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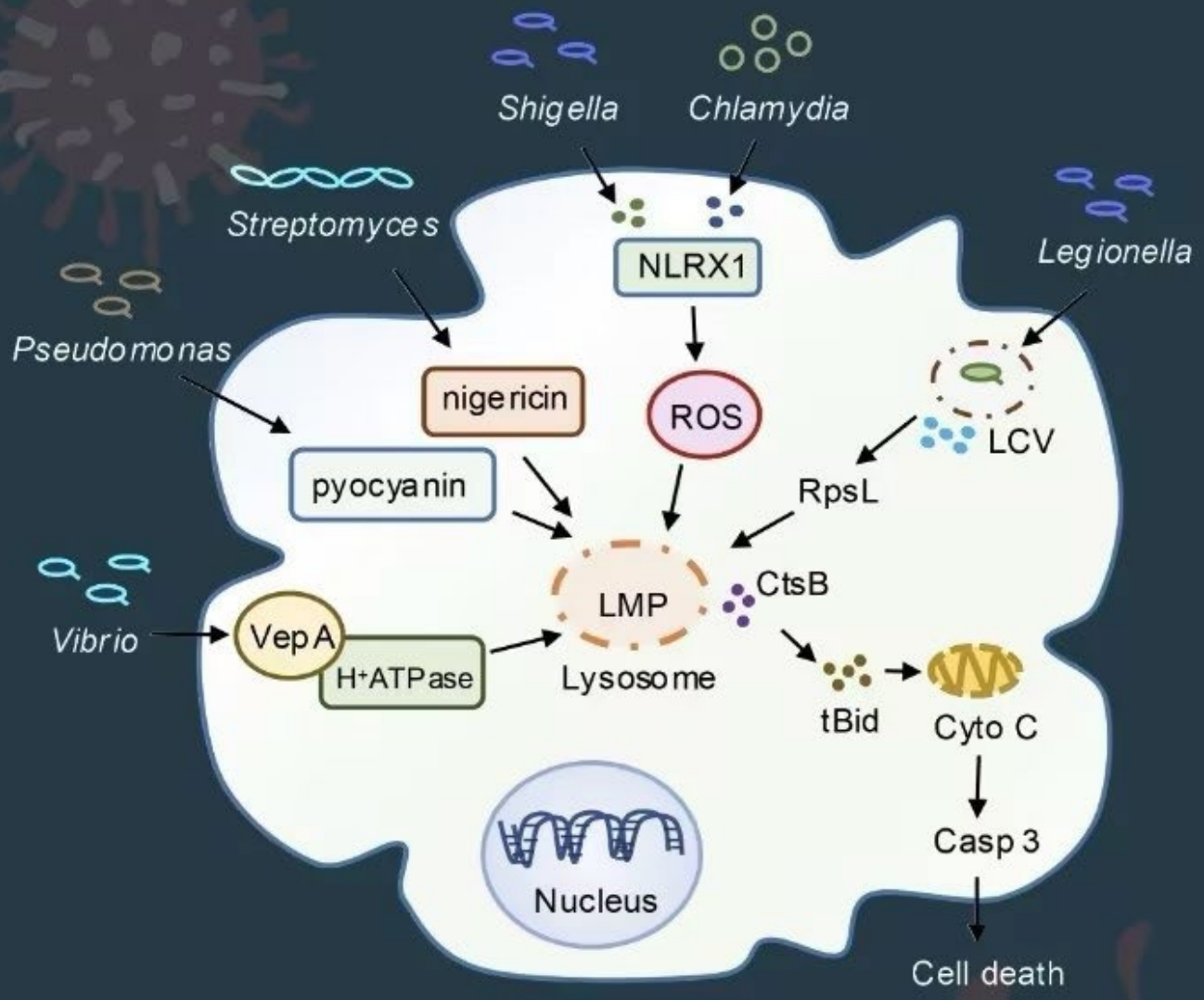

Interplays between host pattern-recognition receptors and pathogen ligands in immunogenic cell death

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EDITORIAL

Exploring natural products: Novel insights and therapeutic potential of plant-based compounds

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(This article belongs to the *Special Issue: Natural Products in the Prevention and Treatment of Microbiological, Immunological, and Infectious Diseases: Integrating Wild Edible Plants and Beyond*)

