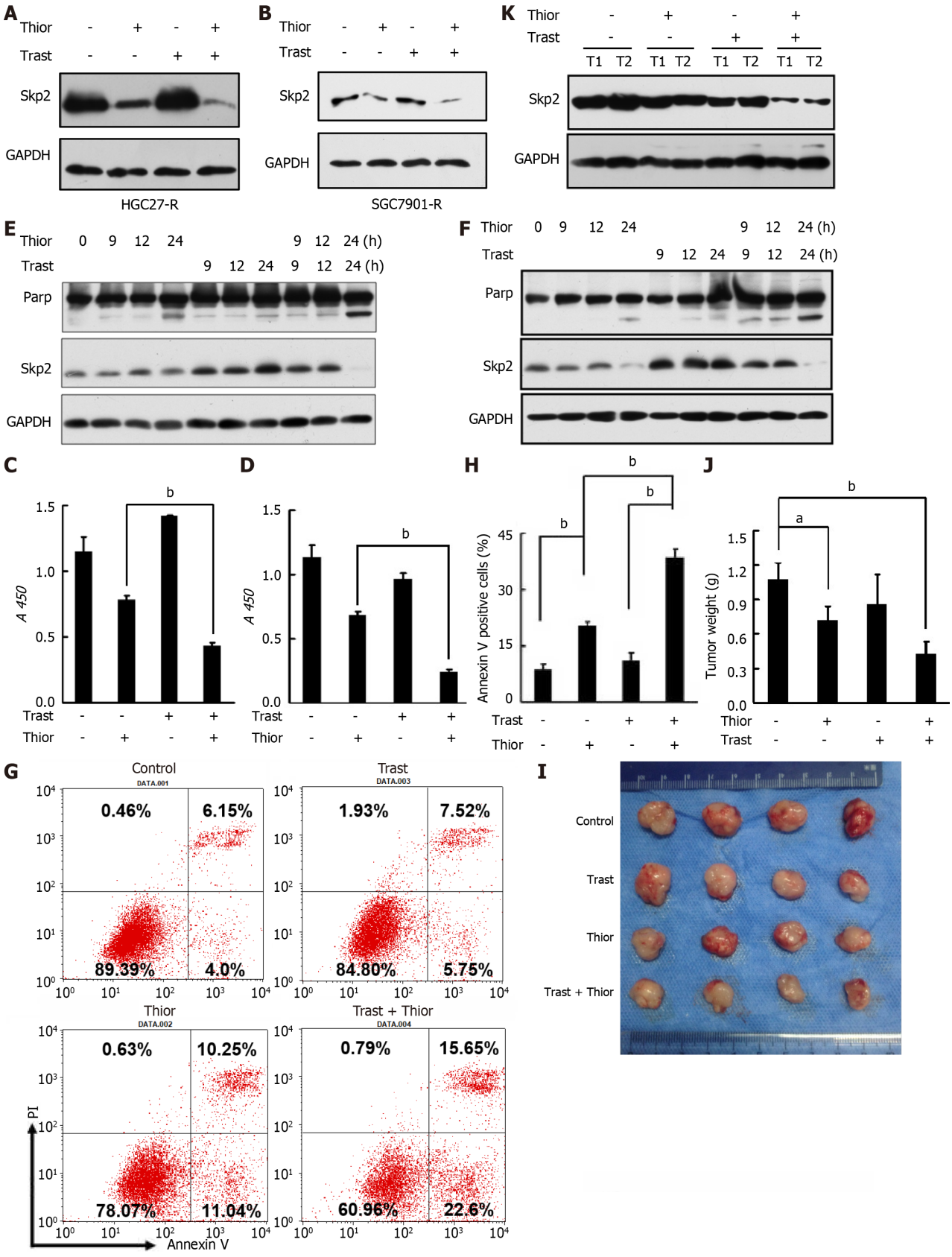

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Figure 3 Thioridazine increases the primary susceptibility of gastric cancer cells to trastuzumab *in vitro* and *in vivo*. A and B: HGC27-R and SGC7901-R cells were treated with phosphate-buffered saline (PBS), thioridazine (5 μM), trastuzumab (5 μg/mL), or thioridazine (5 μM) plus trastuzumab (5 μg/mL) for 24 h. S-phase kinase associated protein 2 (Skp2) expression was determined by western blotting; C and D: HGC27 and SGC7901 cells were treated with trastuzumab (10 μg/mL) or thioridazine (2.5 μM) alone or in combination for 48 h. Proliferation activity was evaluated by cell counting kit-8 assays; E and F: HGC27 and SGC7901 cells were treated with trastuzumab (10 μg/mL) or thioridazine (2.5 μM) alone or in combination for various times. The expression of Skp2 and Parp

was analyzed by western blotting; G and H: HGC27 cells were treated with trastuzumab (10 μg/mL) or thioridazine (2.5 μM) alone or in combination for 24 h, followed by an apoptosis assay, quantitative results; I-K: Nude mice were injected s.c. with 0.1 mL of an HGC27 cell suspension (3×10^7 cells/mL) in the right upper flank. The mice were treated with PBS, trastuzumab (0.5 mg/kg), thioridazine (25 mg/kg), or trastuzumab (0.5 mg/kg) plus thioridazine (25 mg/kg) daily by intraperitoneal (i.p.) injection beginning 7 d after cell implantation. After two weeks of drug administration, the mice were sacrificed. Primary tumors were excised, photographed, and weighed. Western blot analysis was used to assess Skp2 expression in primary tumors. $^aP < 0.05$; $^bP < 0.01$. Thior: Thioridazine; Trast: Trastuzumab.

Thioridazine combined with lapatinib more effectively suppressed glycolysis mediated by Skp2

Trastuzumab-resistant advanced breast cancer responds to lapatinib, a dual tyrosine kinase inhibitor (TKI), according to the results of the phase III trial EGF104900 and the HER2CLIMB Randomized Clinical Trial[30,31]. However, the addition of lapatinib to a regimen of capecitabine and oxaliplatin did not improve overall survival (OS) in patients with HER2-amplified gastroesophageal adenocarcinoma[32]. In addition, lapatinib plus paclitaxel demonstrated activity in the second-line treatment of patients with HER2 FISH-positive IHC3+ advanced GC but did not significantly improve OS in the intent-to-treat population[33]. Here, we explored the effect of combination treatment with lapatinib and thioridazine on the glycolytic phenotype in GC cells. The combination of thioridazine and lapatinib completely abolished Skp2 expression (Figure 4A-C). It has been reported that Skp2 regulates glycolysis by inducing Glut1 expression and Akt/mTOR pathway activation[34,35]. The decreased protein levels of p-Akt, p-mTOR, and Glut1 were consistent with the downregulation of Skp2 (Figure 4A-C). Furthermore, both glucose uptake and lactate production were decreased more apparently in HGC27-R and SGC7901-R cells treated with thioridazine and lapatinib together than in those treated with either alone (Figure 4D-G). Compared to siRNA-NC-transfected cells, HGC27 cells transfected with SKP2-2 siRNA did not show inhibitory effects of thioridazine on glucose uptake or lactate production (Figure 4H and I). These results indicate that in combination, thioridazine and lapatinib can markedly suppress glycolysis by downregulating Skp2 in trastuzumab-resistant GC cells.

Thioridazine improves the inhibitory effect of lapatinib on GC cells

We further examined the anticancer effect of thioridazine combined with lapatinib. As shown in Figure 5A-C, thioridazine significantly enhanced the anti-proliferative activity of lapatinib in GC cells (HGC27-R, SGC-7901, and NCI-N87-R). Thioridazine (5 μM) in combination with lapatinib (1 or 2 μM) markedly increased apoptosis in HGC27-R and SGC7901-R cells (Figure 5D-F).

Moreover, Figure 6A-C shows that combined administration of thioridazine and lapatinib strongly decreased the growth, volume, and weight of tumors in mice bearing SGC7901-R xenografts. No toxic or side effects were observed during the administration period, and the weight of the mice did not significantly decrease (Figure 6D). Lower expression of Skp2 was found in the combined thioridazine and lapatinib treatment group than in the other groups (Figure 6E). The protein levels of Skp2, p-Akt, and Glut1 were greatly decreased in xenograft tissues from mice treated with both thioridazine and lapatinib (Figure 6F). These data demonstrate that thioridazine enhances the antitumor activity of lapatinib by inhibiting Skp2 expression *in vitro* and *in vivo*.

Our study demonstrates that thioridazine can overcome trastuzumab resistance by blocking glycolysis, growth, and apoptosis resistance by downregulating the expression of Skp2 in GC cells.

DISCUSSION

It has been confirmed that HER-2 has the most outstanding clinical significance in advanced GC. However, targeting HER-2 in advanced GC remains challenging due to the high heterogeneity and subsequent resistance caused by prolonged therapy. Despite the development of numerous HER2-targeted drugs, including antibody-drug conjugates, TKIs, bispecific antibodies, vaccines, and immune checkpoint inhibitors, to combat trastuzumab resistance in HER2-positive breast cancer, the efficacy of these treatments in HER2-positive GC remains uncertain.

Trastuzumab combined with palbociclib, a CDK4/6 inhibitor, was demonstrated to yield favorable survival outcomes in patients with advanced breast cancer[36,37]. Multiple studies have demonstrated that the simultaneous administration of supplementary inhibitors, including figitumumab (an insulin-like growth factor 1 receptor inhibitor), ipatasertib (an AKT inhibitor), and MK2206 (another AKT inhibitor), in individuals with HER2-overexpressing tumors increases the efficacy of trastuzumab[38-40]. However, additional extensive research is needed for clinical incorporation of these agents. In contrast to the aforementioned drugs currently under development, the expedited introduction of approved drugs for new therapeutic applications could be facilitated in the clinical market. For example, the potential of combining trastuzumab with metformin as an innovative adjuvant therapy for HER-2-positive breast cancer is being investigated in an ongoing phase II clinical trial[41]. Our findings in the current study highlight the ability of thioridazine to augment the effect of trastuzumab in GC.

The antipsychotic drug thioridazine was first discovered as a phenothiazine-type piperidine drug. In 2013, Sachlos *et al*[42] first discovered that thioridazine can induce the differentiation of acute myeloid leukemia cells and breast cancer stem cells and increase sensitivity to doxorubicin without affecting the function of normal hematopoietic stem cells. It has been shown that thioridazine can inhibit the expression of a multidrug resistance protein (P-gp) and increase sensitivity to chemotherapy drugs in glioblastoma[43]. In addition to inducing reactive oxygen species accumulation and DNA damage, thioridazine can increase autophagy and apoptosis in ovarian cancer cells[44]. It reduces ovarian cancer angiogenesis by inhibiting vascular endothelial growth factor receptor-2, PI3K, and mTOR signaling[45]. Recent studies

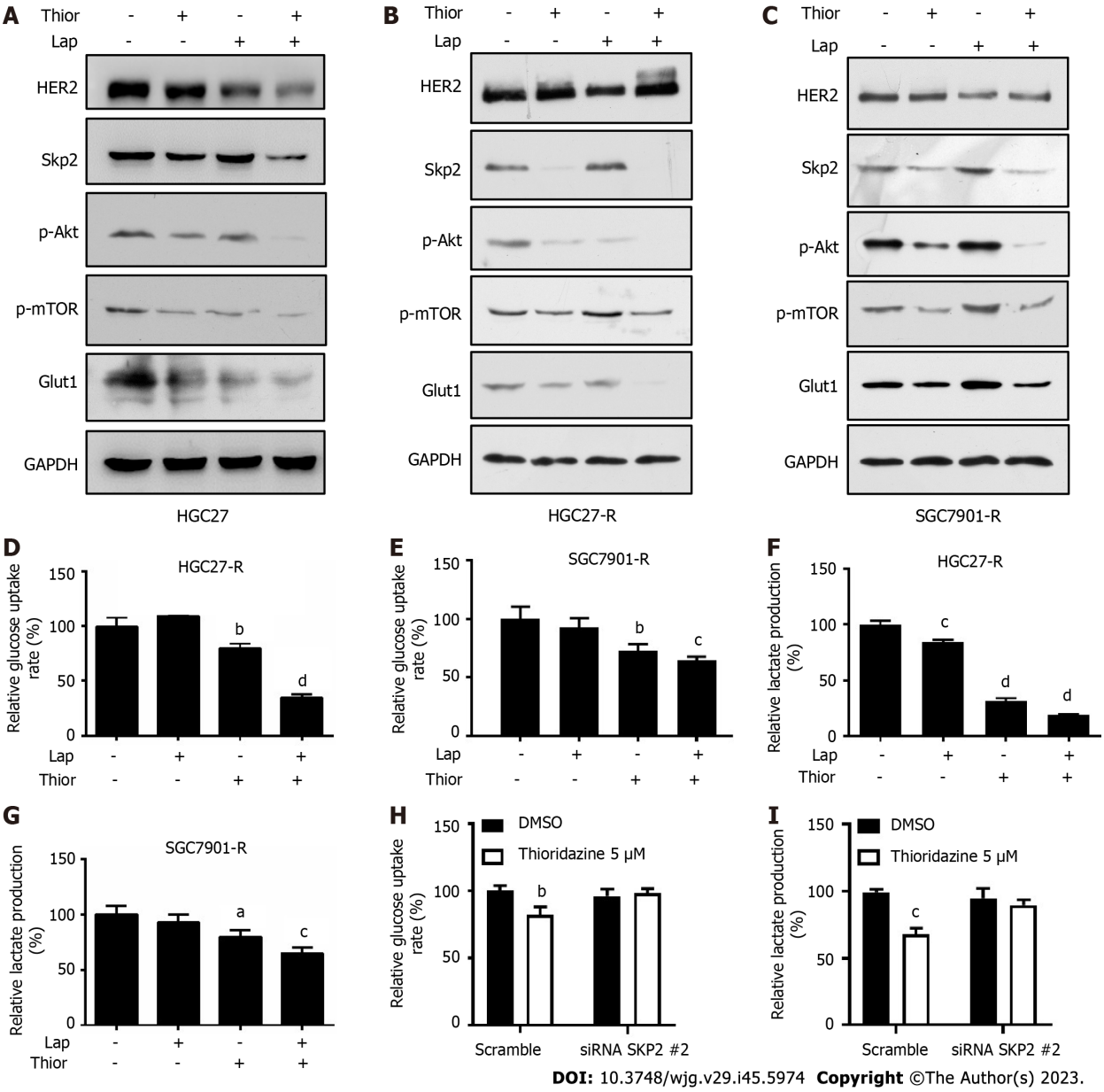

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Figure 4 Thioridazine combined with lapatinib decreases S-phase kinase associated protein 2/p-protein kinase B/p-mammalian target of rapamycin/glucose transporter type 1 protein levels and glycolysis in gastric cancer cells. A-C: HGC27, HGC27-R and SGC7901-R cells were treated with thioridazine (5 μM) alone or in combination with lapatinib (5 μM) for 24 h. S-phase kinase associated protein 2 (Skp2), p-protein kinase B (Akt), Akt, p-mammalian target of rapamycin, and glucose transporter type 1 protein levels were analyzed by western blotting; D-G: HGC27-R cells were treated with thioridazine (5 μM) and lapatinib (5 μM) alone or in combination for 24 h. The glucose uptake rate and lactate production rate were measured following the manufacturer's instructions; H and I: HGC27-R cells were divided into two groups that were transfected with small interfering RNA (siRNA) negative control or siRNA SKP2 2 for 48 h. Each group was treated without or with thioridazine (5 μM) for another 24 h. The glucose uptake rate and lactate production rate in all groups were measured. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; $^{d}P < 0.0001$. Thior: Thioridazine; Lap: Lapatinib.

have demonstrated that thiolidazine exhibits the potential to augment the susceptibility of glioblastoma cells towards temozolomide through the inhibition of autophagy[46]. Furthermore, the concurrent administration of thiazidine and oxaliplatin has been found to stimulate immunogenic cell death in colon cancer by inducing endoplasmic reticulum stress [47]. In this study, we first revealed that thioridazine can inhibit glycolysis in GC by downregulating Skp2 expression at both the transcriptional and translational levels. However, further evidence is needed to determine whether the main regulatory mechanism by which thioridazine regulates the expression and function of Skp2 is mediated through transcriptional inhibition, posttranslational inhibition, or blockade by protein interactions. The effectiveness of combining thioridazine with other HER-2- or non-HER-2-targeted drugs, such as neratinib, tucatinib, and apatinib, in treating trastuzumab-resistant GC remains uncertain.

Based on our findings, Skp2 may serve as a predictor of the trastuzumab response in patients with HER-2-positive GC, thereby aiding in identifying the patient subgroups most likely to benefit from HER-2-targeted therapies. Additionally, our study underscores the advantageous impacts of thioridazine when used in combination with synergistic drugs for the management of GC. These findings offer valuable support for future clinical investigations aimed at exploring the

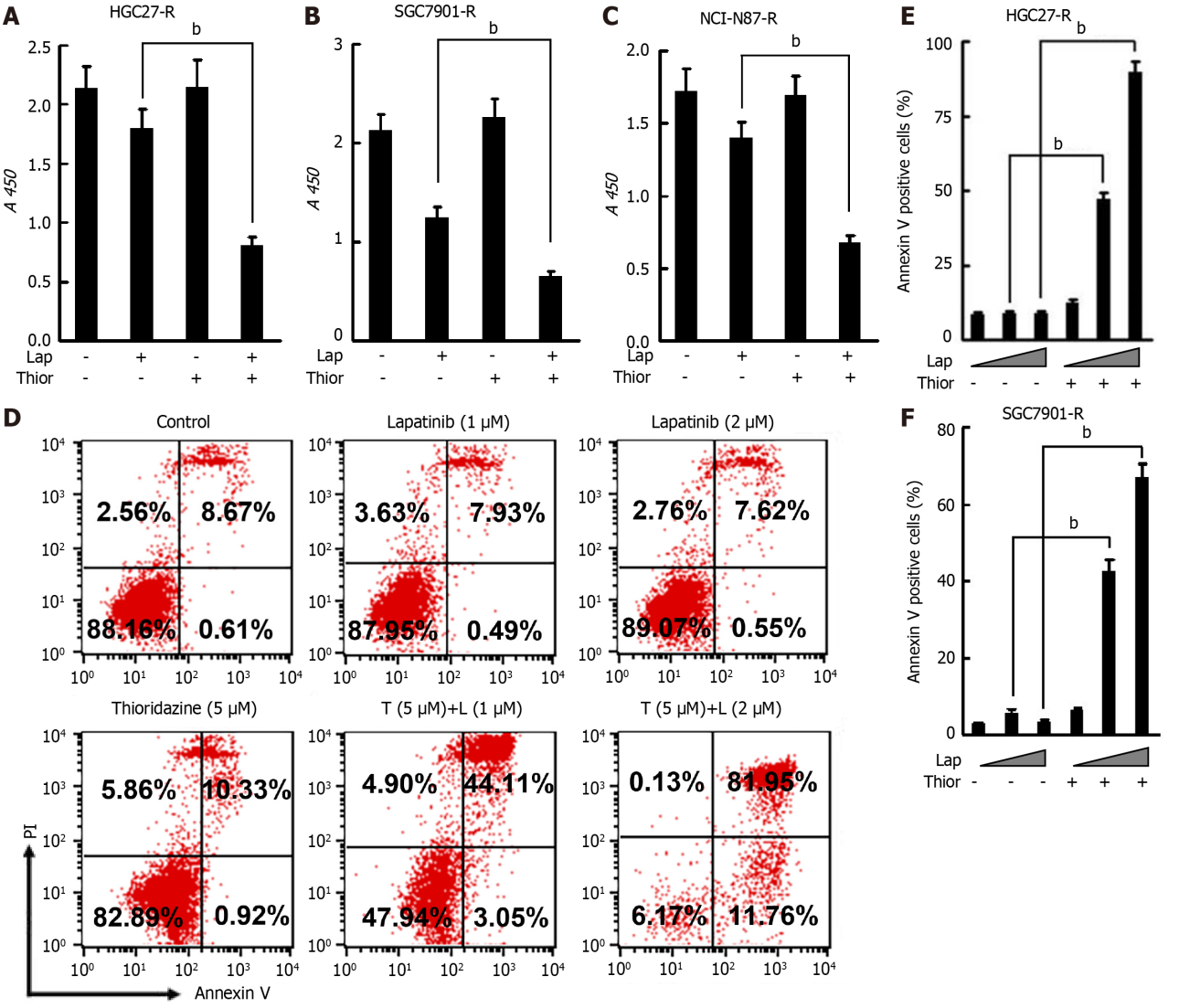

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Figure 5 The combination of thioridazine and lapatinib exhibits more pronounced antitumor activity than either alone in vitro against trastuzumab-resistant gastric cancercells. A-C: To HGC27-R, SGC7901-R, and NCI-N87-R cells were treated with trastuzumab (10 μg/mL) or thioridazine (5 μM) alone or in combination for 48 h. Proliferation activity was evaluated by cell counting kit-8 assays; D-F: HGC27-R cells were treated with lapatinib (1 μM or 2 μM) in the presence or absence of thioridazine (5 μM) for 24 h, followed by an apoptosis assay. Similar experiments were performed and results were quantified in SGC7901-R cells. The experiments were repeated three times. $^{b}P < 0.01$. Thior: Thioridazine; Lap: Lapatinib.

potential of thioridazine as a viable treatment option for GC. Notably, HER-2-targeted drugs possess dosage-independent potential for cardiac toxicity. In addition, thioridazine has been reported to be associated with arrhythmias in a minority of schizophrenia patients. Consequently, when considering the administration of these drugs individually or in combination, it becomes imperative to exclude individuals with preexisting heart conditions and implement vigilant cardiac surveillance.

CONCLUSION

In conclusion, our data demonstrate that thioridazine combined with lapatinib exhibits synergistic effects in impeding cell proliferation, inducing apoptosis, and suppressing tumor growth in GC. This study provides an experimental foundation for the potential utilization of thioridazine in overcoming trastuzumab resistance in GC. The objective of this study is to offer potential drug selection and administration strategies for patients with advanced GC who exhibit resistance to trastuzumab. Furthermore, these findings offer novel insights for the future investigation of Skp2 inhibitors.

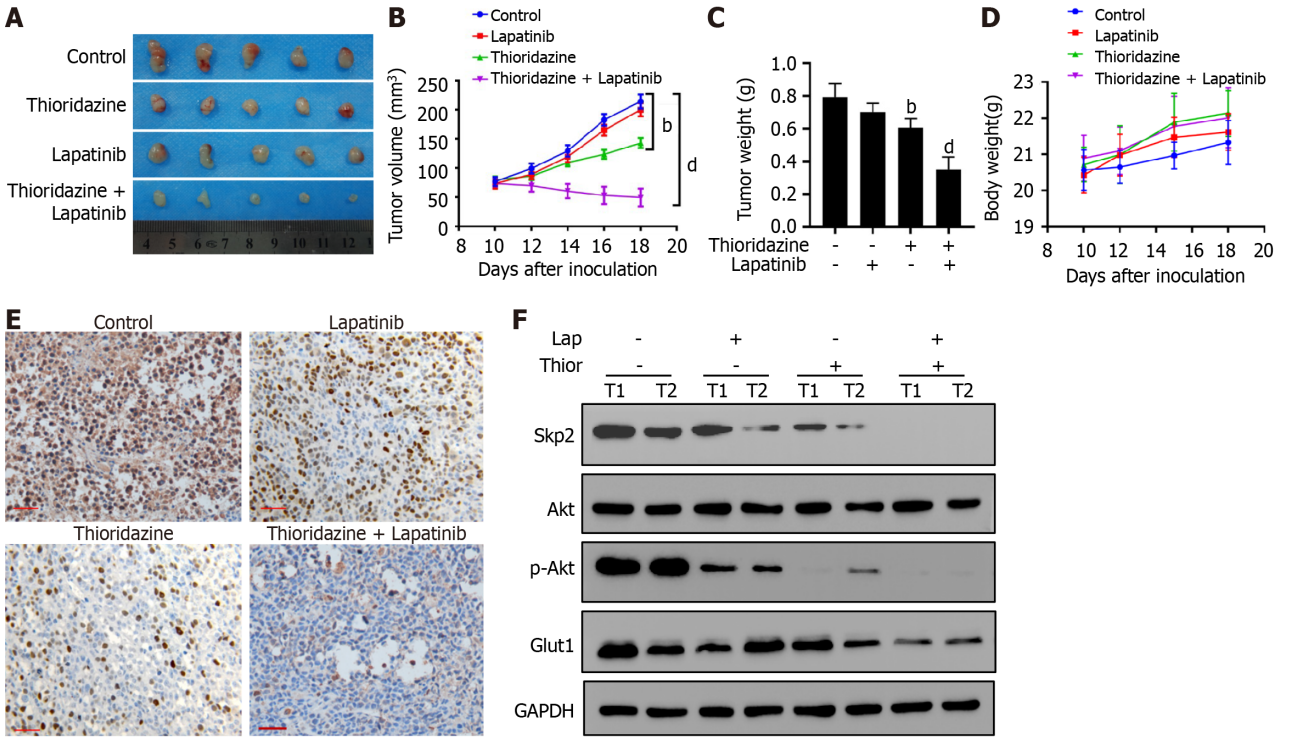

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Figure 6 Coadministration of thioridazine and lapatinib demonstrates potent anticancer activity in mice harboring gastric cancer xenografts. A: SGC7901-R cells (1 × 10^6) were injected s.c. into the flanks of nude mice. Ten days post-implantation, the mice were randomized into four groups of five mice each. Then, the mice were treated by i.p. injection with phosphate-buffered saline, lapatinib (70 mg/kg), thioridazine (25 mg/kg), or lapatinib (70 mg/kg) plus thioridazine (25 mg/kg) for 14 d. Gross morphology of the final excised xenograft tumor masses; B: Tumor volumes following treatments were recorded; C: Wet tumor weights; D: Body weights of mice; E: S-phase kinase associated protein 2 (Skp2) expression was evaluated by immunohistochemistry in primary tumors (scale bar = 100 μm); F: The protein levels of Skp2, protein kinase B (Akt), p-Akt, glucose transporter type 1, and GAPDH in two primary tumors from each group were measured by western blotting. $^bP < 0.01$, $^dP < 0.0001$. Thior: Thioridazine; Lap: Lapatinib.

ARTICLE HIGHLIGHTS

Research background

The drug resistance observed in patients with human epidermal growth factor receptor 2 (HER-2)-positive advanced gastric cancer (GC) treated with trastuzumab is a significant concern, as no established targeted therapy regimen for use after the development of drug resistance is available. S-phase kinase associated protein 2 (Skp2) has been identified as a crucial target for GC treatment; however, the development of new drugs targeting Skp2 remains a considerable challenge.

Research motivation

To investigate potential pharmacological interventions targeting Skp2 to increase the efficacy of subsequent therapies for patients with HER-2-positive GC who have developed resistance to trastuzumab.

Research objectives

This study aims to elucidate the inhibitory effect of thioridazine on Skp2 expression and to preliminarily assess the potential of thioridazine in reversing the resistance of HER2-positive GC cells to trastuzumab through both *in vivo* and *in vitro* experiments.

Research methods

The impact of altering the Skp2 protein expression level through overexpression or knockdown on the sensitivity of HER2-positive GC cells to trastuzumab was assessed using a cell counting kit-8 assay. The influence of thioridazine on Skp2 protein expression was demonstrated through computational docking analysis and Cellular Thermal Shift Assay. Flow cytometry, a glucose uptake assay, a lactate production assay, and xenograft experiments in nude mice were employed to evaluate the effects of thioridazine alone or in combination with trastuzumab and lapatinib on the cell cycle, apoptosis, glucose metabolism, and tumor growth.

Research results

Trastuzumab sensitivity can be increased in HER-2-positive GC cells through negative modulation of Skp2 expression. Thioridazine can selectively inhibit Skp2 expression and the protein kinase B/mammalian target of rapamycin signaling

pathway. Thioridazine combined with lapatinib effectively reverses trastuzumab resistance in GC cells by diminishing glycolysis.

Research conclusions

Combining thioridazine with lapatinib is a potential strategy to reverse trastuzumab resistance in GC by suppressing Skp2 expression.

Research perspectives

Further investigation into the optimal combination ratio, initial dosage, and dose-response correlation between thioridazine and lapatinib in GC xenograft models will contribute to the development of more precise drug reference protocols for subsequent clinical trials. A new therapeutic strategy for the management of GC by simultaneous targeting of Skp2 and HER-2 could be introduced.

FOOTNOTES

Co-first authors: Zheng-Yan Yang and Yi-Wei Zhao.

Co-corresponding authors: Zhi-Guang Ren and Ming Shi.

Author contributions: Yang ZY drafted the manuscript and conducted the experiments of immunoblotting, qPCR, and apoptosis analysis in this study; Zhao YW completed the glucose metabolism analysis and animal experiments; Xue JR contributed to the CCK-8 assay and animal experiments; Guo R assisted in the construction and amplification of recombinant plasmid vectors; Liu HD participated in the data collection of docking; Zhao Z assisted in immunohistochemical analysis of xenograft tumor tissue samples; Ren ZG supervised the experiments, corrected the data, and revised the manuscript; Shi M conceived and designed the main content of this study and was responsible for analyzing the research results; all authors contributed to the article and approved the submitted version.

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ORCID number: Zheng-Yan Yang 0000-0002-2294-1907; Zhi-Guang Ren 0000-0002-4011-5334; Ming Shi 0000-0001-9263-6468.

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ORIGINAL ARTICLE

Basic Study

Pien Tze Huang alleviates Concanavalin A-induced autoimmune hepatitis by regulating intestinal microbiota and memory regulatory T cells

Xin Zeng, Miao-Hua Liu, Yi Xiong, Lin-Xin Zheng, Kai-En Guo, Hai-Mei Zhao, Yu-Ting Yin, Duan-Yong Liu, Bu-Gao Zhou

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Xin Zeng, Miao-Hua Liu, Yi Xiong, Lin-Xin Zheng, Kai-En Guo, Department of Postgraduate, Jiangxi University of Chinese Medicine, Nanchang 330004, Jiangxi Province, China

Hai-Mei Zhao, College of Traditional Chinese Medicine, Jiangxi University of Chinese Medicine, Nanchang 330004, Jiangxi Province, China

Yu-Ting Yin, College of Pharmacy, Jiangxi University of Chinese Medicine, Nanchang 330004, Jiangxi Province, China

Duan-Yong Liu, Formula-Pattern Research Center, Jiangxi University of Chinese Medicine, Nanchang 330004, Jiangxi Province, China

Bu-Gao Zhou, Office of Academic Research, Jiangxi University of Chinese Medicine, Nanchang 330004, Jiangxi Province, China

Corresponding author: Bu-Gao Zhou, PhD, Professor, Office of Academic Research, Jiangxi University of Chinese Medicine, No. 1688 Meiling Road, Nanchang 330004, Jiangxi Province, China. 57161998@qq.com

Abstract

BACKGROUND

Traditional Chinese medicine has used the drug Pien Tze Huang (PTH), a classic prescription, to treat autoimmune hepatitis (AIH). However, the precise mode of action is still unknown.

AIM

To investigate the mechanism of PTH in an AIH mouse model by determining the changes in gut microbiota structure and memory regulatory T (mTreg) cells functional levels.

METHODS

Following induction of the AIH mouse model induced by Concanavalin A (Con A), prophylactic administration of PTH was given for 10 d. The levels of mTreg cells were measured by flow cytometry, and intestinal microbiota was analyzed by 16S rRNA analysis, while western blotting was used to identify activation of

the toll-like receptor (TLR)2, TLR4/nuclear factor-κB (NF-κB), and CXCL16/CXCR6 signaling pathways.

RESULTS

In the liver of mice with AIH, PTH relieved the pathological damage and reduced the numbers of T helper type 17 cells and interferon-γ, tumor necrosis factor-alpha, interleukin (IL)-1β, IL-2, IL-6, and IL-21 expression. Simultaneously, PTH stimulated the abundance of helpful bacteria, promoted activation of the TLR2 signal, which may enhance Treg/mTreg cells quantity to produce IL-10, and suppressed activation of the TLR4/NF-κB and CXCL16/CXCR6 signaling pathways.

CONCLUSION

PTH regulates intestinal microbiota balance and restores mTreg cells to alleviate experimental AIH, which is closely related to the TLR/CXCL16/CXCR6/NF-κB signaling pathway.

Key Words: Pien Tze Huang; Autoimmune hepatitis; Intestinal microbiota; Memory regulatory T cell; Toll-like receptor signaling

Core Tip: Intestinal microbiota disorder plays an important role in the pathogenesis of autoimmune hepatitis (AIH), in which one of the main characteristics is the hypofunction of memory regulatory T (mTreg) cells to maintain immune tolerance. The interaction between Treg cells and intestinal microbiota may provide a feasible therapeutic strategy for AIH. As a well-known traditional Chinese medicine, Pien Tze Huang (PTH) can effectively treat AIH in the clinic. However, its mechanism is still unclear. In the present study, PTH regulated intestinal microbiota balance and restored mTreg cells to alleviate experimental AIH, which was closely related to the toll-like receptor/CXCL16/CXCR6/nuclear factor-κB signaling pathway.

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INTRODUCTION

Autoimmune hepatitis (AIH) is an interfacial hepatitis characterized by lymphocyte infiltration and the production of autoantibodies; however, its etiology is unknown. Some studies have suggested that AIH is associated with abnormal intestinal permeability[1]. In the case of increased intestinal permeability or intestinal leakage, abnormal intestinal microbiota can enter the submucosa to activate immune cells and produce inflammatory factors or metabolites, which can invade the liver through the portal vein and cause liver injury. Among the many mechanisms that the immune system uses to recognize microbiota are the toll-like receptors (TLRs), which directly promote regulatory T (Treg) cell proliferation and interleukin (IL)-10 production, thereby preventing imiquimod's ability to induce psoriasis-like symptoms on the skin[2]. Moreover, in the peripheral blood of patients with AIH, some studies have discovered a reduced frequency of Treg cells, essential agents which maintain immune homeostasis, indicating that a deficiency in Treg cell quantity and abnormal Treg cell function may play a vital role in the pathogenesis of AIH[3].

The treatment for AIH is usually high doses of a steroid, which can suppress the immune system. Recent studies have suggested using intestinal microbiota as a potential strategy to treat AIH[4,5]. In their study it was shown that compound probiotics may regulate intestinal microbiota and intestinal permeability, reduce the level of T helper type 1 (Th1) and Th17 cells, improve the level of Treg cells, suppress activation of the TLR4/nuclear factor-κB (NF-κB) pathway, and promote the emergence of AIH[6]. Therefore, for patients with AIH, the interaction between Treg cells and intestinal microbiota may provide a feasible therapeutic strategy. However, the activation degree of effector Treg cells is closely related to memory Treg (mTreg) cells[7], suggesting that mTreg cells could quickly transform into effector Treg cells, playing a crucial part in preserving immunological tolerance.

As a well-known traditional Chinese medicine, Pien Tze Huang (PTH) contains Moschus, Bovis calculus, Snake bile, Notoginseng radix et rhizoma, and other ingredients[8,9] and is regularly used to treat liver conditions, including AIH [10-14]. PTH controls the NF-κB signaling pathway and the NLRP3 inflammasome to reduce IL-1, IL-6, and IL-17 production in blood and to reduce joint inflammation in mice with collagen-induced arthritis[10]. However, the definite mechanism of PTH is not fully understood. PTH has a physiological basis for controlling Treg cells and intestinal microbiota. Bile acid, the active component of Bovis calculus and Snake bile, and notoginsenoside R1, is a potent saponin that is extracted from Panax notoginseng. The immune system's signals to the microbiota may be mediated by bile acids [15]. Song *et al*[16] reported that dietary adjustment and flora transplantation could improve a significant population of colonic Foxp3$^+$ Treg cells with highly-expressed RORγ, and optimize the constituents of the gut bile acids pool. Moreover,

Gong *et al*[17] found that notoginsenoside R1 modulates NF-κB and MAPK signaling to exert anti-inflammatory effects to reduce carbon tetrachloride-induced liver fibrosis. Based on the above evidence, we hypothesized that PTH could treat intestinal microbiota imbalance and a lack of mTreg cells. Thus, this research aimed to determine the mechanism of PTH in the AIH mouse model by observing the changes in intestinal microbiota structure and mTreg functional levels.

MATERIALS AND METHODS

Mice

Forty male specific pathogen-free C57BL/6J mice (aged 8-9 wk; 20-22 g) were bought from GemPharmatech Co., Ltd. (Nanjing, China) (Animal Certificate Number: SCXK (Su)2018-0008). The mice were housed at a temperature of 22 ± 1 °C, relative humidity of 50% ± 10%, and a light/dark cycle of 12/12 h. All animal experiments (including euthanasia of mice) were performed in accordance with the Institutional Animal Care and Use Committee's guidelines (Nanchang, China). The experimental protocols (Permit Number: JZLLSC20210090) were approved by The Jiangxi University of Chinese Medicine's Animal Care and Use Committee.

Materials and reagents

Concanavalin A was bought from Sigma-Aldrich Trading Co., Ltd. (CA, United States, No. C2010-250MG). Dexamethasone (DXM) was obtained from Henan Runhong Pharmaceutical Co., Ltd. (Henan, China, No. 2103111). PTH was acquired from Zhangzhou PTH Pharmaceutical Co., Ltd. (Zhangzhou, China, No. 2108123). As mentioned in previous studies by other researchers, the online pressurized liquid extraction-ultra-high-performance liquid chromatography-ion trap-time-of-flight mass spectrometry (online PLE-UHPLC-IT-TOF-MS) method was used for rapid and comprehensive analysis of the components of PTH. PTH produced a total of 73 signals, of which 71 components could only be inferred from the data. Bovis calculus and Snake bile worked together in a synergistic way to contribute to the occurrence of the remaining 11 components, which included 36 from Notoginseng radix et rhizoma, 15 from Snake bile, and 9 from Bovis calculus (Supplementary Table 1)[18,19].

Induction and treatment of experimental AIH

In order to determine the optimal dose of PTH in treating AIH, an animal experiment was carried out on the treatment of AIH with high, middle, and low doses of PTH.

According to earlier research[20,21], the mice were randomly allocated into 6 groups (10 mice in each group): Control group (control), Concanavalin A (Con A) group (model), Con A + low-dose PTH group (PTH-L), Con A + middle-dose PTH group (PTH-M), Con A + high-dose PTH group (PTH-H), and Con A + DXM group (DXM). Mice in the Con A + PTH-L, Con A + PTH-M, and Con A + PTH-H groups received PTH (117 mg/kg, 234 mg/kg, and 468 mg/kg) every day for 10 d by gavage, respectively, while mice in the normal group and Con A group received the same amount of normal drinking water. The DXM group was intraperitoneally injected with DXM (3 mg/kg) every day. On the 10th d, the AIH mouse model was established, three hours after the last administration of PTH, the mice in the normal control group were injected with 0.2 mL of 0.9% sodium chloride solution through the tail vein, and mice in the other 5 groups were injected with 15 mg/kg Con A through the tail vein. At 8 h after Con A injection, fresh blood was collected from the mice after deep anesthesia with 2% sodium pentobarbital (20 mg/kg i.p.). The abdominal cavity was then quickly opened to collect mouse liver, spleen and colon tissues.

Mice in the Con A + PTH-M group received PTH (234 mg/kg) every day for 10 days by gavage, while mice in the control group and Con A group received the same amount of normal drinking water. The DXM group was intraperitoneally injected with DXM (3 mg/kg) every day. On the 10th day, the AIH mouse model was established as originally stated; three hours after the last administration of PTH, 0.2 mL of a 0.9% sodium chloride solution was injected into the tail vein of the mice in the normal control group, and the mice in the other three groups received an intravenous injection of 15 mg/kg Con A into the tail vein. At 8 h after Con A injection, fresh blood was collected from the mice after deep anesthesia with 2% sodium pentobarbital (20 mg/kg i.p.). The abdominal cavity was swiftly opened to collect mouse colon, spleen, and liver tissues.

Serum measurements

Mouse peripheral blood was centrifuged at 1123 g for 15 min to extract the serum. An automated chemistry analyzer was used to measure the concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin (ALB), globulin (GLB), total protein (TP), indirect bilirubin (IBIL), triglyceride (TG), and high-density lipoprotein (HDL) IBIL in mice (AU480, Beckman Coulter, Pasadena, CA, United States).

Pathological observation

After one week of fixing in 4% buffered paraformaldehyde, the sample was dehydrated in a 50% to 100% alcohol gradient, made completely transparent in dimethyl benzene, and embedded in paraffin. Following deparaffinization and rehydration, the samples were cut into sections 4 μm thick and stained with hematoxylin-eosin (Solarbio, Beijing, China). Thereafter, the slices were randomly and double-blindly observed, and scored by pathologists using a biomicroscope (Lecia, Wetzlar, Germany). Using the Ishak scoring system[22] shown in Supplementary Table 2, liver damage was graded with regard to portal and intralobular inflammation, degeneration, and necrosis. The degree of pathology was graded from 0 (no pathology) to 6 (severe pathology).

Immunofluorescence staining of colon tissue

Immunofluorescence of intestinal mucosae was carried out to evaluate occludin expression and distribution. The tissues were quickly deparaffinized, rehydrated, and cleaned in 1% phosphate buffered saline. The samples were then blocked with 5% bovine serum albumin, and subsequently incubated with occludin (1:100, Abcam, MA, United States) at 4 °C overnight. After three washes, at room temperature, the samples were incubated with anti-FITC IgG secondary antibody for two hours. Finally, the slides were then counterstained with DAPI for 5 min. Images were captured using a fluorescent microscope (Leika, Wetzlar, Germany).

Enzyme-linked immunosorbent assay

Mouse liver tissues were removed and cut into pieces in lysis buffer at the proportion of 1:10, homogenized in ice water using an electric homogenizer, incubated at 4 °C for 30 min, and the supernatants were then removed by centrifugation at 15493 *g* for 15 min. The expression levels of IL-4 (No. MM-0165M1), IL-17A (No. MM-0170M1), interferon (IFN)-γ (No. MM-0182M1), IL-1β (No. MM-0040M1), IL-2 (No. MM-0701M1), IL-6 (No. MM-0163M1), IL-10 (No. MM-0176M1, MM-0176M1), IL-21 (No. MM-0688M1) and tumor necrosis factor-alpha (TNF-α) (No. MM-0132M1) were detected by a commercial enzyme-linked immunosorbent assay (ELISA) kit (Jiangsu Meimian Industry Co., Ltd., Jiangsu, China). According to the instructions the absorbance was measured at 450 nm by a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, United States).

Flow cytometry

Mouse spleen was ground in a sterile culture dish and filtered through nylon mesh to prepare a single cell suspension. RPMI 1640 tissue medium was used to suspend the splenocytes, 10% heat-inactivated fetal bovine serum, 50 units per milliliter of penicillin, and 50 nanograms per milliliter of streptomycin were added, and cultivated for three hours at 37 °C with Cell Stimulation Cocktail after erythrocyte lysis (BD Biosciences, Franklin Lakes, NJ, United States). Extracellular antigens were stained for 20 min at 4 °C in RPMI 1640 medium following FcγR blockage with anti-CD16/32 antibodies (BD Biosciences, Franklin Lakes, NJ, United States). Foxp3/Transcription Factor Buffer Set (BD Biosciences, Franklin Lakes, NJ, United States) was used to fix and permeabilize the cells before staining for intracellular cytokines. CellQuest (BD Biosciences, CA, United States), and a BD FACSCalibur flow cytometer were used to collect the samples, and FlowJo software (BD Biosciences) was used to analyze the data. Cell populations were identified by staining with APC-H7 rat anti-mouse CD4 (1:200, BD Biosciences, NJ, United States), PE rat anti-mouse IL-17A (1:100, BD Biosciences, NJ, United States) FITC-CD25 (1:100, BD Biosciences, NJ, United States), BV421 rat anti-mouse Foxp3 (1:100, BD Biosciences, NJ, United States), APC rat anti-mouse programmed cell death ligand 1 (PD-L1)+(1:100, BD Biosciences, NJ, United States), Alexa Fluor 647 rat anti-mouse CCR7 (1:100, BD Biosciences, NJ, United States), and BV510 rat anti-mouse CD45RA (1:100, BD Biosciences, NJ, United States).

Western blot

According to the instructions above, the liver tissue supernatant utilized for western blotting was produced (see the ELISA section). The traditional BCA protein determination method was used to measure the protein concentration (Cwbiotech, Beijing, China). Sodium-dodecyl sulfate gel electrophoresis was used to separate the same amount of protein by 10%-15% before being transferred to polyvinylidene fluoride membranes. After being sealed with 5% skimmed milk, the membranes were incubated with primary antibodies at 4 °C overnight. The antibodies were anti-GAPDH (1:10000, Proteintech, Wuhan, China), Tubulin (1:1000, Abclonal, Wuhan, China), TLR2 (1:1000, Abcam, MA, United States), TLR4 (1:1000, Abcam, MA, United States), CXCR6 (1:1000, Boster, Wuhan, China), NF-κB p65 (1:1000, Cell Signaling Technology, Boston, United States), CXCL16 (1:1000, Bioss, Beijing, China),and Occludin (1:1000, Abcam, MA, United States). The membranes were then rinsed with Tris buffered saline and Tween (TBST) and incubated at 37 °C for 1 h with the secondary antibody (1:10000, Proteintech, Wuhan, China). The tagged protein bands were scanned using the ChemiDoc XRS+Gel Imaging System (Hercules, CA, United States). after a second TBST wash.

Isolation of fecal bacterial microbiota and 16S rRNA gene sequencing analysis

According to the directions on the DNA extraction kit (Omega Bio-tek, Norcross, GA, United States), the DNA of the microbial community was collected. DNA concentration and purity were assessed using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, United States), and the quality of the DNA extracted was determined using 1% agarose gel electrophoresis. The hypervariable region V3-V4 of the bacterial 16S rRNA gene was amplified with primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, United States). The 16S rRNA gene was amplified using PCR in the following manner: A first denaturation was performed at 95 °C for three minutes, followed by 27 cycles of denaturation at 95 °C for thirty seconds, annealing at 55 °C for thirty seconds, and extension at 72 °C for forty-five seconds, followed by a third extension at 72 °C for ten minutes, before finishing at 4 °C. Purified PCR products were quantified after removal from 2% agarose gels using a Quantus TM Fluorometer (Promega, United States) after purification using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States).

Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) used an Illumina MiSeq PE300 platform/NovaSeq PE250 platform (Illumina, San Diego, United States) to sequence the purified amplicons in a paired-end manner using equimolar pooling. The NCBI Sequence Read Archive database received the raw reads (Accession Number: SRP392420). In addition, I-sanger was used to conduct co-occurrence network analysis, correlation analysis, and microbiological difference analysis (Majorbio Bio-Pharm Technology Co., Ltd.; www.i-sanger.com).