Natural products, derived from plants, animals, and microorganisms, are compounds that have been used for centuries in traditional medicine and are now increasingly recognized for their therapeutic potential. These substances, which include a wide array of bioactive molecules such as alkaloids, flavonoids, and terpenoids, offer a rich source of novel treatments for various diseases. Their diverse mechanisms of action and safety profiles make them valuable in developing new pharmaceuticals and nutraceuticals. As research advances, natural products continue to play a pivotal role in drug discovery, offering innovative ways to address health challenges and improve well-being.[1,2]

A diet based on natural products and plants has long been a fundamental source of both nourishment and medicine for humanity. While plant-based foods are a staple for nutritional support, many people worldwide also turn to botanical remedies to address their health needs, whether through traditional practices or complementary and alternative medicine. Today, there is a global resurgence in the interest and use of plant-based therapies and botanical health products. This renewed enthusiasm for herbal medicine has prompted increased scientific investigations into the pharmacologically active compounds found in medicinal plants, deepening our understanding of their potential health benefits.[1,2]

Wild edible plants, rich in secondary metabolites such as polyphenols and terpenoids, are excellent candidates for use in nutraceuticals and functional foods. For instance, the Mediterranean region is celebrated for its diverse array of wild edible plants, which are integral to the local diet. These plants have long been recognized by local communities for their nutritional, protective, and medicinal benefits, well before these advantages were scientifically proven. In the eastern Mediterranean region, wild edible plants remain highly valued as sources of healthy food and are frequently harvested by women in rural areas, providing both sustenance and a source of income in economically constrained regions.[3,4]

A diet rich in medicinal plants supports balanced immune function, which is crucial for defending the body against microbial invaders. Immunomodulators, which can either enhance or suppress the immune response, play a critical role in this process. Plant-based secondary metabolites have shown significant potential as natural immunomodulators, offering a promising alternative to conventional immunosuppressants and immunostimulant drugs that often elicit severe side effects. Many plant species exhibit strong immunomodulating properties due to their ability to interact with the immune

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system through various mechanisms and molecular targets. Phytochemicals such as alkaloids, flavonoids, terpenoids, carbohydrates, and polyphenols are particularly noted for their immunomodulatory effects in numerous medicinal plants. Many edible plants are important sources of antimicrobial compounds exhibiting high activity against both Gram-positive and Gram-negative bacteria. Cultivated vegetables, fruits, nuts, herbs, and spices have been investigated more thoroughly than wild species; thus, they dominate the list. Although more than 7000 species of wild edible plants are encompassed in human diets, their immunomodulatory properties are poorly investigated, and most of them still need to be studied.[3]

Recent advancements in medicine and molecular biotechnology have significantly improved the containment and, in some cases, eradication of certain pathogens, particularly in developed countries. Nonetheless, the emergence of evolving pathogens has given rise to new infectious diseases. Technological and socioeconomic changes have accelerated global movement, further facilitating the spread of these diseases, as evidenced by the rapid global dissemination of the 2009 influenza pandemic and the 2014 Ebola outbreak.[5]

In this context, natural products from plants are emerging as promising candidates for next-generation antibacterial and antiviral agents. In developed countries, where 80% of the population depends on traditional medicine for primary health care, and in countries such as India, renowned for its extensive collection of medicinal herbs, the potential of plant-based treatments for bacterial and viral diseases is substantial. In this regard, Mediterranean wild edible plants and their antimicrobial properties have been known since ancient times, and rediscovering them as natural remedies for common infections is gaining traction in modern times. Current strategies for combating bacterial infections heavily rely on antibiotics and preservatives, which often have limited efficacy and can cause serious side effects. This underscores the urgent need for novel antimicrobial agents and food preservatives with enhanced efficacy and reduced toxicity.[6]

Recent years have seen a surge in research on herbal medicines worldwide, with both developed and developing nations intensifying efforts to scientifically assess and validate these treatments through rigorous clinical trials. This special issue aims to compile original research articles that investigate the efficacy of active constituents or extracts from natural products in preventing and treating microbiological, immunological, and infectious diseases. We also welcome review articles that offer comprehensive insights into the current state of research in this field. Some of the highlights of this special issue include:

(1) Evidence-based herbal medicine and natural products are used for the prevention and management of microbiological, immunological, and infectious diseases
(2) Pharmaceutical formulations of pharmacologically active metabolites in managing chronic, microbiological, immunological, and infectious diseases
(3) Different pharmacologically active metabolites are used in the management of human microbiological, and immunological diseases
(4) Recent advances in the discovery of natural drugs for addressing microbiological, immunological, and infectious diseases
(5) Compounds from the Mediterranean diet and medicinal plants.