Statistical analysis

Data were expressed as the mean ± SEM. The Student *t*-test or one-way analysis of variance (ANOVA), followed by the Wilcoxon rank-sum test and Kruskal-Wallis's test were used for multiple comparisons. $P < 0.05$ or $P < 0.01$ was considered significantly different. GraphPad Prism 8.0 and SPSS 21.0 were used for statistical analysis.

RESULTS

Dose screening of PTH relieved liver injury induced by Con A in mice

Con A-induced AIH is a confirmable model for studying human AIH, as it simulates many aspects of this disease, including liver damage, abnormal elevation of serum transaminase level and excessive generation of pro-inflammatory cytokines[23]. The liver weight (Figure 1A) and liver weight index (Figure 1B), spleen weight (Figure 1C), colonic weight (Figure 1D), and colonic weight index (Figure 1E), serum ALT (Figure 1F) and AST (Figure 1G), and the levels of IL-17A (Figure 1J) were raised in the Con A group compared with mice not treated with Con A ($P < 0.01$), while following PTH-L, PTH-M, PTH-H or DXM treatment, these results were the opposite ($P < 0.05$ or $P < 0.01$). The expression of IL-4 (Figure 1H) and IL-10 (Figure 1I) in Con A-induced AIH mice without treatment was lower than in control mice and AIH mice treated with PTH-L, PTH-M, PTH-H or DXM ($P < 0.01$). Compared with the normal control group, mice in the Con A group showed interfacial hepatitis, death of a large number of liver cells, structural disorder of liver lobules, increased infiltration of inflammatory cells, focal (or spotted) lytic necrosis or apoptosis (Figure 1K), and higher Ishak scores (Figure 1L) ($P < 0.01$). After 10 d of PTH treatment, compared with mice in the Con A group, mice in the PTH-L, PTH-M, PTH-H groups showed less interfacial hepatitis and mild focal (or spotted) lytic necrosis or apoptosis, more complete liver lobular structure, reduced inflammatory cell infiltration (Figure 1K), and lower Ishak scores (Figure 1L) ($P < 0.01$); thus, relieving AIH. However, pathological damage of the liver and the expression level of inflammatory factors were further reduced and liver function was further improved following the middle dose of PTH compared with the high dose of PTH and the low dose of PTH in AIH mice. The middle dose of PTH was the best dose to relieve AIH, and this dose was selected for the follow-up study.

PTH relieves liver injury induced by Con A in mice

Compared with the control group, the livers in the Con A group were dark red and swollen. Compared with the Con A group, the livers in the Con A + PTH-M and Con A + DXM groups were bright red with smooth surfaces (Figure 2B). Moreover, the liver weight (Figure 2C) and liver weight index (Figure 2D) were increased in the Con A group compared with mice not treated with Con A ($P < 0.01$), while PTH or DXM treatment decreased the increase in liver weight (Figure 2C) and liver weight index (Figure 2D) ($P < 0.05$). Compared with the normal control group, mice in the Con A group showed interfacial hepatitis, death of a large number of liver cells, structural disorder of liver lobules, increased infiltration of inflammatory cells, focal (or spotted) lytic necrosis or apoptosis (Figure 2F), and higher Ishak scores (Figure 2E) ($P < 0.01$). After 10 d of PTH treatment, compared with mice in the Con A group, mice in the Con A + PTH-M group and Con A + DXM groups showed less interfacial hepatitis and mild focal (or spotted) lytic necrosis or apoptosis, more complete liver lobular structure, reduced inflammatory cell infiltration (Figure 2F), and lower Ishak scores (Figure 2E) ($P < 0.01$). These findings demonstrate that PTH efficaciously relieves the pathological liver injury in Con A-induced AIH mice.

PTH improves liver function in AIH mice

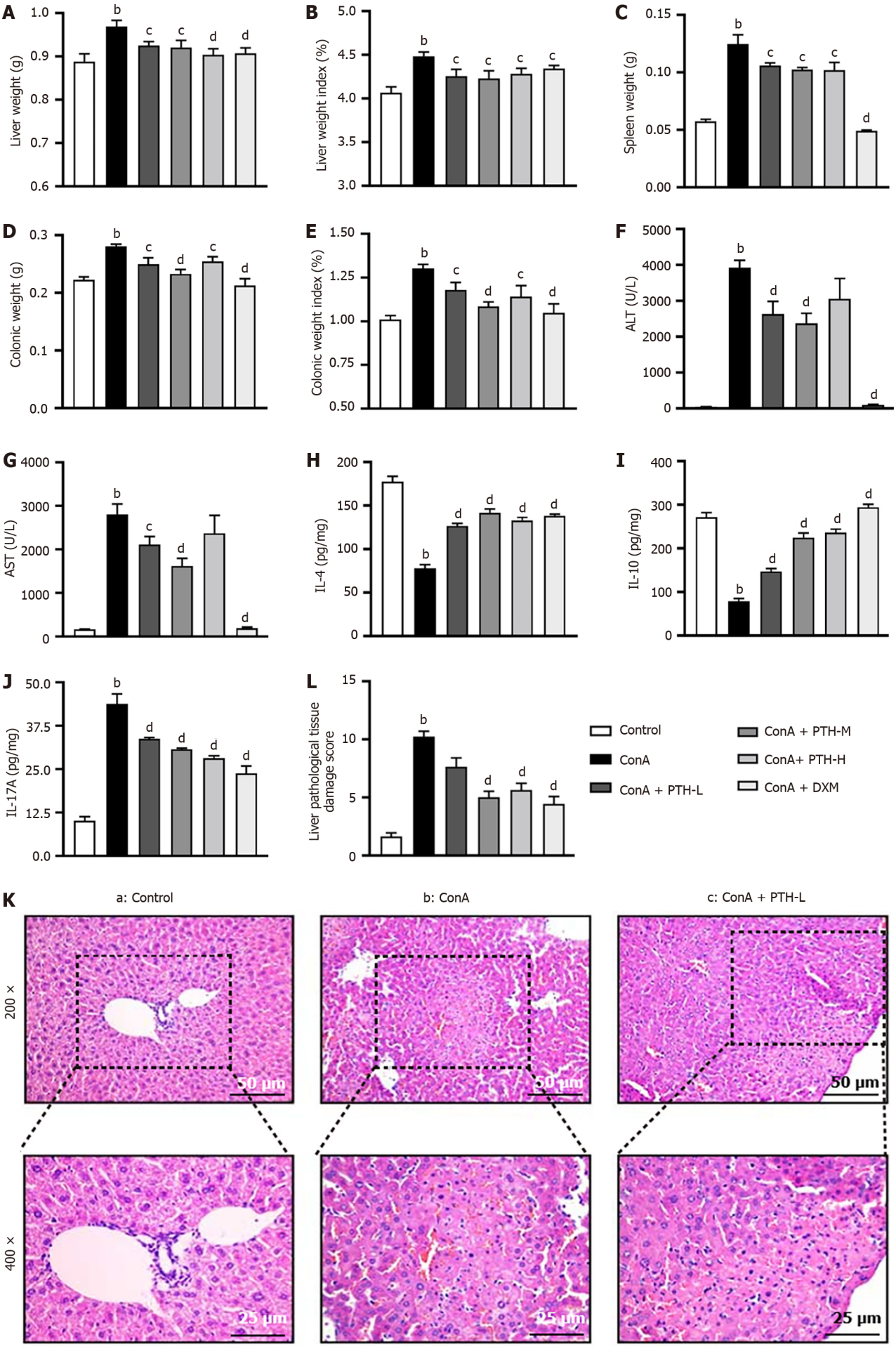
A biochemical liver test is an important method to judge whether there is liver damage, evaluate the severity of liver illness, and judge the treatment effect and prognosis[24-26]. The levels of serum enzymes, including ALT (Figure 3A), AST (Figure 3B), ALP (Figure 3C), IBIL (Figure 3D), and TG (Figure 3E) were raised in mice who received Con A compared with control mice ($P < 0.01$), and mice with Con A-induced AIH treated with PTH and DXM. In addition, decreased levels of TP (Figure 3F), ALB (Figure 3G), GLB (Figure 3H), and HDL (Figure 3I) were detected in serum from mice with Con A-induced AIH treatment ($P < 0.01$), and these levels were increased after treatment with PTH and DXM ($P < 0.05$ or $P < 0.01$). The above results show that PTH can improve liver function in mice with Con A-induced AIH.

PTH regulates the expression of cytokines in liver tissue

An essential aspect of immunological damage in AIH is abnormal expression of inflammatory cytokines, such as IFN-γ, IL-1β, IL-2, IL-6, IL-21, TNF-α, and IL-10. Th17, Treg, and mTreg cells thereafter undergo transformation and migration into the liver. The secretion of IFN-γ (Figure 4A), IL-1β (Figure 4B), IL-2 (Figure 4C), IL-6 (Figure 4D), IL-21 (Figure 4E), TNF-α (Figure 4F) in liver tissues from AIH mice in the Con A group was higher than that in the control, Con A + PTH-M and Con A + DXM groups ($P < 0.01$). In contrast, the expression of IL-10 (Figure 4G) in Con A-induced AIH mice without treatment was lower than in control mice and AIH mice treated with PTH or DXM ($P < 0.01$). According to these findings, PTH modulates the equilibrium of pro- and anti-inflammatory cytokines in AIH mice.

PTH regulates Th17 and Treg differentiation in AIH mice

Production of the pro-inflammatory cytokine IL-17A is a characteristic of Th17 cells, the phenotype of Treg cells is $CD4^+$ $CD25^+Foxp3^+$, and they play a significant role in autoimmune disease such as AIH and so on[27,28]. Programmed death-1 (PD-1) is a member of the CD28/B7 superfamily of costimulatory molecules that plays an inhibitory effect in the periphery. PD-L1 (also known as B7-H1) and PD-L2 (also known as B7-DC) are ligands for PD-1[29]. In the present study,


A
Liver weight (g)
B
Liver weight index (%)
C
Spleen weight (g)
D
Colonic weight (g)
E
Colonic weight index (%)
F
ALT (U/L)
G
AST (U/L)
H
IL-4 (pg/mg)
I
IL-10 (pg/mg)
J
IL-17A (pg/mg)
L
Liver pathological tissue damage score
Control
ConA
ConA + PTH-L
ConA + PTH-M
ConA+ PTH-H
ConA + DXM
K
a: Control
b: ConA
c: ConA + PTH-L
200 ×
400 ×
50 μm
25 μm

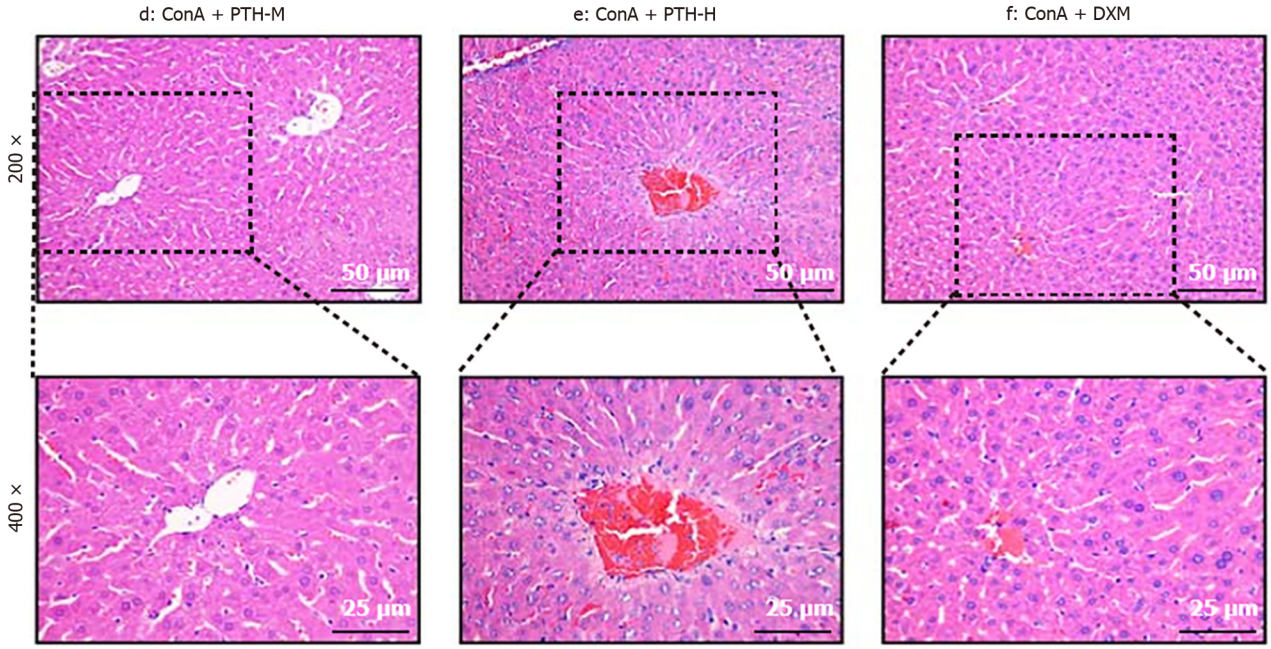


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Figure 1 Dose screening of Pien Tze Huang relieved liver injury induced by Concanavalin A in mice. A: Liver weigh; B: Liver weigh index; C: Spleen weigh; D: Colonic weigh; E: Colonic weigh index; F: Serum alanine aminotransferase; G: Serum aspartate aminotransferase; H-J: The levels of cytokines: Interleukin (IL)-4 (H), IL-10 (I), IL-17A (J) were measured by enzyme-linked immunosorbent assay; K: Representative photographs of hematoxylin and eosin-stained liver sections; L: Scores for liver injury in different groups. According to the Ishak scoring system, liver injury was scored with respect to portal and intralobular inflammation, fragmented necrosis, and bridging necrosis. The extent of the pathology was scored from 0 (no pathology) to 4 (severe pathology). Data are representative images or the mean ± SEM (n = 8). $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* the control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* the Concanavalin A group. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; IL: Interleukin; ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.

the mononuclear cells in peripheral blood and $CD4^+$ lymphocytes were measured by flow cytometry (Figures 5A and B). The percentage of $CD4^+IL\text{-}17A^+$ (Th17) cells (Figure 5C) in the peripheral blood of AIH mice was markedly raised ($P <$ 0.01), while the levels of $CD4^+IL\text{-}17A^+$ (Th17) cells (Figure 5C) were considerably lower in PTH-treated AIH mice than in untreated AIH mice ($P < 0.01$). However, the percentage of $CD4^+CD25^+Foxp3^+$ Treg cells (Figure 5D) and $CD4^+CD25^+Foxp3^+PD\text{-}L1^+$ cells (Figure 5E) in the peripheral blood of mice with Con A-induced AIH were significantly decreased ($P <$ 0.05 or $P < 0.01$), while PTH treatment significantly increased the percentages of $CD4^+CD25^+Foxp3^+$ Treg cells (Figure 5D) and $CD4^+CD25^+Foxp3^+PD\text{-}L1^+$ cells (Figure 5E) in AIH mice ($P < 0.01$). The above results demonstrate that PTH controls the balance of Th17 cells and Treg cells in mice with Con A-induced AIH.

PTH regulates the number of central memory T cells and their transformation into effector T cells in AIH mice

Central memory T (TCM) cells, whose phenotype is $CD4^+CD45RA^-CCR7^+$, mainly exist in lymphoid organs. Following antigen stimulation, TCM cells can rapidly transform into effector T (TEM) cells. The TEM phenotype is $CD4^+CD45RA^-CCR7^-$ and these cells exist in the periphery for a long time to produce IFN-γ, IL-17, and other inflammatory factors. This study used lymph node homing receptors $CD45RA^-$, $CD45RA^+$, and chemokine CC receptor (CCR)7 (Figures 6A-D) to recognize and count memory T cells. The levels of $CD4^+CD45RA^+CCR7^+$ (Figure 6E), $CD4^+CD45RA^+CCR7^-$ (Figure 6F), $CD4^+CD45RA^-CCR7^+$ (TCM) (Figure 6G), and $CD4^+CD45RA^-CCR7^-$ (TEM) (Figure 6H) cells were markedly decreased in untreated AIH mice in contrast to control mice ($P < 0.05$ or $P < 0.01$). After PTH treatment, the levels of $CD4^+CD45RA^+$ $CCR7^+$ (Figure 6E), $CD4^+CD45RA^+CCR7^-$ (Figure 6F), $CD4^+CD45RA^-CCR7^+$ (TCM) (Figure 6G) and $CD4^+CD45RA^-CCR7^-$ (TEM) (Figure 6H) cells were significantly increased ($P < 0.05$ or $P < 0.01$), which suggests that PTH controlled the quantity of TCM cells and TEM cells in AIH mice.

PTH regulates the subgroups of mTreg cells in AIH mice

$CD45RA^+Foxp3^{low}$ cells are static mTreg cells, which become $CD45RA^-Foxp3^{high}$ cells after activation. This study used lymph node homing receptors $CD45RA^-$ and $CD45RA^+$ (Figures 7A-D) to recognize and count memory Treg cells. In contrast to control mice, the number of $CD4^+CD45RA^-Foxp3^{high}$ mTreg (Figure 7E) and $CD4^+CD45RA^+Foxp3^{high}$ (Figure 7F) cells were significantly decreased in untreated AIH mice ($P < 0.05$), which implies that this is one of the primary characteristics in mice with Con A-induced AIH. After PTH treatment, the numbers of $CD4^+CD45RA^-Foxp3^{high}$ mTreg (Figure 7E), and $CD4^+CD45RA^+Foxp3^{high}$ (Figure 7F) significantly increased ($P < 0.05$). These results show that PTH improves mTreg cells level in AIH mice.

PTH improves the composition of gut microbiota in AIH mice

Increasing evidence has suggested that disordered intestinal microbiota is a significant characteristic in mice with Con A-

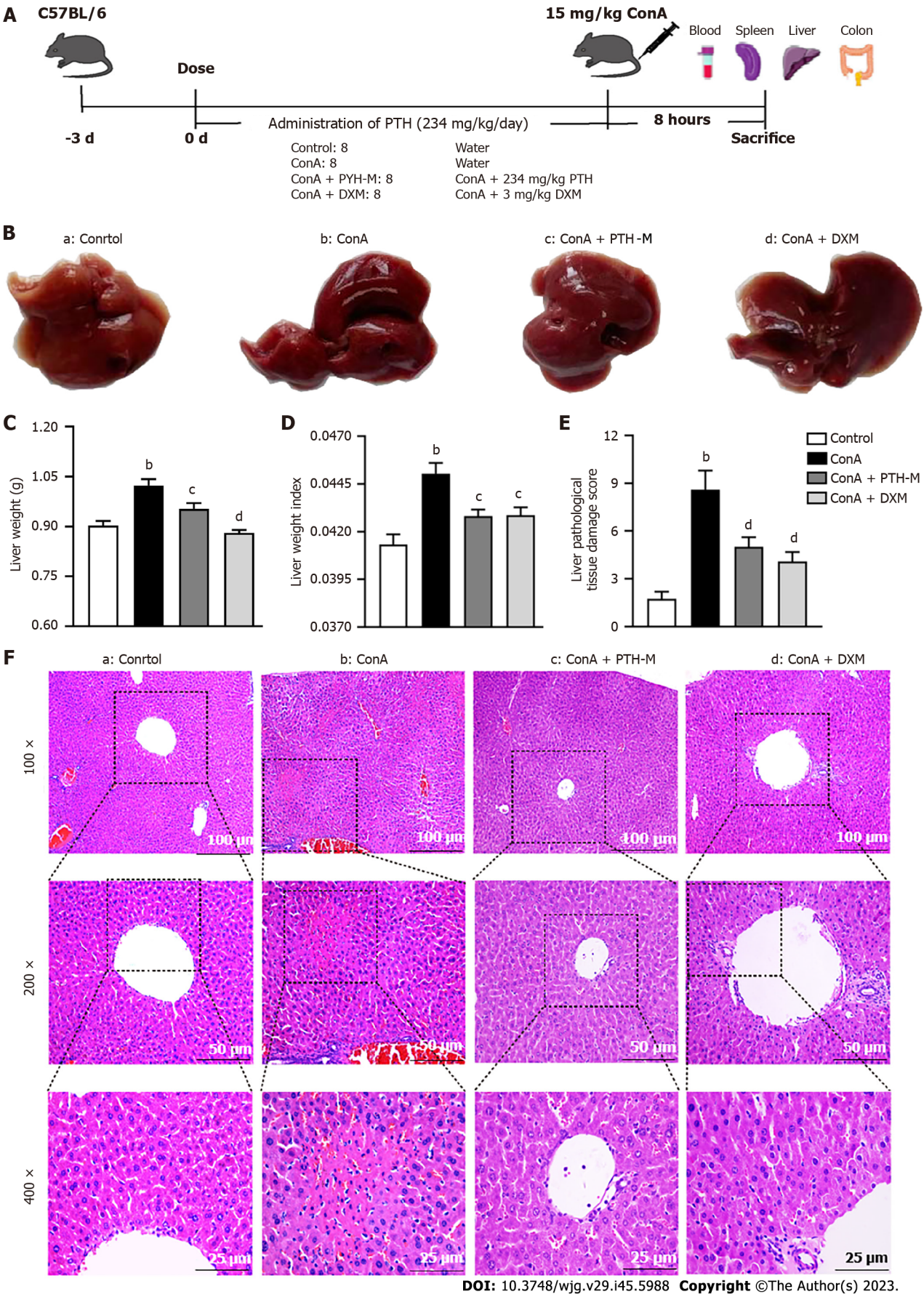

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Figure 2 Pien Tze Huang relieves liver injury induced by Concanavalin A in mice. A: Timeline of the experimental process of treatment and autoimmune hepatitis induction in mice; B-E: Images of mouse livers (B), liver weigh (C), liver weigh index (D), and scores for liver injury (E) in different groups. According to the Ishak scoring system, liver injury was scored with respect to portal and intralobular inflammation, fragmented necrosis, and bridging necrosis. The

extent of the pathology was scored from 0 (no pathology) to 4 (severe pathology); F: Representative photographs of hematoxylin and eosin-stained liver sections. Data are representative images or the mean ± SEM (n = 8). [a]P < 0.05 and [b]P < 0.01 *vs* the control group; [c]P < 0.05 and [d]P < 0.01 *vs* the Concanavalin A group. ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.