Conflict of interest

The author declares that he has no competing interests.

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REVIEW ARTICLE

Helicobacter pylori: A Cause of peptic ulcer disease among Adolescent Girls in Africa

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Abstract

Peptic ulcer disease (PUD) is a major global health concern that often results in hospitalization due to insufficient protective factors in the mucosa. *Helicobacter pylori* contributes to PUD development, causing symptoms such as epigastric pain, bloating, fullness, and nausea. An extensive literature search was conducted using electronic databases such as PubMed and Google Scholar to identify articles published between 2013 and 2024. Relevant cross-sectional studies, systematic reviews, meta-analyses, literature reviews, and case reports were included in the analysis, whereas editorials, perspectives, and commentaries were excluded from the study. Overall, 20% of teenagers and 45% of individuals aged >45 years were infected with *H. pylori*, indicating that transmission can occur at any age during childhood and adolescence. Moreover, 8.4% of adolescents aged 10 – 19 years and 64.6% of those aged <18 years tested positive for *H. pylori*. This bacterium can spread through familial transmission and exposure to oral or fecal matter. Large households and bedroom sharing were associated with *H. pylori* infection, and adolescents from rural areas showed a higher infection rate than those from urban areas. Improving personal hygiene and implementing educational initiatives within families are key to curbing the spread of *H. pylori* infection in Africa. Prioritizing hygiene and social improvements in national and subnational strategies can considerably reduce infection rates. Adopting a whole family-based approach and allocating funds for relevant projects can benefit families and children across the continent. Moreover, prompt implementation of interventions to combat *H. pylori* infection among African adolescents is essential. Promoting cleanliness and raising awareness are key strategies to ensure long-term health among the African youth.

Keywords: Peptic ulcer disease; *Helicobacter pylori*; Adolescent girls; Africa

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1. Introduction

One of the main causes of gastrointestinal diseases, which often require hospitalization, is peptic ulcer disease (PUD).[1] PUD has been reported since the 4th century B.C.,[1] affecting 5 – 10% of the population worldwide.[2] PUD occurs when protective factors within the mucosa, such as mucus secretion, bicarbonate production, and blood flow,

are insufficient to counteract offensive factors, such as stomach acids and pepsins, within the mucosa.[1] *H. pylori*, a type of Gram-negative bacteria, resides in the stomachs of many individuals. This bacterium plays a key role in the development of gastric conditions, such as gastric cancer and PUD.[3] In many cases, PUD symptoms manifest intermittently, with the initial symptom being epigastric pain, followed by bloating, fullness, abdominal distension, early satiety syndrome, and nausea.[4] The mortality rate of complicated perioperative PUD in Africa is high and has been increasing, with perforated PUD and mortality rates showing a stronger relationship ($r = 0.41$, $p < 0.0001$) than bleeding PUD and mortality rates ($r = 0.32$, $p = 0.001$).[5] In the present review, the primary goal was to prioritize the well-being of specific demographic groups, particularly adolescent girls living in Africa. It is widely believed that various sociodemographic and geographical elements contribute to the prevalence of *H. pylori* infection in Africa.[6] The prevalence of *H. pylori* infection is greater in Africa than in other regions worldwide, where a decline in prevalence has been noted.[7] In Nigeria, for example, the highest prevalence of 93.6% was documented.[8] Moreover, in Tunisia, the prevalence of *H. pylori* infection was 82.7%.[9] In addition, the prevalence of *H. pylori* infection in South Africa, Rwanda, Ghana, and Uganda was 77.6%, 77.5%, 58.72%, and 35.7%, respectively.[10-13] Furthermore, Morocco[6] and Sudan[14] had a prevalence of 63.8% and 8.4%, respectively. Unfortunately, *H. pylori* infection in African countries often goes unrecognized, despite its high prevalence. A previous review reported that *H. pylori* is commonly associated with several risk factors, including low income, unclean water sources, overcrowded living conditions, smoking, and increased interferon-gamma levels.[15] This narrative review aimed to bridge the gap between PUD and *H. pylori* infection among adolescent girls in Africa by examining relevant studies. Based on a previous study, the prevalence of *H. pylori* infection increased to 20% among teenagers and reached a peak of 45% in adults aged ≥45 years.[16] A study conducted in Ziway, Ethiopia, involving school-aged children with *H. pylori* infection[17] revealed that close family relationships, particularly in large families living together, contribute significantly to the spread of the infection.[17] Extreme poverty has a profound impact on a substantial portion of the population, as observed in South Africa.[18] This is due to the lack of access to necessities, including clean water, adequate nutrition, effective sanitation, safe housing conditions, vaccinations, quality education, and nurturing during childhood and adolescence.[18]

To reduce the burden of infection in Africa, effective and cost-efficient approaches are required.[15] The primary factors contributing to the scarcity of health-care resources include the government's inadequate financial support for public hospitals, underpaid employees in the public health sector who are more prone to corruption, and a shortage of financial resources.[15] Implementing interventional health programs, including raising awareness and enhancing personal cleanliness, could minimize the likelihood of *H. pylori* infection among females at a young age, thereby promoting overall wellness in adolescents.[14] A previous study conducted in Egypt demonstrated that children aged <18 years who developed *H. pylori* infection were more likely to experience stunted growth than those who were not infected.[19] The present review aimed to evaluate the economic and social consequences of PUD in adolescent girls, considering its potential ramifications for their physical and mental health. Moreover, the possibility of stigma associated with having a chronic health condition was examined, as it may affect their outlook on the future.

2. Methodology

An extensive literature search was conducted using electronic databases such as PubMed and Google Scholar to identify articles published between 2013 and 2024. Cross-sectional studies, systematic reviews, meta-analyses, literature reviews, and case reports related to the study topic were included in the analysis, whereas editorials, perspectives, and commentaries not relevant to this review were excluded.

3. Results

3.1. Etiology and Pathophysiology of PUD

The two main causes of reduced mucosal resistance to injury include non-steroidal anti-inflammatory drug (NSAID) use and *H. pylori* infection.[6] PUD has a complex etiology, with blood group O, long-term NSAID use, and *H. pylori* infection being the most common causative factors.[1] Public health concerns on PUD are significant across Africa, particularly in Nigeria,[1] as reported in a previous study, which provided insights into the disease's risk factors and epidemiological patterns.[1] Approximately two-thirds of the population in Africa is estimated to have *H. pylori* infection, making it a major contributor to the pathophysiology of PUD.[1]

A well-known cause of chronic gastritis is infection caused by *H. pylori*, which colonizes the stomach lining and causes the stomach mucosa to remain inflamed over time, leading to the development of chronic gastritis.[20,21] In addition to *H. pylori* infection, the other factors linked to the pathophysiology of PUD include stress, smoking, NSAID use, and dietary practices.[22] The stomach's protective lining can be damaged by long-term NSAID use, increasing the risk of developing peptic ulcers.[22]

Increased psychological stress levels, frequently worsened by socioeconomic burden, may contribute to the onset and aggravation of PUD among Africans. Prolonged stress can alter the integrity of the mucosa and release of stomach acids, making individuals more susceptible to ulcers.[22] In many African nations, tobacco use is a common habit linked to an increased risk of PUD.[22] Dietary factors that can irritate the stomach mucosa and contribute to PUD development include the consumption of spicy foods and large amounts of alcohol, irregular eating patterns, inadequate nourishment, and poor food choices.[22]

Cigarette smoking is considered one of the main causes of the development of ulcers. Hazardous duodenal contents can reflux back into the stomach as a result of cigarette smoking. Moreover, there seems to be a greater risk of *H. pylori* infection among smokers. This elevated risk could be attributed to the detrimental effects of smoking on the antioxidant levels or immune system, which is locally present in the mucosa of the gastroduodenum. These activities have the potential to compromise the natural defenses of the stomach and duodenum against *H. pylori*.[23] The innermost layer of epithelium forms a continuous layer of defense against harmful substances in the lumen. A detrimental impact of smoking cigarettes is the stimulation of cell apoptosis, which causes tissue damage and gastrointestinal tract malfunction.[23]