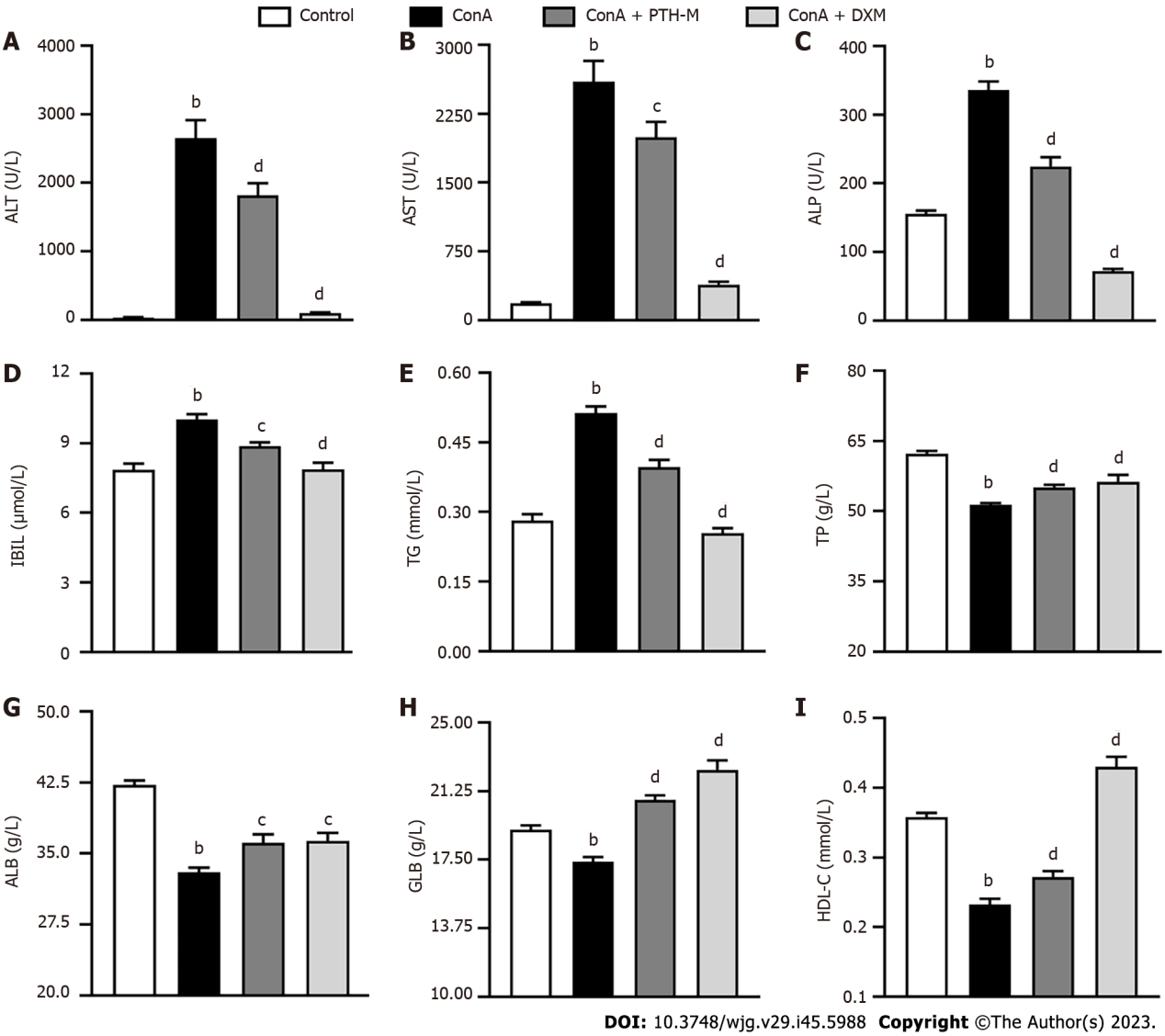

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Figure 3 Pien Tze Huang improves liver function in mice with autoimmune hepatitis. A: Serum alanine aminotransferase; B: Serum aspartate aminotransferase; C: Serum alkaline phosphatase; D: Serum indirect bilirubin; E: Serum triglyceride; F: Serum total protein; G: Serum albumin; H: Serum globulin; I: Serum high-density lipoprotein. Data are representative images or the mean ± SEM (n = 8). [a]P < 0.05 and [b]P < 0.01 *vs* the control group; [c]P < 0.05 and [d]P < 0.01 *vs* the Concanavalin A group. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; IBIL: Indirect bilirubin; TG: Triglyceride; TP: Total protein; ALB: Albumin; GLB: Globulin; HDL: High-density lipoprotein; ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.

induced AIH[30]. The operational taxonomic unit (OTU) level (Figure 8A) rarefaction curve of the Shannon index at the OTU level levelled off when the sequencing data reached saturation, covering the vast majority of species in the gut microbiome community of AIH mice. The abundance grade curve at the OTU level (Figure 8B) showed the species richness and evenness of species diversity in the sample. When the abscissa position of the extension end point of the sample curve is more backward, this implies that the species richness of the sample is higher, the curve is smoother, and the species distribution of the sample is uniform. The abundance and diversity of the microbial population were expressed by single sample diversity (Alpha diversity) analysis, including the microbial richness indices Shannon and Simpson (Figures 8C-D). Figure 8C shows a substantial variation in the Shannon index at the OTU level between the Con A group and Con A + PTH-M group. Although there was no substantial variation in the Shannon index among the control, Con A, and Con A + DXM groups, the Shannon index at the OTU level was higher in the Con A group than in the control mice and Con A + DXM groups. Interestingly, the result of the Simpson index (Figure 8D), which also represented species richness, showed the opposite trend. The Simpson index at the OTU level was lower in the Con A group than in the control, Con A + PTH-M, and Con A + DXM groups.

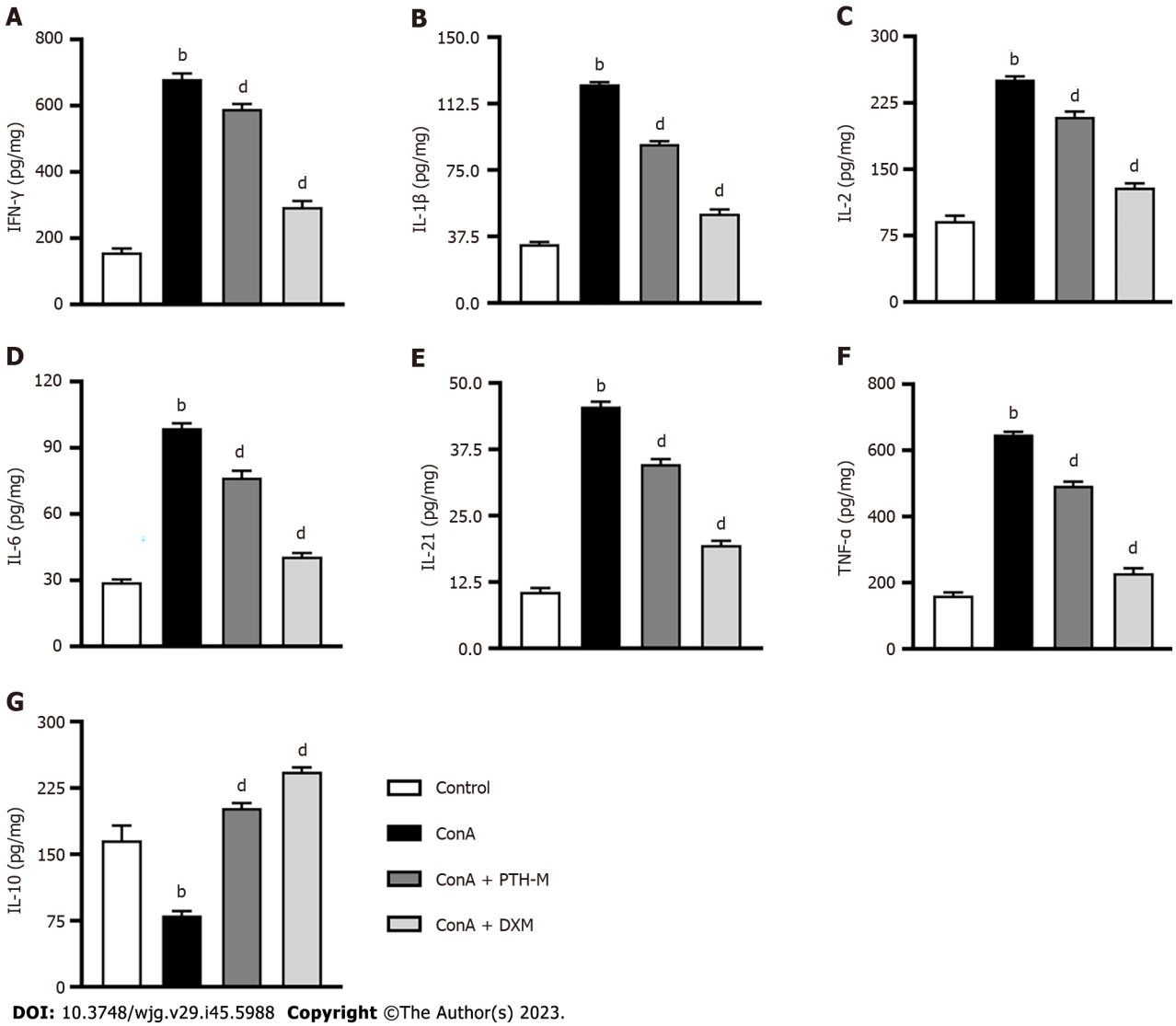

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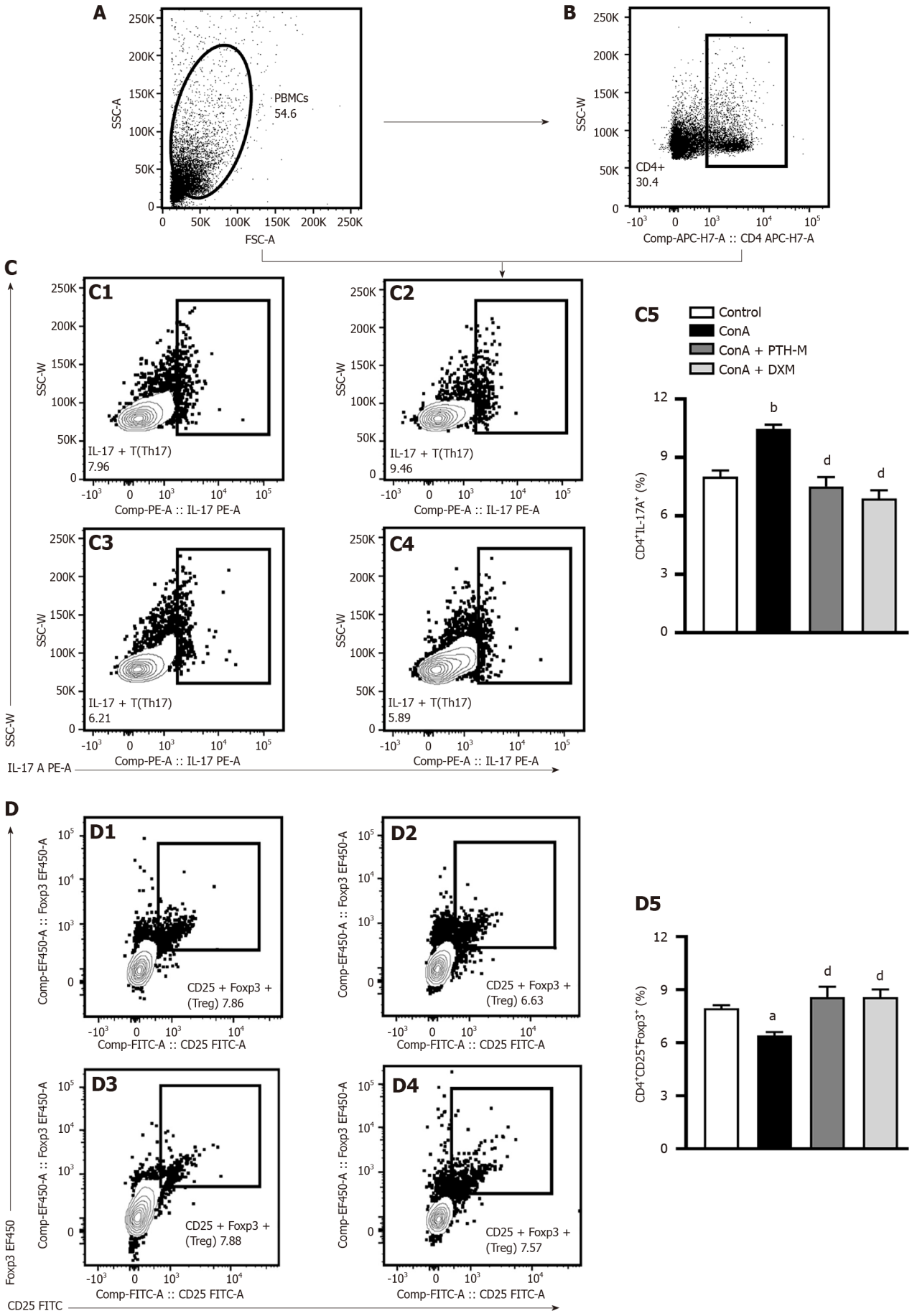
Figure 4 Pien Tze Huang regulates the expression of inflammatory cytokines in mice with autoimmune hepatitis. A-G: The levels of cytokines: Interferon-γ (A), interleukin (IL)-1β (B), IL-2 (C), IL-6 (D), IL-21 (E), tumor necrosis factor-alpha (F), and IL-10 (G), were measured by enzyme-linked immunosorbent assay. Data are representative images or the mean ± SEM (n = 8). $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* the control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* the Concanavalin A group. IFN: Interferon; IL: Interleukin; TNF: Tumor necrosis factor; ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.

Subsequently, in order to compare the diversity of the intestinal microbiota among these four groups, the diversity analyses were used, including principal co-ordinates analysis (PCoA) (Figure 8E). The PCoA analyses proved that the distribution of species in the Con A and Con A + PTH-M groups was distinct from the control group, the Con A + PTH-M group and the control groups were separated from one another by a smaller distance than the Con A and control groups (Figure 8E). Venn diagram analysis at the OTU level revealed additional variations in the intestinal microbiota composition across the four groups (630, 634, 627, and 620 OTUs were seen in the control, Con A, Con A + PTH-M, and Con A + DXM groups, respectively) (Figure 8F).

The percent of community abundance at the genus level (Figure 8G) was used to analyze and compare the species abundance of each group of samples and observe their biological composition and structure. *Lachnospiraceae-NK4A136*, *norank-f-norank-o-Clostridia-UCG-014*, and *Desulfovibrio* were lower in the Con A group than in the control and Con A + PTH-M groups. *Alloprevotella*, *Prevotellaceae-UCG-001*, *Dubosiella*, and *Odoribacter* were superior in the Con A group than in the control and Con A + PTH-M groups.

Figure 8H shows the phylogenetic tree at the genus level. Each branch in the evolutionary tree represented a species. According to the higher taxonomic rank that the species belong to, the branches are colored differently. The bar graph on the right displays the percentage of reads of species in various groups, and the lengths of the branches represent the evolutionary distance between the two species, or the level of species difference. Picrust was used to predict the function of the 16S amplicon sequencing results (Figure 8I). The functions of enrichment mainly include the transport and metabolism of hydrates; transcription; amino acid transport and metabolism; signal transduction mechanism; copy, recombine, repair; translation, ribosome structure, and biogenesis; inorganic ion transport and metabolism; energy production and transformation.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54.6
SSC-A
FSC-A
B
SSC-W
CD4+
30.4
Comp-APC-H7-A :: CD4 APC-H7-A
C
C1
IL-17 + T(Th17)
7.96
C2
IL-17 + T(Th17)
9.46
C3
IL-17 + T(Th17)
6.21
C4
IL-17 + T(Th17)
5.89
Comp-PE-A :: IL-17 PE-A
SSC-W
IL-17 A PE-A
C5
Control
ConA
ConA + PTH-M
ConA + DXM
CD4+IL-17A+ (%)
D
D1
CD25 + Foxp3 +
(Treg) 7.86
D2
CD25 + Foxp3 +
(Treg) 6.63
D3
CD25 + Foxp3 +
(Treg) 7.88
D4
CD25 + Foxp3 +
(Treg) 7.57
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Comp-FITC-A :: CD25 FITC-A
Foxp3 EF450
CD25 FITC
D5
CD4+CD25+Foxp3+ (%)

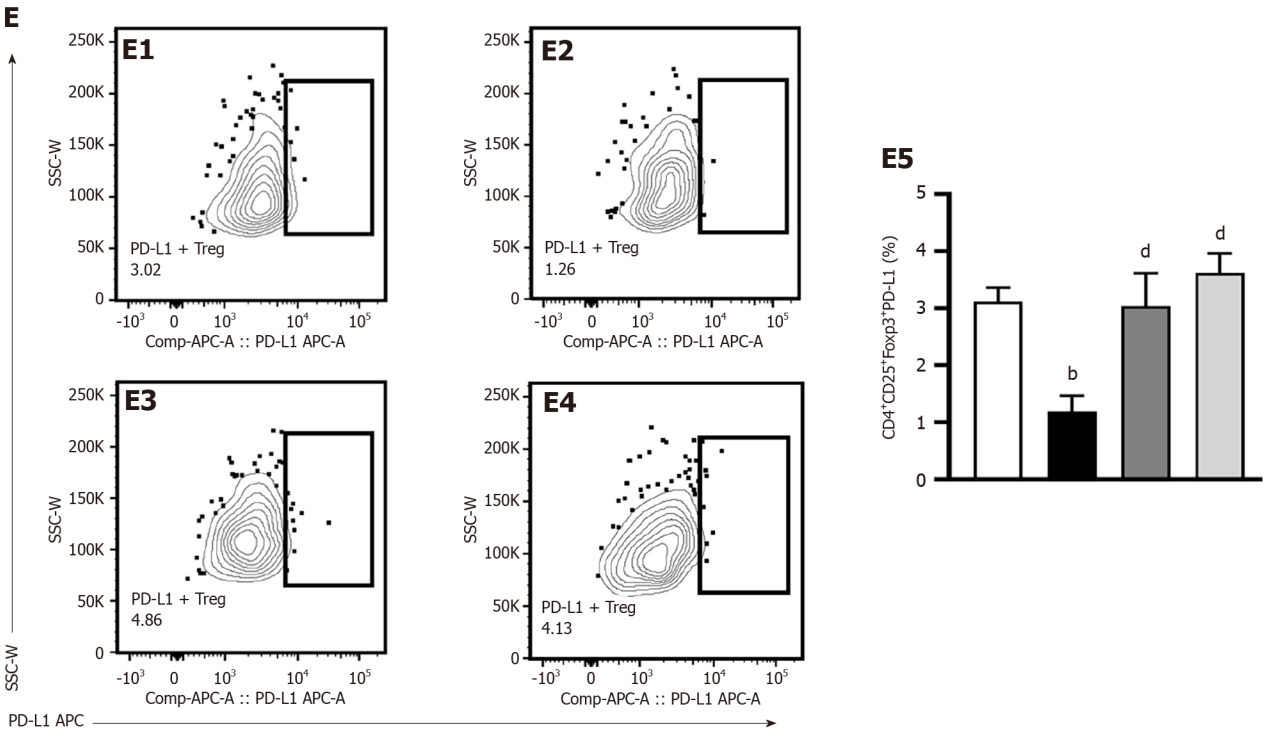

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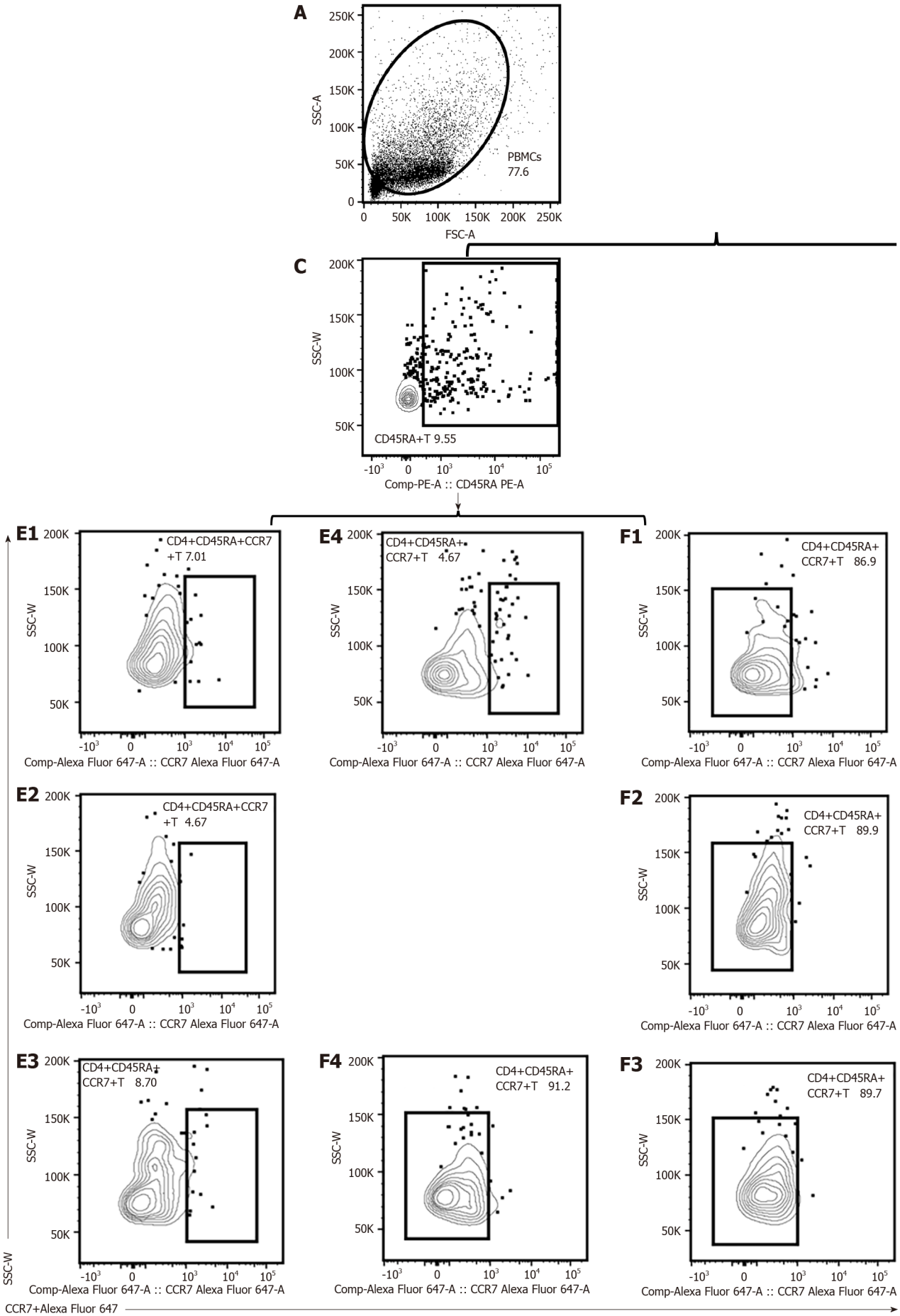
Figure 5 Pien Tze Huang regulates the number of T helper type 17 and regulatory T cells in mice with autoimmune hepatitis. A: Mononuclear cells in peripheral blood; B: $CD4^+$ lymphocytes measured by flow cytometry; C: $CD4^+IL\text{-}17A^+$(T helper type 17) cells. C1-C4: Control, Concanavalin A (Con A), Con A + middle-dose Pien Tze Huang (PTH-M), and Con A + dexamethasone (DXM) group, respectively; C5: Statistical analysis of $CD4^+IL17A^+$ cell frequencies in these four groups; D: $CD4^+CD25^+Foxp3^+$ cells. D1–D4: Control, Con A, Con A + PTH-M, and Con A + DXM group, respectively; D5: Statistical analysis of $CD4^+CD25^+Foxp3^+$ cell frequencies in these four groups; E: $CD4^+CD25^+Foxp3^+PD\text{-}L1^+$ cells. E1–E4: Control, Con A, Con A + PTH-M, and Con A + DXM group, respectively; E5: Statistical analysis of $CD4^+CD25^+Foxp3^+PD\text{-}L1^+$ cell frequencies in these four groups. Data are representative images or the mean ± SEM (n = 8). $^aP < 0.05$ and $^bP < 0.01$ *vs* the control group; $^cP < 0.05$ and $^dP < 0.01$ *vs* the Concanavalin A group. IL: Interleukin; ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.

To identify the species with different abundances in various groups (samples) of microbial communities, hypothesis tests were conducted, and the significance of the observed differences were assessed, and rigorous statistical methods of the Kruskal-Wallis *H* test (Figure 9A) and Wilcoxon rank-sum test (Figures 9B and C) were used. As shown in Figure 9A, *Dubosiella* and *Rikenellaceae-RC9*-gut-group were higher in the Con A group than in the control, Con A + PTH-M, and Con A + DXM groups, while *Desulfovibrio* were lower. As shown in Figures 9B and C, *Alloprevotella*, *Rikenellaceae-RC9*-gut-group and *Prevotellaceae-NK3B31*-group were superior in the Con A group than in the control and Con A + PTH-M groups. The above results show that PTH can improve Con A-induced intestinal microbial composition in AIH mice.

PIH regulates gut microbiota-associated important environmental factors in AIH mice

Based on the actual effects of PTH on Treg/mTreg and intestinal microbiota in AIH mice, the correlation between AST, ALT, HAI, TLR4, $CD4^+CD25^+Foxp3^+$ Treg cells, $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells, and intestinal microbiota was further investigated using a Spearman correlation heat map, redundancy analysis (RDA), and distance-based RDA (db-RDA). In addition, environmental factors such as AST, ALT, HAI, TLR4, $CD4^+CD25^+Foxp3^+$ Treg, and $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells were used to examine their relationship with the intestinal microbiota. On the basis of RDA analysis (Figure 9D), the abundance of AST, ALT, HAI, and TLR4 were accordant with the Con A group's intestinal microbiota, while the abundance of $CD4^+CD25^+Foxp3^+$ Treg and $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells were accordant with the gut microbiota of mice in the control and Con A + PTH-M groups. On the basis of db-RDA analysis (Figure 9E), the abundance of AST, ALT, HAI and TLR4 were compatible with the gut microbiota of the Con A group, while the abundance of $CD4^+CD25^+Foxp3^+$ Treg and $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells were similar to the gut microbiota of mice in the Con A + PTH-M group. According to the Spearman correlation heat map at the genus level (Figure 9F), *Desulfovibrio* was negatively correlated with HAI, AST, ALT, TLR4 and positively connected with $CD4^+CD25^+Foxp3^+$ Treg and $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells; *Prevotellaceae-NK3B31*-group was positively connected with HAI, AST, ALT, TLR4, and negatively connected with $CD4^+CD25^+Foxp3^+$ Treg and $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells; *Rikenellaceae-RC9*-gut-group was positively connected with HAI, AST, ALT, and negatively connected with $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells; *Dubosiella* was negatively connected with $CD4^+CD25^+Foxp3^+$ Treg and $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells.

According to Western blot analysis, AIH mice had higher levels of TLR4 protein expression (Figure 9G) than the control group ($P < 0.01$), and the Con A + PTH-M group had lower levels of TLR4 protein expression (Figure 9G) than AIH mice ($P < 0.01$) (Figure 9G). To sum up, PIH regulates gut microbiota-associated important environmental factors in AIH mice.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SSC-W
CD45RA+T 9.55
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+T 7.01
E4
CD4+CD45RA+
CCR7+T 4.67
F1
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CCR7+T 86.9
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CCR7+T 91.2
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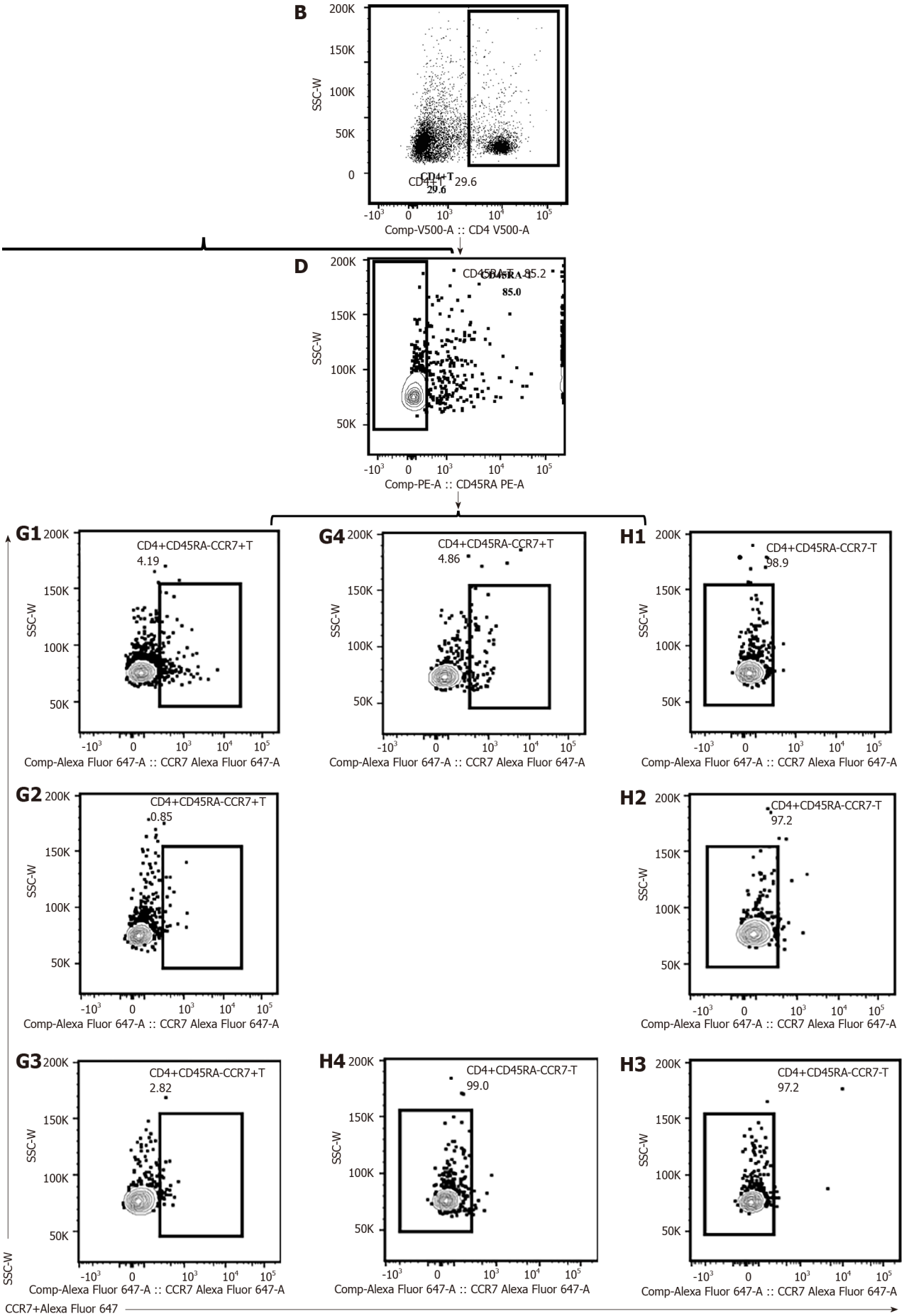
B
SSC-W
CD4+T 29.6
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D
CD45RA-T 85.2
Comp-PE-A :: CD45RA PE-A
G1
CD4+CD45RA-CCR7+T 4.19
G4
CD4+CD45RA-CCR7+T 4.86
H1
CD4+CD45RA-CCR7-T 98.9
G2
CD4+CD45RA-CCR7+T 0.85
H2
CD4+CD45RA-CCR7-T 97.2
G3
CD4+CD45RA-CCR7+T 2.82
H4
CD4+CD45RA-CCR7-T 99.0
H3
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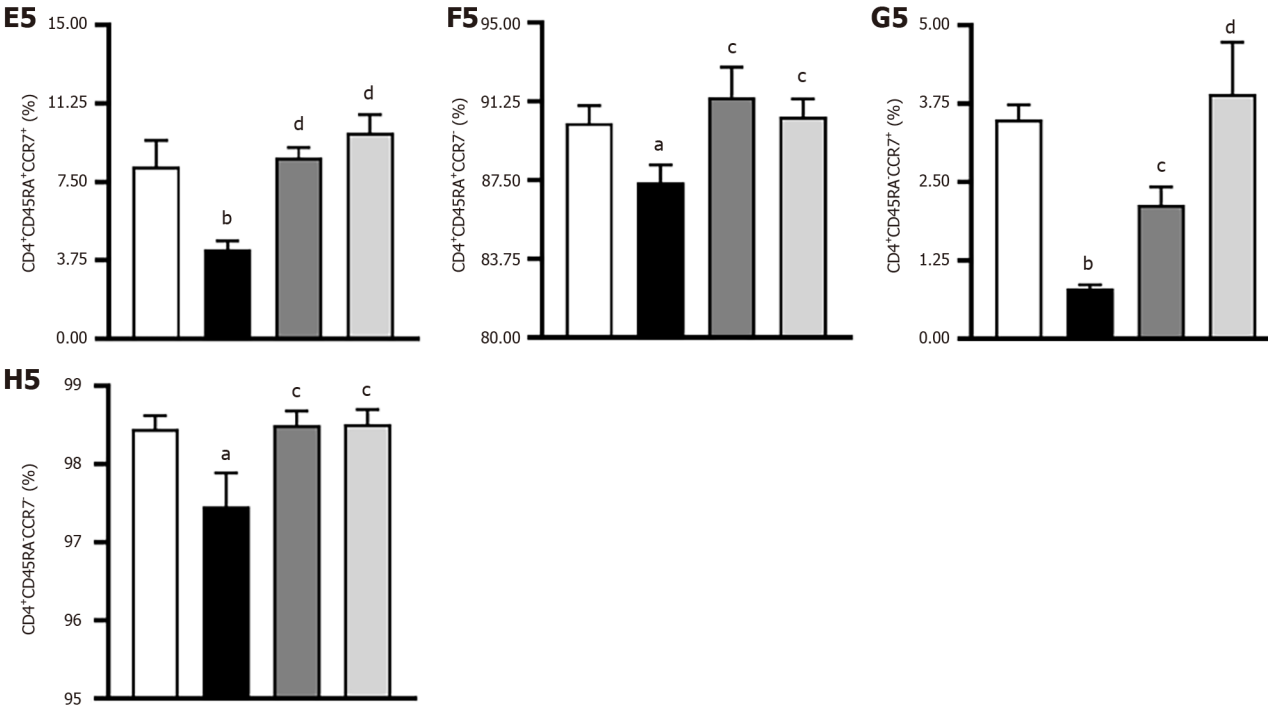

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Figure 6 Pien Tze Huang regulates the number of memory T cells in mice with autoimmune hepatitis. A: Peripheral blood mononuclear cells; B: CD4+ lymphocytes measured by flow cytometry; C: CD45RA$^+$ lymphocytes measured by flow cytometry; D: CD45RA$^-$ lymphocytes measured by flow cytometry; E: CD4$^+$CD45RA$^+$CCR7$^+$ cells. E1-E4: Control, Concanavalin A (Con A), Con A + middle-dose Pien Tze Huang (PTH-M), and Con A + dexamethasone (DXM) group in order; E5: Statistical analysis of CD4$^+$CD45RA$^+$CCR7$^+$ cell frequencies in these four groups; F: CD4$^+$CD45RA$^+$CCR7$^-$ cells. F1-F4: Control, Con A, Con A + PTH-M, and Con A + DXM group, respectively; F5: Statistical analysis of CD4$^+$CD45RA$^+$CCR7$^-$ cell frequencies in these four groups; G: CD4$^+$CD45RA$^-$CCR7$^+$ cells. G1-G4: Control, Con A, Con A + PTH-M, and Con A + DXM group, respectively; G5: Statistical analysis of CD4$^+$CD45RA$^-$CCR7$^+$ cell frequencies in these four groups; H: CD4$^+$CD45RA$^-$CCR7$^-$cells. H1-H4: Control, Con A, Con A + PTH-M, and Con A + DXM group, respectively; H5: Statistical analysis of CD4$^+$CD45RA$^-$CCR7$^-$ cell frequencies in these four groups. Data are representative images or the mean ± SEM (n = 8). $^a P < 0.05$ and $^b P < 0.01$ *vs* the control group; $^c P < 0.05$ and $^d P < 0.01$ *vs* the Concanavalin A group. ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.

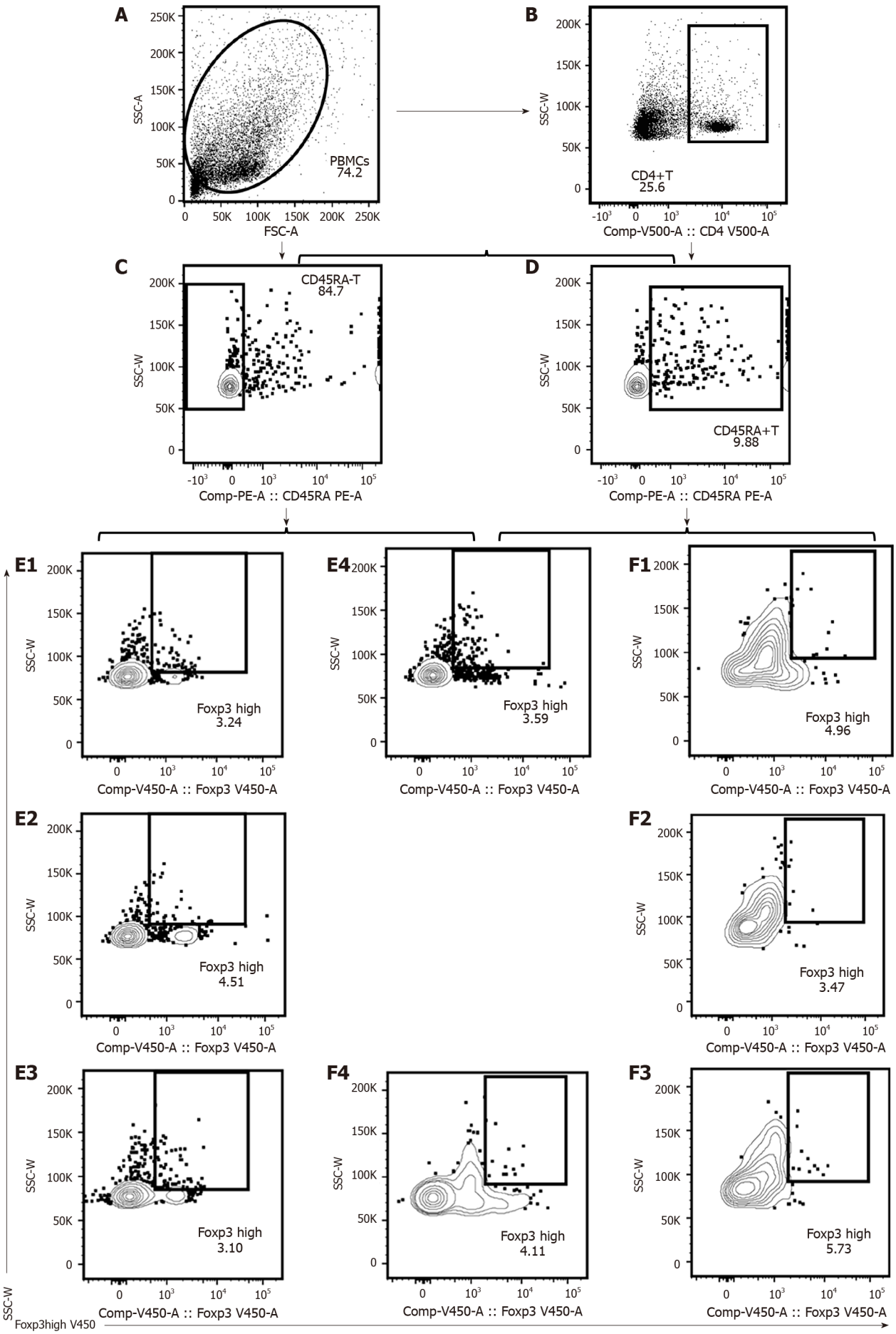
PTH maintains the intestinal barrier in AIH mice

In order to further study whether intestinal microflora and/or other pathogens (or immunocomplexes) can enter the liver through the injured intestinal microbiota barrier and whether PTH can treat AIH by protecting the intestinal microbiota barrier and preventing bacterial translocation, the intestinal microbiota permeability test was performed. In the present study, the colonic weight (Figure 10A), colonic weight index (Figure 10B), and colonic weight/colonic length (Figure 10C) of AIH mice in the Con A group were higher than in the control group ($P < 0.01$), while colonic weight (Figure 10A), colonic weight index (Figure 10B), and colonic weight/colonic length (Figure 10C) of AIH mice in the Con A + PTH-M group were lower than in the Con A group ($P < 0.05$). Also, colonic inflammation was more pronounced in the colonic mucosa and submucosa of AIH model mice than in the control group (Figure 10D). Compared with the untreated Con A group, histological colonic damage was improved following 10-d of PTH and DXM therapy, with fewer inflammatory cells in the colonic mucosa and submucosa (Figure 10D).

Occludin content in tight junction proteins may be an indicator of the intestinal barrier's permeability. The expression of occludin was reduced in the colon tissues of mice in the Con A group than in the control group (Figures 10E and F), while the expression of occludin was elevated in the colon tissues of mice in the Con A + PTH-M group than in the Con A group (Figures 10E and F). The above data suggest that PTH has a protective effect on the intestinal barrier.

PTH regulates TLR/CXCL16/CXCR6 /NF-κB signal activation in AIH mice

Emerging evidence has identified that TLRs are interrelated with autoimmune diseases by recognizing the change in gut microbiota and/or other pathogens (or immunocomplexes). Figures 9D-G show that in the treatment groups, there was a sizable association between the distribution of sample microflora and mTreg cells. The small intestine's epithelial cells are stimulated by *Lactiplantibacillus plantarum 22A-3* to secrete transforming growth factor-beta, and retinoic acid is likely produced through TLR2, resulting in an increase in CD103+ DCs and the Foxp3$^+$ Treg population[31], and the activation of TLR4 by lipopolysaccharide (LPS) leads to the induction of pro-inflammatory Th17 cells and reduces the immunosuppressive function of Treg cells[3,32]. Differentiation of Th17 and Treg cells is adjusted by NF-κB[33,34]. CXCL16 promotes the migration of immune cells to secondary lymphoid organs and inflammatory sites, and CXCL16 is also an effective direct activator of NF-κB. The findings of western blot analysis revealed that the proteins TLR4 (Figure 9G), NF-κB p65 (Figure 11C), CXCL16 (Figure 11D), and CXCR6 (Figure 11E) in the liver were highly expressed in AIH model mice *vs* the control group ($P < 0.01$), while the expression of TLR4 (Figure 9G), NF-κB p65 (Figure 11C), CXCL16 (Figure 11D), and


A
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FSC-A
PBMCs
74.2
B
SSC-W
CD4+T
25.6
Comp-V500-A :: CD4 V500-A
C
CD45RA-T
84.7
SSC-W
Comp-PE-A :: CD45RA PE-A
D
CD45RA+T
9.88
SSC-W
Comp-PE-A :: CD45RA PE-A
E1
Foxp3 high
3.24
SSC-W
Comp-V450-A :: Foxp3 V450-A
E4
Foxp3 high
3.59
SSC-W
Comp-V450-A :: Foxp3 V450-A
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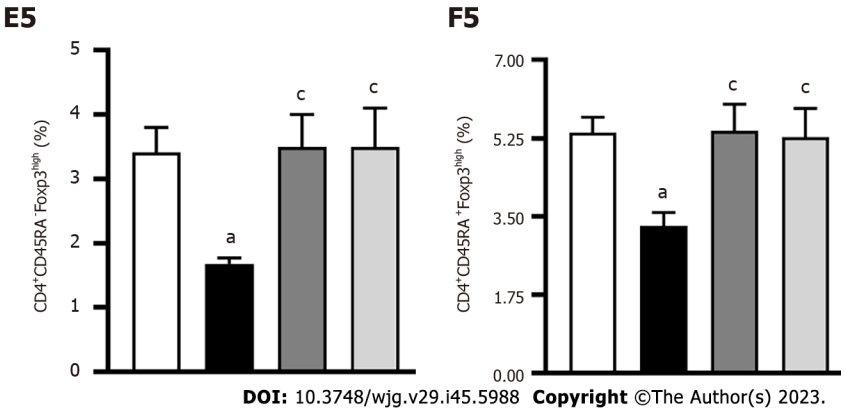

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Figure 7 Pien Tze Huang regulates the number of special memory regulatory T cells in mice with autoimmune hepatitis. A: Peripheral blood mononuclear cells; B: $CD4^+$ lymphocytes measured by flow cytometry; C: $CD45RA^-$ lymphocytes measured by flow cytometry; D: $CD45RA^+$ lymphocytes measured by flow cytometry; E: $CD4^+CD45RA^-Foxp3^{high}$ cells. E1-E4: Control, Concanavalin A (Con A), Con A + middle-dose Pien Tze Huang (PTH-M), and Con A + dexamethasone (DXM) group, respectively; E5: Statistical analysis of $CD4^+CD45RA^-Foxp3^{high}$ cell frequencies in these four groups; F: $CD4^+CD45RA^+Foxp3^{high}$ cells. F1-F4: Control, Con A, Con A + PTH-M, and Con A + DXM group, respectively; F5: Statistical analysis of $CD4^+CD45RA^+Foxp3^{high}$ cell frequencies in these four groups. Data are representative images or the mean ± SEM (n = 8). $^aP < 0.05$ and $^bP < 0.01$ *vs* the control group; $^cP < 0.05$ and $^dP < 0.01$ *vs* the Concanavalin A group. ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.