Urease, which converts urea into ammonia and carbon dioxide to neutralize the acidic pH and shield bacterial cells from stomach acids, is essential for *H. pylori* colonization.[24] *H. pylori* can weaken the mucus gel layer and decrease prostaglandin synthesis, which makes it easier for irritants such as pepsin and gastric acid to enter deeper into the stomach wall layers and cause mucosal damage and ulcer development.[24] Peptic ulcers may occur as a result of a dysregulation in the equilibrium between aggressive agents and defense mechanisms in the duodenum and stomach.[24] Histologically, chronic peptic ulcers exhibit well-defined ulcer bases, granulation tissue formation, fibrosis, and scarring in deeper layers, whereas acute peptic ulcers may exhibit surface erosions, fibrinoid necrosis, and inflammatory cell infiltrates.[24] Hematemesis and melena, perforation leading to peritonitis, and blockage from scarring and fibrosis are certain complications associated with peptic ulcers. Peptic ulcers heal through a combination of mechanisms, including angiogenesis, tissue repair, inflammation resolution, and mucosal regeneration. Ulcer recurrence is caused by various factors, such as NSAID use, defective tissue repair, persistent *H. pylori* infection, and systemic diseases.[24] Figure A1 shows the pathogenesis of PUD, which involves both aggressive and defensive factors.

A polygenic inheritance pattern and genetic predisposition to PUD may contribute to the disease's familial aggregation. Duodenal and stomach ulcers have different patterns of familial aggregation, with first-degree relatives of patients exhibiting a higher prevalence of ulcers.[25] For example, duodenal ulcers are associated with host polymorphism affecting cytokine IL-1β levels. In a previous meta-analysis, Zhang *et al.* conducted subgroup analyses of data from 3,793 participants and revealed that the IL-1β–31 C/C genotype has a protective effect against the development of duodenal ulcers.[25] However, in the same study, no evidence of a significant correlation was observed between the IL-1β–31 C/T polymorphism and duodenal ulcers; thus, it remains unclear whether duodenal ulcers are caused by *H. pylori* infection.[25]

3.2. Understanding PUD occurring in adolescent girls across Africa

In adolescent girls, PUD can present with various clinical manifestations. Abdominal pain is one of the most prevalent symptoms of peptic ulcers in children.[26] Other symptoms may include vomiting, melena, nausea, stomach discomfort, hematemesis, abdominal distension, sour regurgitation, ozostomia, eructation, paleness, bloody feces, poor appetite, and abdominal tenderness.[26] The signs and symptoms of PUD vary depending on the disease location and patient's age. Gastric and duodenal ulcers can be distinguished by the timing of symptom occurrence (before or after meals). Nocturnal pain is frequently observed in cases of duodenal ulcers. Patients with gastric outlet obstruction may report abdominal bloating and/or fullness.[27] Warning or alarm symptoms that should necessitate immediate referral include unintentional weight loss, progressive dysphagia, overt gastrointestinal bleeding, iron deficiency anemia, recurrent emesis, and family history of upper gastrointestinal malignancies.[28,29] Table A1 shows the symptoms of PUD among adolescent girls in Africa, as reported in studies published between 2013 and 2024.

3.3. Prevalence and incidence of PUD

PUD is a common affliction worldwide. Over 300,000 global deaths were attributed to PUD in 2013, according to estimates from the Global Burden of Disease.[5] A previous study conducted in northern Sudan reported a prevalence of *H. pylori* infection of 8.4% among adolescents aged 10 – 18 years.[14] In addition, a study conducted in Egypt demonstrated that 407 (64.6%) adolescents aged ≤18 years tested positive for *H. pylori*, with the highest prevalence of *H. pylori* infection reported among adolescents aged >10 years (32.9%).[19] In another study conducted in Owerri, Nigeria, the prevalence of *H. pylori* infection was

20%, which increased with decreasing socioeconomic status and age; children aged 10 – 15 years showed the highest prevalence.[30] Furthermore, the overall prevalence of *H. pylori* infection among adolescents in Yaoundé, Cameroon, was 78%, with the infection more frequently detected in girls (90.3%) than in boys (72.6%).[31] Table A2 shows the prevalence of *H. pylori* infection among adolescents in Africa, as reported in studies published between 2013 and 2024.