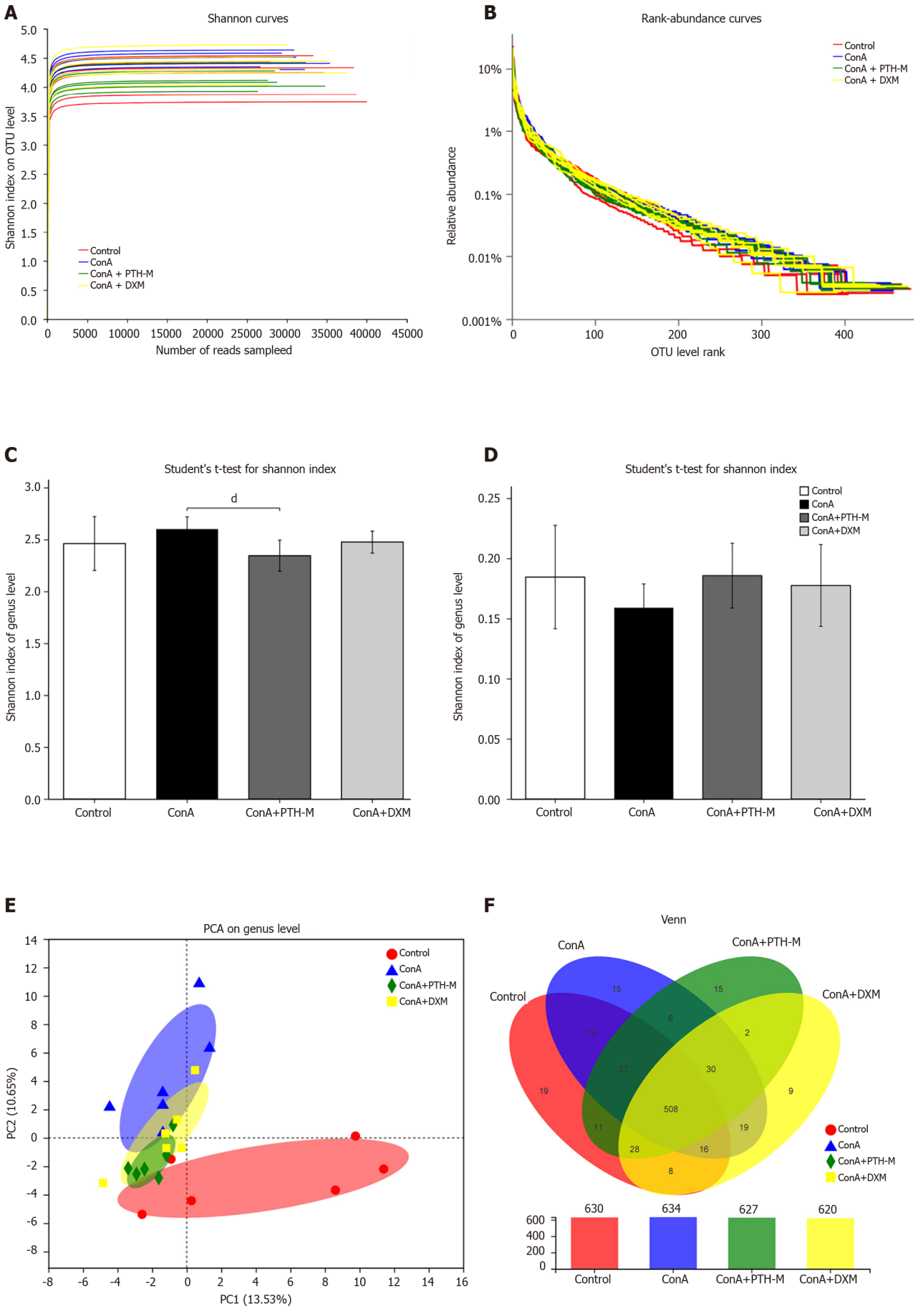
CXCR6 (Figure 11E) proteins were markedly decreased in mice in the Con A + PTH-M group than in mice in the Con A group ($P < 0.01$). However, the protein TLR2 (Figure 11B) in the liver was decreased in AIH model mice compared with the control group ($P < 0.01$). After PTH treatment, the protein TLR2 (Figure 11B) in the liver was lower in AIH model mice than in the Con A group ($P < 0.01$). Hence, these data suggest that PTH regulates Treg cell expression by inhibiting the NF-kB pathway and promoting TLR2 expression.

DISCUSSION

PTH has anti-inflammatory, neuroprotective, and immunoregulatory properties and is used in traditional Chinese medicine[10,35], which can reduce the serum homocysteine level and inhibit endoplasmic reticulum stress and the PERK/eIF2α pathway to reduce the severity of alcohol and high-fat diet in rats[36]. PTH can also inhibit apoptosis of intestinal crypt cells induced by 5-fluorouracil by inhibiting the ratio of Bax/Bcl-2, which can effectively reduce 5-fluorouracil-induced intestinal mucositis[37]. The above results suggest that PTH has the potential to treat immune liver injury and sustain the integrity of the gut mucosal barrier.

In order to further investigate the mechanism of PTH in the treatment of AIH, we analyzed the expression of various cytokines in AIH mice, and found that PTH treatment downregulated the expression of pro-inflammatory cytokines, such as IFN-γ, IL-1β, IL-2, IL-6, IL-21, and TNF-α, and upregulated the expression of anti-inflammatory factors, such as IL-10 in mice with Con A-induced HIA. IFN-γ[38]. TNF-α[39], IL-1β[40], IL-2[41], IL-21[42], and IL-6[43] promote autoimmune hepatic damage, whereas IL-10[44] protects the liver from damage. Thus, these data suggest that PTH could control the proportion of pro- and anti-inflammatory factors to improve Con A-induced AIH.

Previous research demonstrated a link between the poor prognosis of AIH and the low expression and functioning of Treg cells in the liver's inflammatory microenvironment[45-47]. It is known that mTreg cells can control memory effector reactions to induce differentiation and proliferation of Treg cells, and subsequently reduce collateral damage to tissues [48,49]. Treg cells have a significant role in adjusting the diversity of intestinal microbiota. They are also key cells that can inhibit intestinal inflammation, inhibit the abnormally activated immune response, and maintain intestinal immune tolerance[50]. When exposed to a foreign antigen again, TCM cells, which are long-lived T cells with the capacity for self-renewal and proliferation, can quickly develop into effector cells (TEM)[51]. Moreover, in mice lacking Foxp3 enhancer CNS1, *Firmicutes* were less abundant in the gut relative to the control group[52]. Additionally, Zhou *et al*[53] found that $Foxp3^+$ expression could be increased by PD-1/$PD\text{-}L1^+$ to improve its inhibitory effect. In the present research, the expression of $CD4^+CD25^+Foxp3^+PD\text{-}L1^+$, $CD4^+CD25^+Foxp3^+$ Treg, $CD4^+CD45RA^-Foxp3^{high}$, TCM, and TEM cells were lower in the Con A group than in control mice, while the expression of $CD4^+IL\text{-}17A^+$ Th17 cells was higher. These findings imply that the decreased PD-L1 expression in $CD4^+CD25^+Foxp3^+$ Treg cells downregulates the number of $CD4^+CD25^+Foxp3^+$ Treg cells. The decreased immune memory function leads to reduced differentiation of Treg cells and more activation of Th17 cell differentiation. The total number of Treg cells was insufficient to preserve immunological tolerance and stop pathogenic elements-such as heteroantigen and pathogenic bacteria-from continuing to have a milder immunosuppressive effect on $CD4^+CD25^+Foxp3^+$ Treg cells. Following PTH treatment for 10 d, the number of $CD4^+CD25^+Foxp3^+$ Treg and $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells was raised to maintain immune tolerance. In order to activate the immunosuppressive function and limit the severity of excessive immunological damage, PTH additionally enhanced the expression of PD-L1 in $CD4^+CD25^+Foxp3^+$ Treg cells and decreased the number of $CD4^+IL17A^+$ Th17 cells. Also, PTH effectively reduced Con A-induced AIH in mice. These findings imply that the equilibrium between Th17, mTreg, and


A
Shannon curves
Shannon index on OTU level
Number of reads sampleed
Control
ConA
ConA + PTH-M
ConA + DXM
B
Rank-abundance curves
Relative abundance
OTU level rank
C
Student's t-test for shannon index
Shannon index of genus level
d
Control
ConA
ConA+PTH-M
ConA+DXM
D
Student's t-test for shannon index
E
PCA on genus level
PC2 (10.65%)
PC1 (13.53%)
F
Venn
630
634
627
620

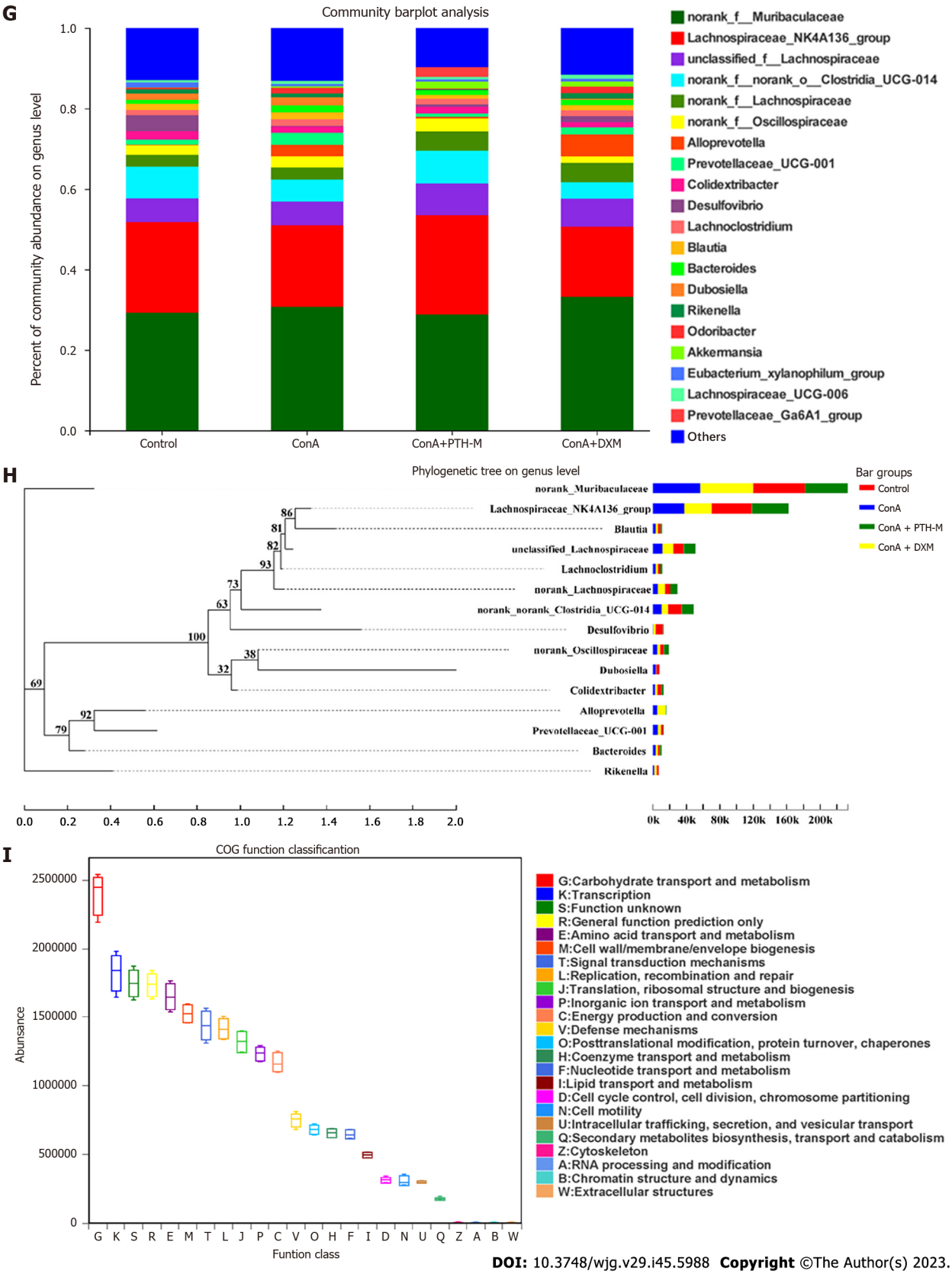


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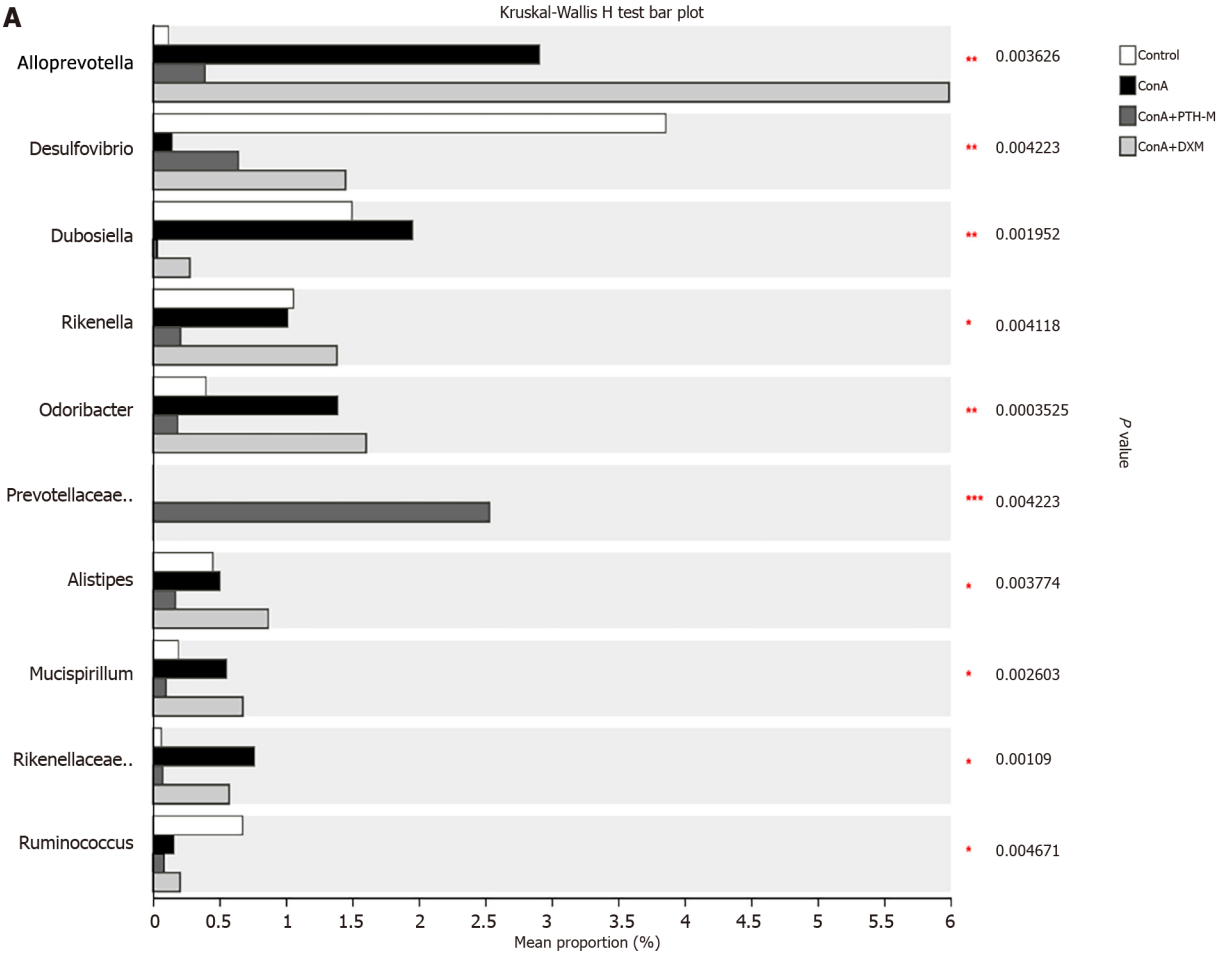
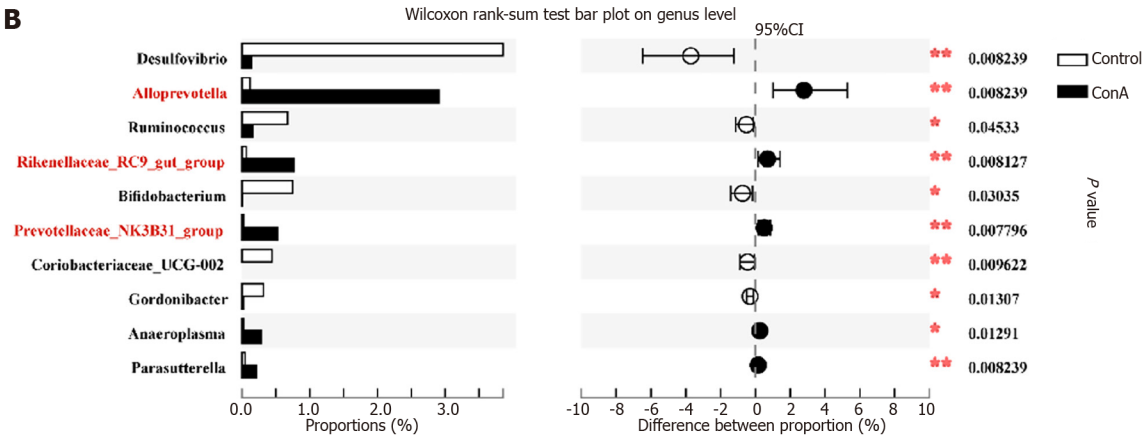
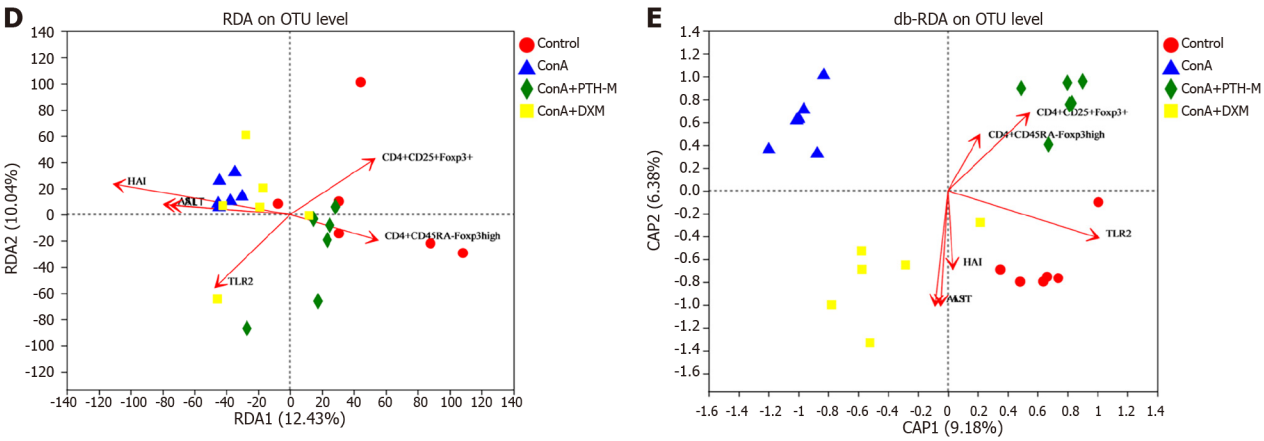
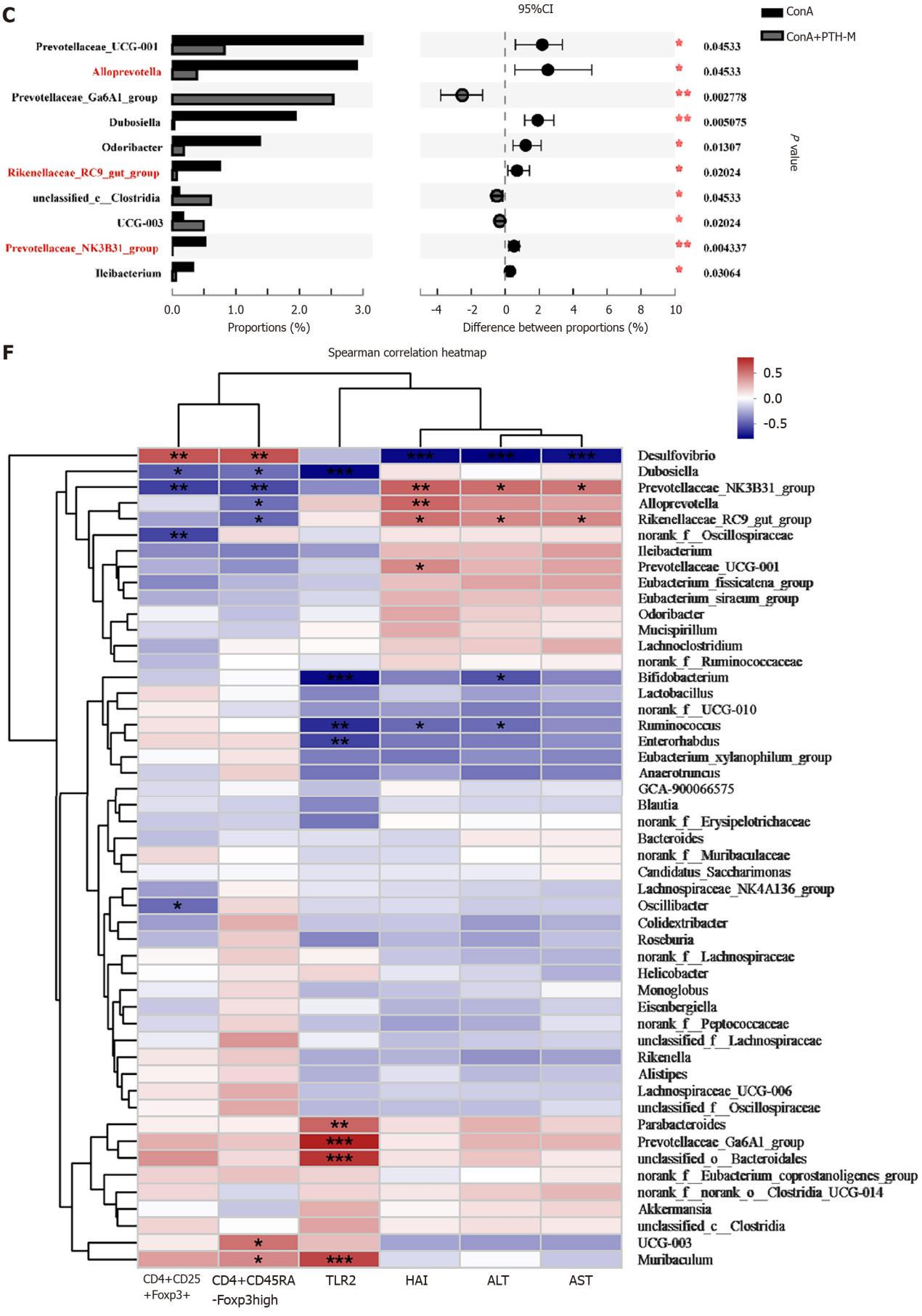
Figure 8 Pien Tze Huang improves the composition of gut microbiota in mice with autoimmune hepatitis. A: The rarefaction curve of the Shannon index at the operational taxonomic unit (OTU) level; B: Rank-Abundance curves at the OTU level; C: Shannon index at the genus level; D: Simpson index at the genus level; E: Partial least squares discriminant analysis score at the genus level; F: The Venn diagram depicts OTUs that differed in each group; G: Community composition bar chart at the genus level; H: Phylogenetic evolutionary tree at the genus level; I: COG function classification. Data are representative images or the mean ± SEM (n = 6). $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* the control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* the Concanavalin A group. ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.


A
Kruskal-Wallis H test bar plot
Alloprevotella
Desulfovibrio
Dubosiella
Rikenella
Odoribacter
Prevotellaceae..
Alistipes
Mucispirillum
Rikenellaceae..
Ruminococcus
0.003626
0.004223
0.001952
0.004118
0.0003525
0.004223
0.003774
0.002603
0.00109
0.004671
P value
Control
ConA
ConA+PTH-M
ConA+DXM
Mean proportion (%)


B
Wilcoxon rank-sum test bar plot on genus level
95%CI
Desulfovibrio
Alloprevotella
Ruminococcus
Rikenellaceae_RC9_gut_group
Bifidobacterium
Prevotellaceae_NK3B31_group
Coriobacteriaceae_UCG-002
Gordonibacter
Anaeroplasma
Parasutterella
0.008239
0.008239
0.04533
0.008127
0.03035
0.007796
0.009622
0.01307
0.01291
0.008239
P value
Control
ConA
Proportions (%)
Difference between proportion (%)


D
RDA on OTU level
Control
ConA
ConA+PTH-M
ConA+DXM
CD4+CD25+Foxp3+
CD4+CD45RA-Foxp3high
HAI
ALT
TLR2
RDA2 (10.04%)
RDA1 (12.43%)
E
db-RDA on OTU level
CD4+CD25+Foxp3+
CD4+CD45RA-Foxp3high
TLR2
HAI
AST
CAP2 (6.38%)
CAP1 (9.18%)


C
95%CI
ConA
ConA+PTH-M
Prevotellaceae_UCG-001
Alloprevotella
Prevotellaceae_Ga6A1_group
Dubosiella
Odoribacter
Rikenellaceae_RC9_gut_group
unclassified_c__Clostridia
UCG-003
Prevotellaceae_NK3B31_group
Ileibacterium
0.04533
0.04533
0.002778
0.005075
0.01307
0.02024
0.04533
0.02024
0.004337
0.03064
P value
0.0
1.0
2.0
3.0
Proportions (%)
-4
-2
0
2
4
6
8
10
Difference between proportions (%)
F
Spearman correlation heatmap
0.5
0.0
-0.5
Desulfovibrio
Dubosiella
Prevotellaceae_NK3B31_group
Alloprevotella
Rikenellaceae_RC9_gut_group
norank_f__Oscillospiraceae
Ileibacterium
Prevotellaceae_UCG-001
Eubacterium_fissicatena_group
Eubacterium_siraeum_group
Odoribacter
Mucispirillum
Lachnoclostridium
norank_f__Ruminococcaceae
Bifidobacterium
Lactobacillus
norank_f__UCG-010
Ruminococcus
Enterorhabdus
Eubacterium_xylanophilum_group
Anaerotruncus
GCA-900066575
Blautia
norank_f__Erysipelotrichaceae
Bacteroides
norank_f__Muribaculaceae
Candidatus_Saccharimonas
Lachnospiraceae_NK4A136_group
Oscillibacter
Colidextribacter
Roseburia
norank_f__Lachnospiraceae
Helicobacter
Monoglobus
Eisenbergiella
norank_f__Peptococcaceae
unclassified_f__Lachnospiraceae
Rikenella
Alistipes
Lachnospiraceae_UCG-006
unclassified_f__Oscillospiraceae
Parabacteroides
Prevotellaceae_Ga6A1_group
unclassified_o__Bacteroidales
norank_f__Eubacterium_coprostanoligenes_group
norank_f__norank_o__Clostridia_UCG-014
Akkermansia
unclassified_c__Clostridia
UCG-003
Muribaculum
CD4+CD25+Foxp3+
CD4+CD45RA-Foxp3high
TLR2
HAI
ALT
AST

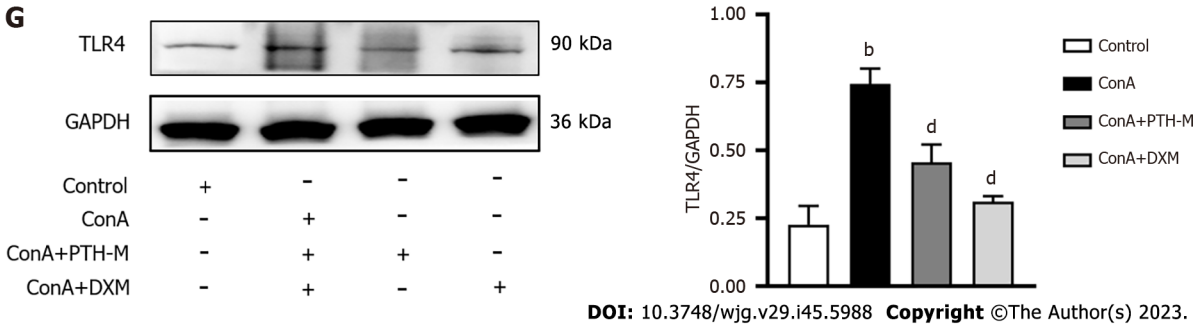


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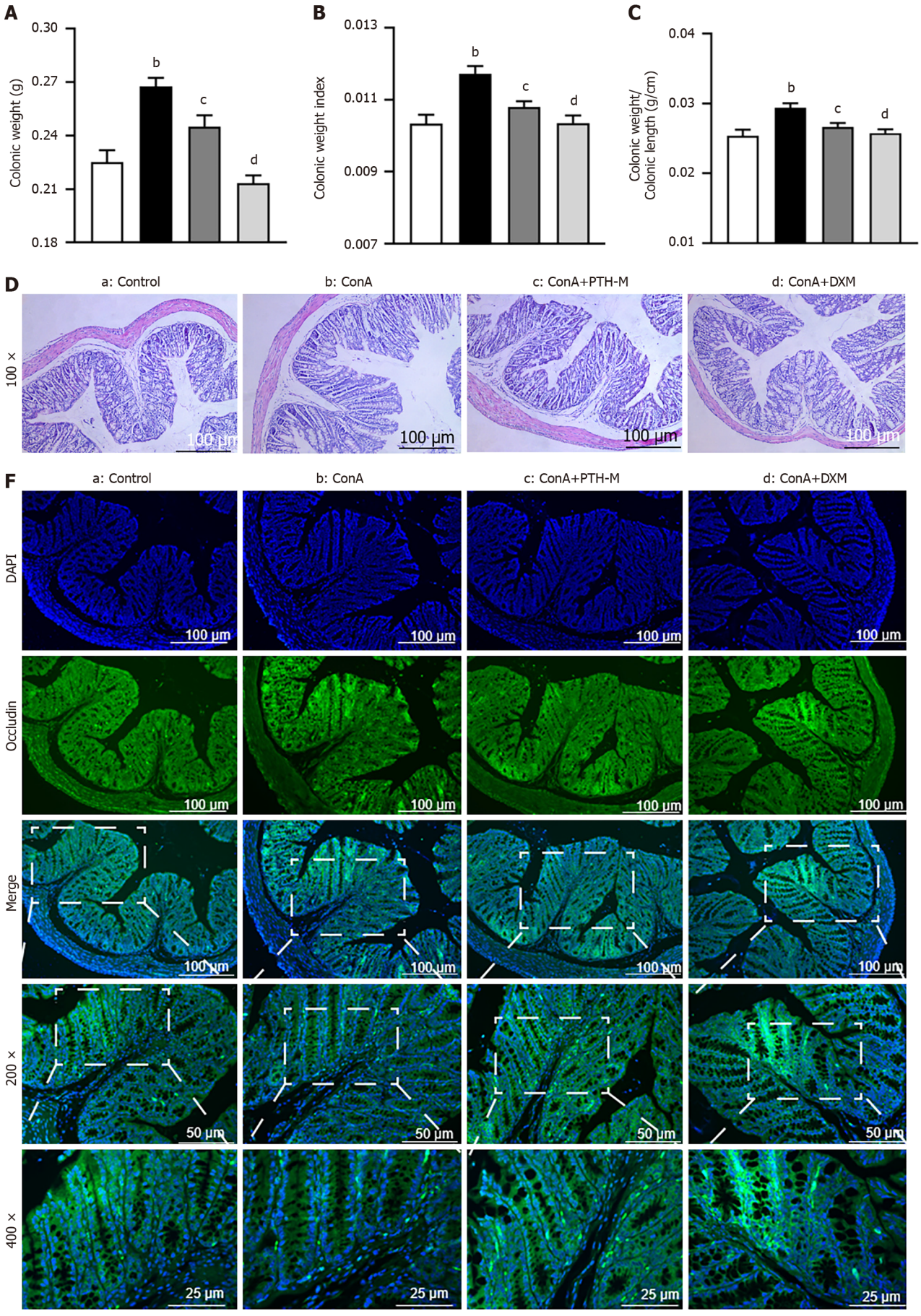
Figure 9 Species differences in intestinal microbiota and correlation analysis. A: Differential analysis among the four groups at the genus level; B: Phylotypes significantly different between the control and Concanavalin A (Con A) groups at the genus level; C: Phylotypes significantly different between Con A and Con A + middle-dose Pien Tze Huang groups at the genus level; D: Redundancy analysis of relationship between toll-like receptor (TLR)4, aspartate aminotransferase (AST), alanine aminotransferase (ALT), HAI, $CD4^+CD25^+Foxp3^+$, $CD4^+CD45RA^-Foxp3^{high}$ and intestinal microbiota; E: Distance-based redundancy analysis of relationship between TLR4, AST, ALT, HAI, $CD4^+CD25^+Foxp3^+$(regulatory T cells) cells, $CD4^+CD45RA^-Foxp3^{high}$ (memory regulatory T cells) cells, and intestinal microbiota; F: Spearman's correlation heatmap of TLR4, AST, ALT, HAI, $CD4^+CD25^+Foxp3^+$, $CD4^+CD45RA^-Foxp3^{high}$ and intestinal microbiota; G: The quantitative analysis of TLR4 protein expression. Data are representative images or the mean ± SEM (n = 6). $^aP < 0.05$ and $^bP < 0.01$ *vs* the control group; $^cP < 0.05$ and $^dP < 0.01$ *vs* the Concanavalin A group. ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.

Treg cells may be controlled by PTH to provide its therapeutic impact in mice with Con A-induced AIH.

It is widely recognized that an imbalance in intestinal microbiota is a key trait and pathogenic component of AIH. Intestinal bacteria and/or other pathogens (or immunocomplexes) can enter the liver through the damaged gut mucosal barrier. PTH has a protective role in the liver of mice with Con A-induced AIH by encouraging the growth of particular helpful bacteria and repressing the growth of some potentially harmful bacteria. In this study, lower abundances of *Alloprevotella*, *Prevotellaceae-UCG-001*, *Dubosiella,* and *Odoribacter*, and higher abundances of *Lachnospiraceae-NK4A136*, *norank-f-norank-o-Clostridia-UCG-014,* and *Desulfovibrio* were seen in the PTH group. Moreover, psyllium elicits a broader microbial shift, increasing the abundance of *Parasutterella* and *Akkermansia*, and decreasing *Enterorhabdus* and *Odoribacter* [54]. Chen *et al*[55] found that chondroitin sulfate can ameliorate diet-induced insulin resistance, improve non-alcoholic fatty liver disease *via* the AKT pathway, and modulate gut microbiota composition, increasing the abundance of *Desulfovibrio* and the levels of hydrogen sulfide (H2S). *Lachnospiraceae_NK4A136*, *Parabacteroides,* and *Clostridium* likely strengthened the availability of *Bifidobacterium pseudocatenulatum LI09* against D-GalN-induced rat liver injury[56]. In this study, species differences and correlation analysis of intestinal microbiota showed that PTH could reduce the abundance of *Bacteroides-caecimuris* and *uncultured-bacterium-g-Prevotellaceae-NK3B31*-group and increase the abundance of *unclassified-g-norank-f-Muribaculaceae*. Simultaneously, correlation analysis showed that *Desulfovibrio* was negatively related to HAI, AST, ALT, TLR4, and positively related to $CD4^+CD25^+Foxp3^+$ Treg and $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells; *Prevotellaceae-NK3B31*-group was positively related to HAI, AST, ALT, TLR4, and negatively related to $CD4^+CD25^+Foxp3^+$ Treg and $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells; *Rikenellaceae-RC9*-gut-group was positively related to HAI, AST, ALT, and negatively related to $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells; *Dubosiella* was negatively related to $CD4^+CD25^+Foxp3^+$ Treg and $CD4^+CD45RA^-Foxp3^{high}$ mTreg cells.

The destruction of intestinal mucosa was the main reason for induced bacterial translocation. Tight junction proteins have a prominent role in maintaining the intestinal barrier. Consequently, it is particularly important to reverse the deterioration of the intestinal barrier, bacterial translocation, and/or other pathogens (or immunocomplexes) to the liver. The present results showed that Con A increases intestinal permeability in mice by decreasing occludin protein expression; intestinal microbiota and/or other pathogens (or immunocomplexes) arrive at the liver through the portal vein to induce AIH. In order to treat mice with Con A-induced AIH, PTH controlled the interreaction between the intestinal microbiota and the number of mTreg cells.

Correlation analysis of environmental factors showed that Treg/mTreg cells were positively correlated with intestinal microbiota, and TLR4 was negatively correlated with intestinal microbiota. Treg/mTreg cells could affect the intestinal microbiota, which in turn could stimulate pattern recognition receptors on the surface of immune cells or metabolites of flora to affect Treg cells. The immune system can sense the intestinal microbiota through the TLRs mechanism[57]. Treg cells mainly express TLR1, TLR2, TLR4, TLR5, and TLR8. It has been demonstrated that the stimulation of TLR2 by the intestinal microbiota or polysaccharide A from *Bacteroides fragilis* plays a defensive role in the gut, lowering inflammation by raising IL-10 production by dendritic and B cells and encouraging Treg proliferation[58]. Moreover, the activation of TLR4 by LPS causes the emergence of pro-inflammatory Th17 cells and reduces the immunosuppressive function of Treg cells[3]. Lai *et al*[59] found that indirubin downregulates LPS-induced inflammation by blocking TLR4 and NF-κB and MAPK activation, which is used to treat mastitis. Studies have also suggested that CXCL16/CXCR6 are elevated in some autoimmune diseases. Treg cells could be inhibited by CXCL16[60]; LPS was discovered to enhance the transcription of CXCL16 *via* the NF-κB signaling pathway, thus promoting the pathogenesis of acute lung injury[61]. In the current study, TLR4, NF-κB p65, CXCR6, and CXCL16 proteins in the liver were markedly expressed in AIH mice, and the protein TLR2 in the liver showed low expression. However, after PTH treatment, the expression of TLR4, NF-κB p65, CXCR6, and CXCL16 was significantly reduced, and TLR2 protein was significantly increased. However, it is still unclear which precise PTH constituents collectively control the interaction between the gut microbiota, TLR/CXCL16/CXCR6/NF-κB signal pathway, and mTreg cells, and which specific bacterium and their metabolites would be significantly enhanced or


A
Colonic weight (g)
0.30
0.27
0.24
0.21
0.18
b
c
d
B
Colonic weight index
0.013
0.011
0.009
0.007
b
c
d
C
Colonic weight/ Colonic length (g/cm)
0.04
0.03
0.02
0.01
b
c
d
D
a: Control
b: ConA
c: ConA+PTH-M
d: ConA+DXM
100 ×
100 μm
F
a: Control
b: ConA
c: ConA+PTH-M
d: ConA+DXM
DAPI
Occludin
Merge
200 ×
400 ×
100 μm
50 μm
25 μm

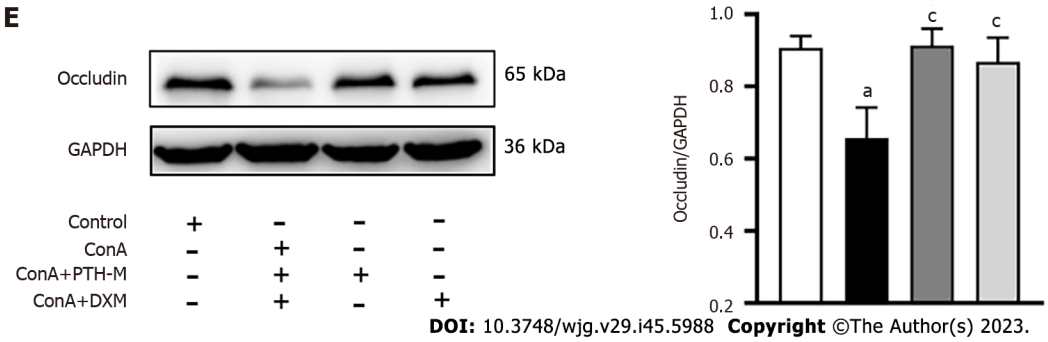


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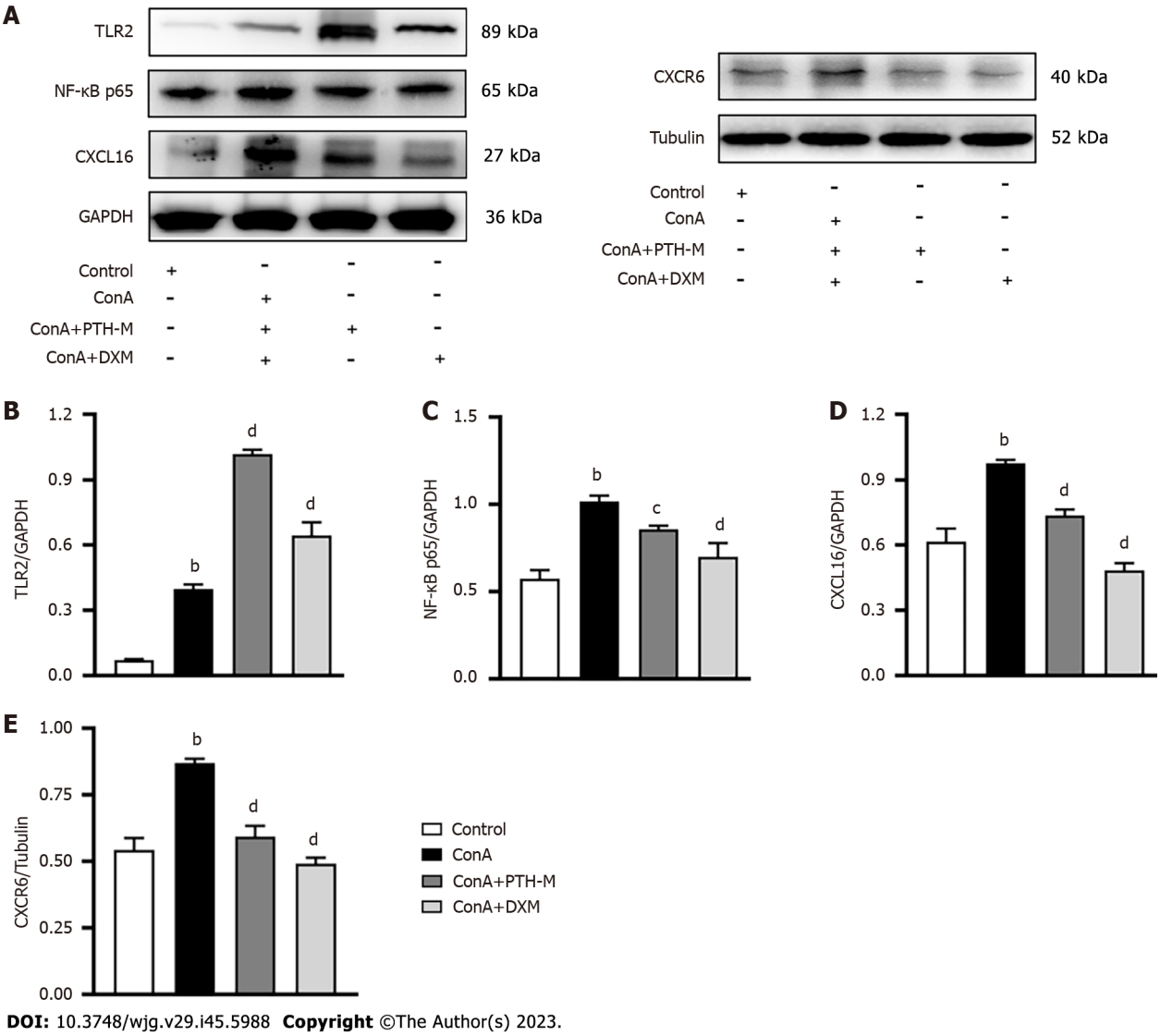
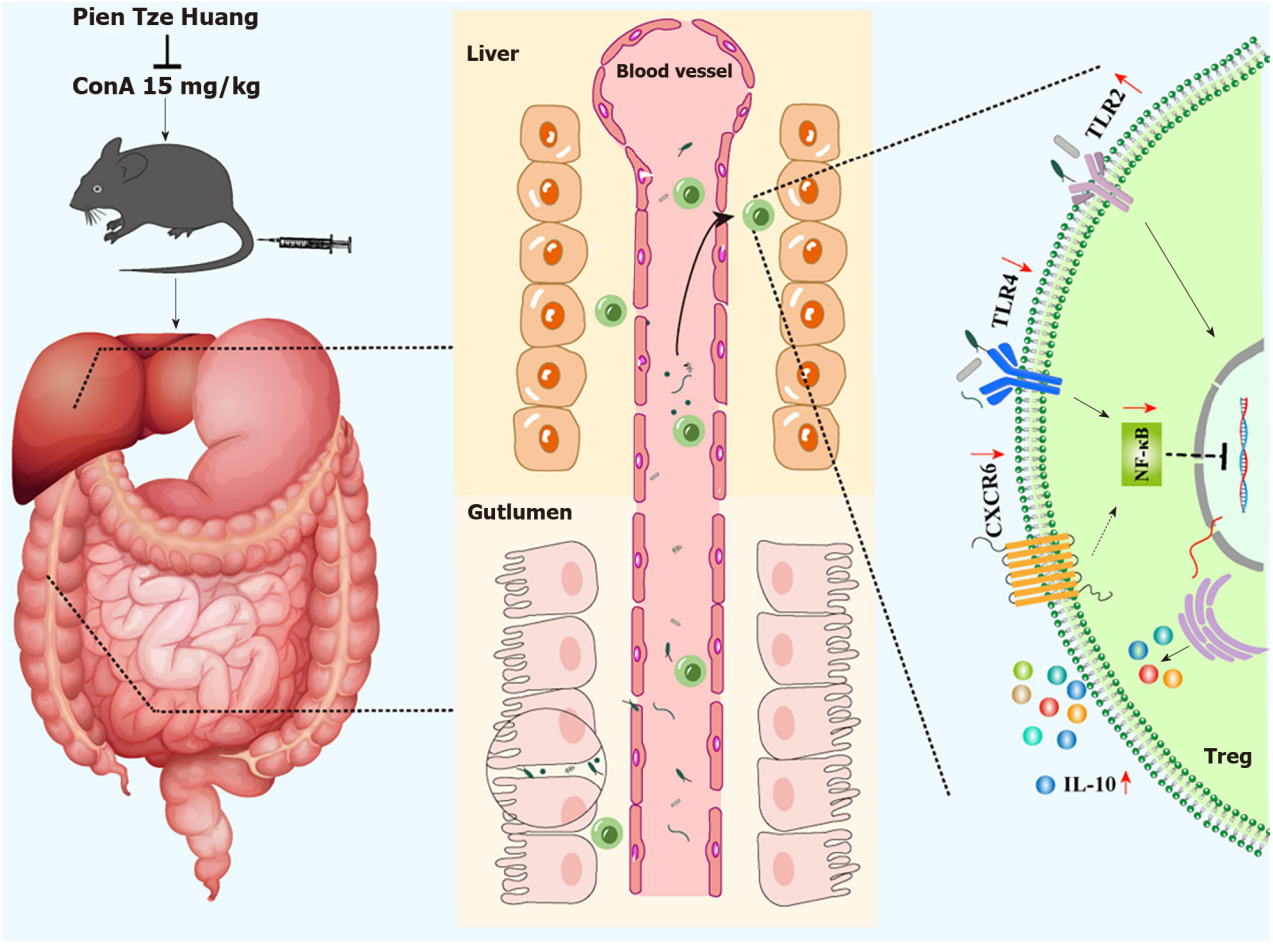
Figure 10 Pien Tze Huang restores the function of the intestinal barrier in mice with autoimmune hepatitis. A: Colonic weight; B: Colonic weight index; C: Colonic weight/Colonic length; D: Colonic hematoxylin and eosin staining; E: The quantitative analysis of occludin protein expression; F: Representative images of occludin in the colon (IF, 200 ×, 400 ×). Data are representative images or the mean ± SEM (n = 8). [a]P < 0.05 and [b]P < 0.01 *vs* the control group; [c]P < 0.05 and [d]P < 0.01 *vs* the Concanavalin A group. ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.