3.4. Risk factors contributing to *H. pylori* infection among adolescent girls in Africa

In the 21st century, although the prevalence of *H. pylori* has declined in Western industrialized countries, it remains stable the same in developing and newly industrialized nations, with notable consequences for global sequelae, such as PUD and gastric cancer.[10] These variations in prevalence may result from various factors, including urbanization, sanitation, and socioeconomic status.[10] The risk of *H. pylori* infection in African children may increase with aging.[32,33] The prevalence of *H. pylori* infection among symptomatic patients at Dessie Referral Hospital in Northeast Ethiopia, as measured using a stool antigen test, was 7% in individuals aged <20 years.[34]

The prevalence of *H. pylori* infection is often high in developing countries and is typically linked to factors such as socioeconomic status and hygiene levels.[10] However, in many cases, transmission within families is the primary means of spread.[10] The prevalence of *H. pylori* infection was higher in large households, women, younger individuals, individuals practicing open-air defecation, and those drinking water from sources other than a pipe or borehole.[35] *H. pylori* infection can occur through exposure to the bacterium through the oral–oral or fecal–oral route. A previous study revealed that the fecal–oral route is the most significant means of transmission.[34] A cross-sectional study conducted among symptomatic Egyptian children showed that children aged >10 years had the highest infection rate at 32.9%.[19] Factors contributing to this increased infection rate included increased exposure to community and outdoor environments as well as the adoption of poor dietary habits, such as consuming food from street vendors.[19] A significant proportion of the participants (88%) who tested positive for *H. pylori* lacked access to clean drinking water from a pipe or borehole.[35] In addition, 20.1% of individuals who practiced open-air defecation tested positive for *H. pylori*.[35] Among adolescents in Ethiopia, the presence of *H. pylori* was significantly linked to having a large household with more than three family members and three or more individuals sharing a bedroom.[34] The infection rate among adolescents from rural areas was significantly higher than that among adolescents from urban areas (60.4% *vs*. 39.6%, $P < 0.004$).[19] Gastroesophageal reflux disease, having multiple sexual partners, likelihood of contracting *H. pylori*, and role of gut microbiota in intrafamilial transmission of the bacterium were also proposed as a cause of PUD.[36] Maternal illiteracy displayed a remarkably strong correlation with *H. pylori* infection.[19] In a previous study conducted in Sudan among adolescents, no association was found between *H. pylori* infection and father's occupation.[14] In Nigeria, consuming unpasteurized milk was significantly correlated with *H. pylori* infection.[37] The detection of *H. pylori* stool antigen among adolescents from East Africa did not show a significant correlation with coffee consumption.[34] Several vegetables, such as *Ocimum gratissimum*, *Carica papaya*, and *Allium*, which are commonly consumed in the southeast and south-south regions of Africa, reportedly confer protection against *H. pylori* infection.[33] Adolescent girls with PUD frequently exhibit various psychosocial traits, such as neurotic personality traits, irregular eating patterns, e-cigarette use, and psychological stress.[38] Moreover, poor socioeconomic status, psychological stress, risky health behaviors, analgesic use, and physically demanding work may increase the risk of ulcer development in populations with a low educational level.[39]

3.5. Diagnostic evaluation of *H. pylori* infection

Laboratory diagnostic and clinical evaluations are two methods utilized to determine the relationship between *H. pylori* infection and PUD. For patients with dyspepsia, the *Helicobacter* rapid urease test is a cost-effective method to diagnose *H. pylori* infection within a reasonable amount of time. This test has a 57.1% sensitivity and 98.9% specificity in diagnosing *H. pylori* infection, along with an 80% positive predictive value and a 96.7% negative predictive value. The gold standard procedures for diagnosing *H. pylori* infection are histopathologic examination and culture; however, they are time-consuming and unsuitable for routine use. Urease tests, including Pronto Dry and Helicotec, can identify urease from tissue biopsy samples, allowing for a rapid diagnosis of *H. pylori* infection.[40] The urea breath test measures the amount of tagged carbon dioxide in breath samples to identify the presence of *H. pylori*.[41] Stool antigen test aids in detecting *H. pylori* antigens in stool samples, which is helpful for post-treatment confirmation and preliminary diagnosis.[41] In individuals with PUD, molecular techniques such as polymerase chain reaction tests can be employed to identify *H. pylori* and associated virulence factors. These techniques have high sensitivity and specificity, which helps determine the effectiveness of eradication after therapy.[42]