DOI: 10.3748/wjg.v29.i45.5988

Figure 11 Pien Tze Huang regulates the expression of toll-like receptor 2, nuclear factor-κB p65, CXCR6, and CXCL16 in mice with autoimmune hepatitis. A: The protein expression of toll-like receptor (TLR)2, nuclear factor-κB (NF-κB) p65, CXCR6, and CXCL16 analyzed by western blot; B: Quantitative analysis of TLR2; C: Quantitative analysis of NF-κB p65; D: Quantitative analysis of CXCL16; E: Quantitative analysis of CXCR6. Data are representative images or the mean ± SEM (n = 8). [a]P < 0.05 and [b]P < 0.01 *vs* the control group; [c]P < 0.05 and [d]P < 0.01 *vs* the Concanavalin A group. TLR: Toll-like receptor; NF-κB: Nuclear factor-κB; ConA: Concanavalin A; PTH: Pien Tze Huang; DXM: Dexamethasone.



DOI: 10.3748/wjg.v29.i45.5988

Figure 12 Protective mechanisms of Pien Tze Huang against autoimmune hepatitis. TLR: Toll-like receptor; NF-κB: Nuclear factor-κB; ConA: Concanavalin A; IL: Interleukin.

have a significant role in PTH treatment of AIH mice.

CONCLUSION

In conclusion, these findings suggest that PTH controls interactions between mTreg cells and intestinal microbiota to reduce experimental AIH. The effect of PTH was similar to that of DXM; yet, in the PTH group, there was obvious immunoregulation. PTH may enhance the colon barrier effect and the variety of intestinal microbiota, in order to prevent the movement of potentially hazardous intestinal substances to the liver, suppression of the TLR4/NF-κB, CXCL16/CXCR6 pathway, and activation of the TLR2 pathway could increase the expression of mTreg cells to secrete more Treg cells, thus limiting the expression of Th17 cells, reducing the creation of inflammatory substances, and achieving the purpose of treating AIH. These results suggest that PTH may balance intestinal microbiota and interfere with mTreg cells to regulate differentiated Treg cells, which is a natural alternative therapy for AIH (as shown in Figure 12).

Although our studies hoped to determine the mechanism of PTH treatment in mice with AIH, as a compound preparation with multiple and complex components, the definite effective ingredients of PTH in the treatment of AIH are ambiguous. Although specific bacteria had interactions with mTreg cells, these interactions were not defined. Crosstalk between the gut microbiota, mTreg cells, the TLR/CXCL16/CXCR6/NF-κB signaling pathway and PTH requires further investigation in future studies. It is very fortunate that PTH regulates intestinal microbiota balance and restores mTreg cells to alleviate experimental AIH, which is closely related to the TLR/CXCL16/CXCR6/NF-κB signaling pathway. These results will have directive significance and help to determine more immunomodulatory effects of PTH, to widen the scope of the clinical application of PTH treatment in AIH, and to develop new drugs related to PTH. In the future, we will plan to separate and co-cultured mTreg cells with PTH, or culture medium from PTH and the specific bacteria using a transwell microfluidic control chip, and to compare the effect of the inhibitor or agonist of the TLR/CXCL16/CXCR6/NF-κB signaling pathway on mTreg cells, and ultimately discover the definite action target and effective ingredients of PTH in the treatment of AIH.

ARTICLE HIGHLIGHTS

Research background

Autoimmune hepatitis (AIH) is a common and frequently-occurring interfacial hepatitis, and its etiology remains unknown. The interaction between regulatory T (Treg) cells and intestinal microbiota may provide a feasible therapeutic strategy for AIH.

Research motivation

As a well-known traditional Chinese medicine, Pien Tze Huang (PTH) is regularly used to treat liver conditions, including AIH. PTH has a physiological basis for controlling Treg cells and intestinal microbiota. However, it is unknown whether PTH can regulate intestinal microbiota and memory Treg (mTreg) cells to treat AIH.

Research objectives

To explore the mechanism of PTH treated AIH by determining the changes in the structure of intestinal microbiota and the level of mTreg cells.

Research methods

Following establishment of the mouse model of Concanavalin A (Con A)-induced AIH, prophylactic administration of PTH was given for 10 d. The levels of mTreg cells were measured by flow cytometry, and intestinal microbiota was analyzed by 16S rDNA analysis, while western blotting was used to identify the activation of the Toll-like receptor (TLR)2, TLR4/nuclear factor-κB (NF-κB), and CXCL16/CXCR6 signaling pathways.

Research results

In the present study, after PTH administration in mice with AIH, the expression of pro-inflammatory factors [such as tumor necrosis factor-alpha, interferon-γ, interleukin (IL)-1β, IL-2, IL-6, and IL-21] and Th17 cells was decreased, followed by improved pathological liver injury. In addition, the number of Treg/mTreg cells and IL-10 level were markedly increased, and the diversity of intestinal microbiota was regulated. Furthermore, activation of the TLR4/NF-κB, CXCL16/CXCR6 signaling pathway was suppressed in hepatic tissue.

Research conclusions

PTH effectively relieved pathological liver injury in mice with Con A-induced AIH, which was realized by improving intestinal microbiota balance and mTreg levels, and inhibiting the TLR/CXCL16/CXCR6/NF-κB signaling pathway.

Research perspectives

This research indicated that the mechanism of PTH treated mice with AIH was closely related to the regulation of intestinal microbiota balance and improving mTreg cell levels. These results provide scientific evidence for the application of PTH in AIH treatment and for the development of new drugs for AIH.

FOOTNOTES

Author contributions: Zeng X and Liu MH contributed to methodology, visualization, and validation of this study; Zeng X, Liu MH, Xiong Y, Zheng LX, and Guo KE were involved in the investigation of this manuscript; Xiong Y, Yin YT, Liu DY, and Zhou BG participated in the supervision of this manuscript; Xiong Y, Zhao HM, Yin YT, Liu DY, and Zhou BG contributed to the project administration and funding acquisition; Zhou BG and Yin YT contributed equally to this work as co-corresponding authors to collectively design, perform, analyze and complete the study and the paper, and to contribute efforts of equal substance throughout the research process.

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Institutional animal care and use committee statement: All experiments on animals (including the euthanasia of mice) were performed in accordance with the Institutional Animal Care and Use Committee's guidelines (Nanchang, China). The experimental protocols (Permit Number: JZLLSC20210090) were approved by The Jiangxi University of Chinese Medicine's Animal Care and Use Committee.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to

the ARRIVE guidelines.

Country/Territory of origin: China

ORCID number: Hai-Mei Zhao 0000-0001-8767-5251; Duan-Yong Liu 0000-0003-2855-2811; Bu-Gao Zhou 0009-0002-9138-7206.

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LETTER TO THE EDITOR

Intestinal barrier in inflammatory bowel disease: A bibliometric analysis

Wen-Yu Luan, Zhe Yang, Xiao-Dong Chen, Tong-Tong Zhang, Fang Zhang, Yan-Dong Miao

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Wen-Yu Luan, Zhe Yang, Xiao-Dong Chen, Tong-Tong Zhang, Fang Zhang, Yan-Dong Miao, Cancer Center, Yantai Affiliated Hospital of Binzhou Medical University, The 2nd Medical College of Binzhou Medical University, Yantai 264100, Shandong Province, China

Corresponding author: Yan-Dong Miao, MD, Associate Chief Physician, Associate Professor, Cancer Center, Yantai Affiliated Hospital of Binzhou Medical University, The 2nd Medical College of Binzhou Medical University, No. 717 Jinbu Street, Muping District, Yantai 264100, Shandong Province, China. miaoyd_22@bzmc.edu.cn

Abstract

The primary objective of this investigation was to examine the evolving trajectories and pivotal focal points within the domain of research on intestinal barriers with regard to inflammatory bowel disease (IBD). Publications germane to the intestinal barrier in the context of IBD were procured from the Science Citation Index Expanded within the Web of Science Core Collection database. Bibliometric scrutiny and visualization were executed employing the R package "bibliometrix" through the R software platform (version: 4.3.0). A comprehensive compilation of 7344 English-language articles spanning from January 1, 2001 to December 31, 2021 was meticulously identified and included in the analysis. Remarkably, China emerged as the preeminent force in the realm of intestinal barrier research in relation to IBD. The significance of the intestinal barrier in the context of IBD has been progressively and comprehensively acknowledged. This recognition has ushered in a fresh therapeutic perspective that offers the promise of enhancing the management of inflammation and prognostication.

Key Words: Intestinal barrier; Inflammatory bowel disease; Bibliometric analysis; Bibliometrix; Gastroenterology

Core Tip: The intricate architecture and operation of the intestinal mucosal barrier assume a pivotal role in maintaining the delicate equilibrium of both physiological and immune processes within the confines of the intestinal milieu. This study employed advanced bibliometric methodologies to investigate evolving paradigms and conspicuous research domains within the ambit of research on intestinal barriers with a specific focus on inflammatory bowel disease. The results reveal swift progress within the realm of intestinal barrier research characterized by a proliferation of expansive collaborative endeavors.

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TO THE EDITOR

We have recently examined the scholarly work authored by Zhou *et al*[1] titled "Intestinal barrier in inflammatory bowel disease (IBD): A bibliometric and knowledge-map analysis". The primary objective of this study was to consider the evolving trends and focal points within the realm of intestinal barrier research in the context of IBD. Given that barrier surfaces, which are composed of specialized epithelial cells, serve as the critical demarcation between the host's internal milieu and the external environment and are indispensable for the preservation of proper physiological and immunological equilibrium, we acknowledge the profound significance of this investigation[2-4]. Nevertheless, we wish to proffer a series of thoughtful recommendations pertaining to the information retrieval methodologies employed in this study.

First and foremost, within the domain of bibliometric inquiries, the meticulous formulation of search strategies assumes paramount importance. The authors have duly noted their utilization of the Web of Science Core Collection (WoSCC) as the primary source of original data, a judicious choice in our estimation. However, it is worth noting that WoSCC encompasses at least 10 subdatabases, including the Science Citation Index Expanded (SCI-Expanded), Social Sciences Citation Index, Emerging Sources Citation Index, Arts and Humanities Citation Index, Conference Proceedings Citation Index-Science, Current Chemical Reactions, Conference Proceedings Citation Index-Social Science and Humanities, Book Citation Index-Science, Index Chemicus, and Book Citation Index-Social Sciences and Humanities. In our view, it may not be appropriate to incorporate all of these subdatabases for the retrieval of eligible articles. For instance, utilizing the retrieval formula employed by the authors, no relevant studies could be located in AHCI, CCR-EXPANDED, and IC[5,6]. Consistent with this perspective, some scholars also contend that it is inappropriate to employ such a diverse array of databases that vary in type and level within a singular bibliometric analysis[7,8]. Among these, SCI-Expanded stands out as the most fitting and widely accepted choice for conducting bibliometric analyses[9,10].

Another pivotal aspect that warrants careful consideration pertains to the appropriateness of topic search (TS) for bibliometric endeavors. The "TS" approach, by design, designates a publication as relevant when the search term appears in the "Title (TI)," "Abstract (AB)," "Author keywords (AK)," or "Keywords plus (KP)." It is imperative to determine that "KP" is generated through WoSCC's automated computational algorithms independent of author input. Consequently, the inclusion of "KP" during the search process may inadvertently include a multitude of extraneous publications[11]. In our experience, the prudent course of action involves relying on "TI," "AB," and "AK" as qualifiers to ensure a more precise and semantically meaningful dataset for bibliometric analysis.

Furthermore, it is of paramount importance to recognize that the effectiveness of a search formula hinges on its comprehensiveness because an overly simplistic approach may unintentionally omit pertinent publications. In the study by Zhou *et al*[1], the authors' reliance on the phrases "IBD" OR "ulcerative colitis" OR "Crohn's disease" is insufficient for capturing the entirety of relevant research. Additionally, in Zhou *et al*'s study, the publication period spanned from 2001 to 2021[1]; however, the exact commencement and conclusion dates were not provided. Moreover, there is some inconsistency in the description of the screening timeframe, with the Methods section indicating 2001-2021 while the AB and results sections mention 2002-2022. Hence, further clarification regarding the precise timeframe for the inclusion of literature is warranted.

As part of our contribution to refining the retrieval strategy, we posit that it is prudent to incorporate synonymous terms and nomenclature associated with "IBD". In our opinion, the authors should additionally incorporate the subsequent terms into the search equation: "Intestinal inflammation" and "gut inflammation." Similarly, for "intestinal barrier," the search formula included "intestinal barrier", "gut barrier", "mucosal barrier," "intestinal permeability", "epithelial barrier", "intestinal tight junction", "intestinal epithelium", and "intestinal integrity". Furthermore, numerous terms exhibit both plural and singular variations. The authors could consider employing various wildcards, such as "*", which signifies that it can replace any number of characters. For instance, "IBD*" would return the terms "IBD" and "IBDs". The flowchart of the literature screening is indicated in Figure 1. Our suggested retrieval formula is summarized in Supplementary material.

The refined search query, incorporating a more extensive array of relevant terminologies, undeniably facilitated a more exhaustive exploration of the pertinent literature. Our search, conducted from January 1, 2001 to December 31, 2021, retrieved a total of 9831 records. After the careful exclusion of literature of diverse types and non-English studies, we ultimately preserved 7344 articles for our analysis. The annual publication trends are elucidated in Figure 2 and the corresponding author's countries and country scientific production are manifested in Figure 3.

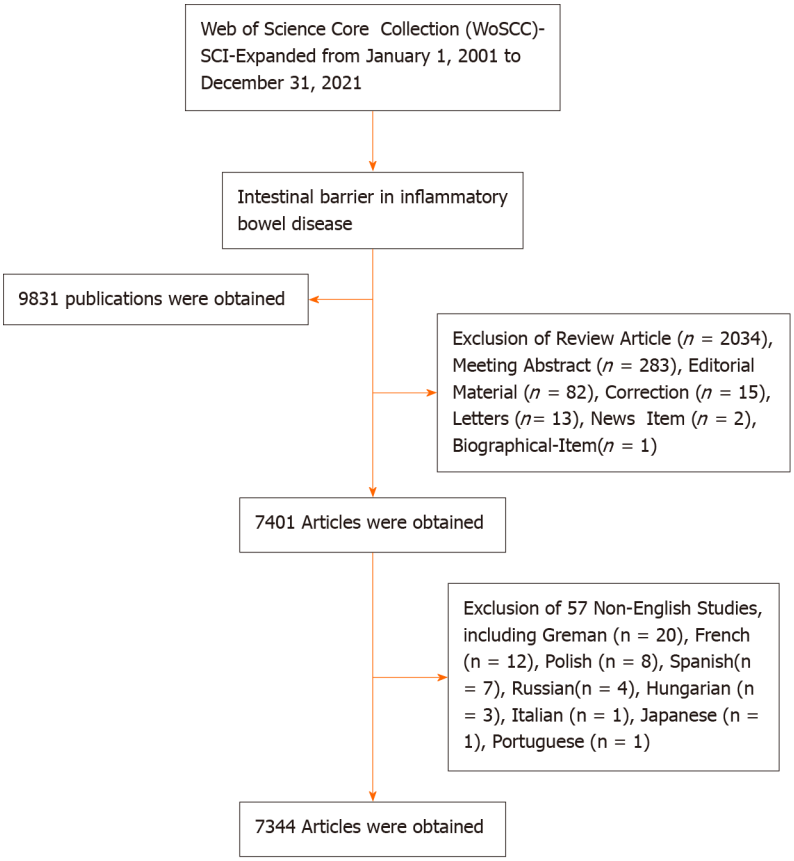

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Figure 1 Flowchart of literature screening.

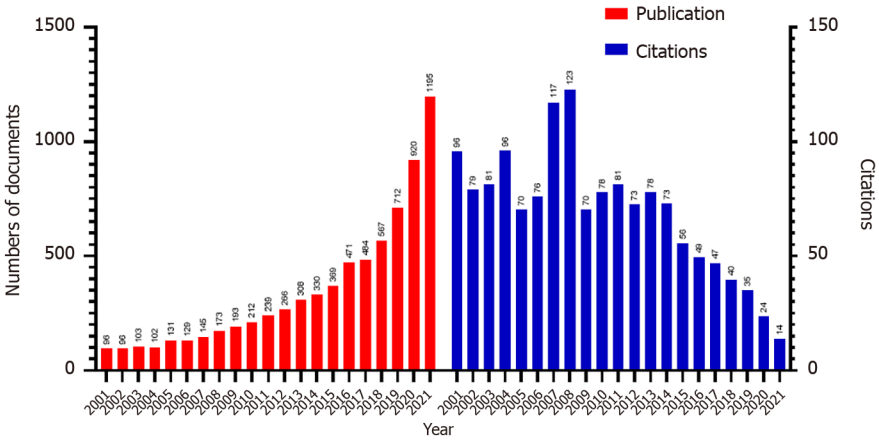

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Figure 2 Annual publication trends and country publications.

In contrast to the findings presented by Zhou *et al*[1], our study, which used a more refined retrieval scope focusing on "TI/AK/AB," identified a larger pool of related studies on the intestinal barrier in IBD (7344 *vs* 4482). It is noteworthy that such a substantial change in the number of publications can exert a significant impact on various quantitative data points, including those related to the most prolific countries (as depicted in Figure 3), institutions, and authors. Therefore, to mitigate potential bias, it is imperative to carefully formulate an appropriate retrieval formula when conducting bibliometric analyses. From our perspective, seeking expert consultation to tailor search keywords to the specific domain of study is an essential and prudent step.

It is crucial to underscore that the significant fluctuations in publication numbers can wield a profound influence on various quantitative aspects, including publication counts, citations, the most prolific countries/regions, institutions, authors, cited authors, journals, cited academic journals, keyword co-occurrence, clusters, and bursts. This underscores the critical importance of meticulously devising an appropriate retrieval formula, which serves as the bedrock for unbiased bibliometric analysis. In our considered view, soliciting expert consultation on search keywords tailored to the

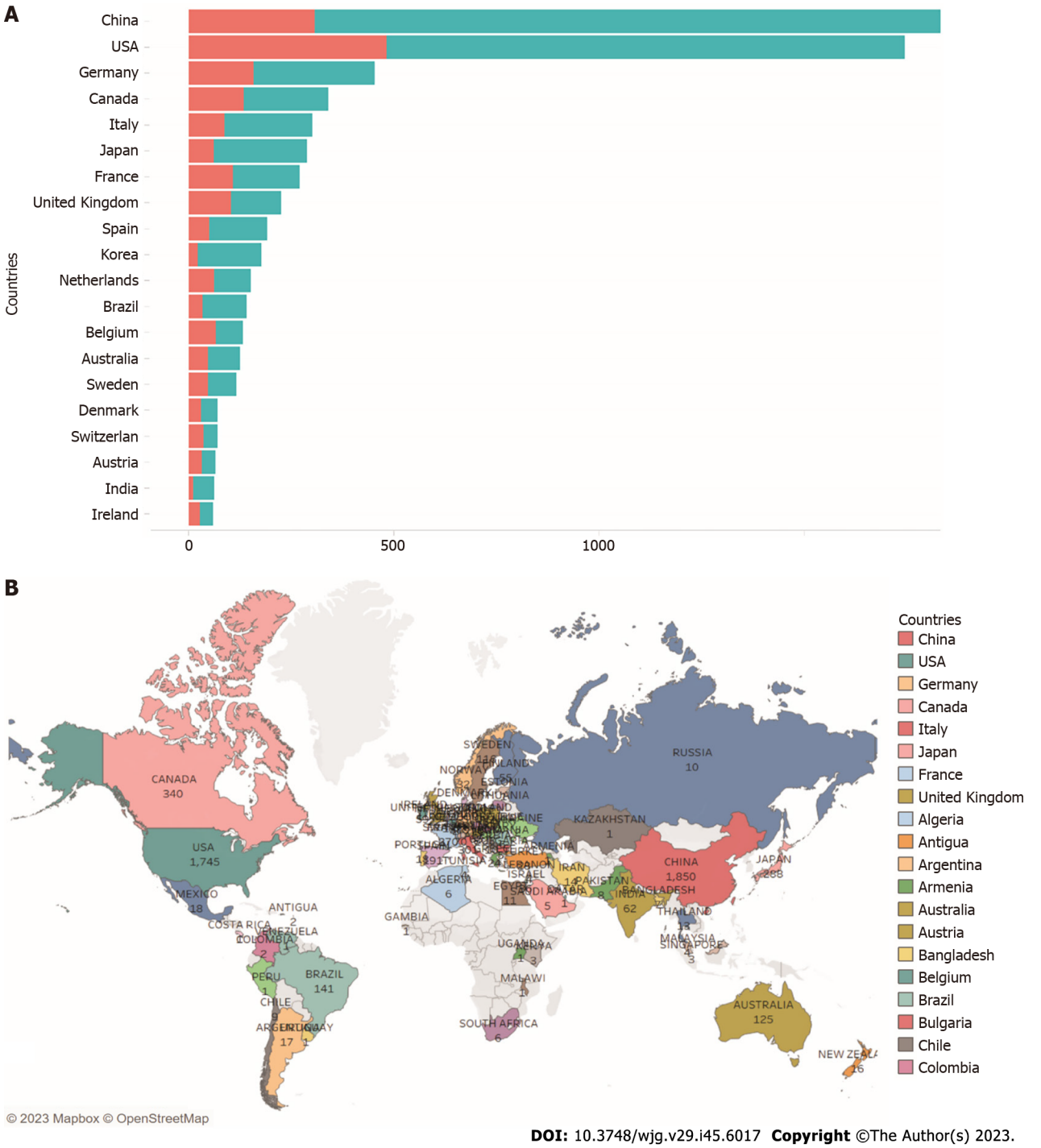

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Figure 3 Corresponding author's countries and country scientific production. A: Corresponding author's countries; B: Country scientific production. The data were analyzed using R package "bibliometrix" by R software (version: 4.3.0).

specific domain of study is an indispensable step in this process.

In conclusion, while we extend our commendations to Zhou *et al*[1] for their meticulous work, we firmly believe that our proposal complements the precision and accuracy of the data analysis of trends in research on "intestinal barrier in IBD" during the last two decades.

FOOTNOTES

Co-corresponding authors: Yan-Dong Miao and Fang Zhang.

Author contributions: Miao YD contributed to conceptualization, manuscript writing, review, and editing, funding acquisition, and project administration; Zhang F contributed to conceptualization, manuscript writing, review, and editing, and supervision; Luan WY wrote the original draft; Yang Z, Chen XD, and Zhang TT performed data analysis and prepared the figures. Miao YD and Zhang F were

designated as co-corresponding authors. Miao YD was responsible for the formulation or evolution of overarching research goals and aims, specifically critical review, commentary, or revision in both pre- or post-publication stages, management and coordination for the research activity planning and execution, and acquisition of the financial support for the project leading to this publication, while Zhang F was responsible for reviewing and editing the draft, oversight, and leadership for the research activity planning and execution, including mentorship external to the core team. This designation reflects their equal contributions and shared responsibility in overseeing the project and correspondence related to this research. All authors approved the final manuscript.

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Country/Territory of origin: China

ORCID number: Wen-Yu Luan 0009-0007-8093-1356; Zhe Yang 0009-0005-1864-2938; Xiao-Dong Chen 0009-0003-9537-7598; Tong-Tong Zhang 0009-0004-6234-5458; Fang Zhang 0000-0001-5120-0991; Yan-Dong Miao 0000-0002-1429-8915.

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