3.6. Public health impact of PUD on Adolescent girls across Africa

From a critical point of view, there may be inadequate medical facilities or resources in many African communities, particularly in rural regions, for accurately diagnosing and treating PUD in adolescent girls. This infrastructural gap may prevent prompt access to health-care services and appropriate care. Poor hygiene and sanitation habits, such as not having access to sanitary facilities and clean water, might further increase the risk of PUD among adolescent girls and facilitate the spread of *H. pylori* infection. Effective disease prevention and management depend on addressing these environmental factors. Specialist care from pediatricians or gastroenterologists may be necessary for adolescent girls with severe or complex PUD. Complex case management may be difficult in many African communities due to restricted access to these specialized health-care services. The other possible public health implications of PUD among adolescent girls in Africa include psychological problems, such as depression, reduced self-esteem, poor concentration, and frustration. Another speculation is that persistent complaints of PUD symptoms among adolescent girls can affect their relationships with their family, peer groups, and spouses, which may hinder their overall well-being. All these issues can lead to stigmatization for these girls.

4. Recommendation and Future Directions

The prevalence of *H. pylori* infection among adolescents is relatively high in Africa.[43] The infection risk increases with aging, school attendance, and sharing sleeping spaces with multiple individuals.[43] To mitigate this, educating families and promoting personal hygiene among adolescent girls are crucial, as reported in a study conducted in Cameroon.[31] Ding proposed a new approach for addressing *H. pylori* infections in China.[44] This approach emphasizes screening, identifying, treating, and monitoring all high-risk family members to save costs in the later stages of treatment.[44] Moreover, the approach aims to prevent bacterial transmission, progression of gastric mucosal lesions, and incidence of gastric cancer.[44] In addition to traditional strategies, such as "test and treat" and "screen and treat," Ding's study suggests adopting a novel whole family-based *H. pylori* prevention and intervention strategy. This comprehensive approach, which is termed the "whole family- or household-based *H. pylori* precision and integrative eradication strategy," is deemed practical not only for communities with high infection rates but also for those with low infection rates.[44] After refinement and discussion, this strategy could significantly reduce the sources of transmission, enhance public awareness, and alleviate the burden of *H. pylori*-related diseases and gastric cancer.[44] To adopt the strategies suggested by Ding, interventions for educating communities on how to treat *H. pylori* infections in Africa should be initiated, beginning with community education and awareness, followed by screening programs and identifying high-risk families, treating infected individuals, monitoring family members, and performing follow-up and surveillance to monitor the effectiveness of treatment. Moreover, the proposed approach should be integrated with existing health-care infrastructure and ultimately customized according to the specific cultural, social, and economic context of each African country or community.[44]

At the local level in Africa, implementing educational initiatives and promoting personal hygiene practices among families can help prevent the spread of *H. pylori* infection, as reported in an Ethiopian study.[43] At the national and subnational levels, strategies similar to those used in a Cameroonian study,[31] particularly focusing on hygienic and social improvements, can be instrumental in safeguarding children against *H. pylori* infection. Finally, at the international level, adopting whole family-based approaches, such as the approach proposed by Ding,[44] can contribute to reducing the global burden of *H. pylori* infection and associated diseases. In Africa, teaching families about hygiene and sanitary habits can prevent the spread of *H. pylori* infections.[43] Ensuring children drink clean water and have good living conditions and utilizing the whole family approach introduced in China are also helpful. Furthermore, African governments should disburse funds for projects and ensure that children, especially adolescent girls, receive the right support when they feel worried or upset about being infected with *H. pylori.*

5. Limitations

The major limitation of our research is that the data available on PUD and *H. pylori* infections in adolescent girls in Africa is limited. Therefore, to determine whether *H. pylori* is a contributing factor in PUD among adolescent girls in Africa, additional original studies, such as quantitative and qualitative studies and case studies, are needed.

6. Conclusion

The increasing prevalence of *H. pylori* infection among adolescents in Africa requires immediate attention. The risk of acquiring this infection is higher for adolescent girls than for their male counterparts. Implementing preventive health measures, such as raising awareness and promoting better hygiene practices, can help reduce the likelihood of developing *H. pylori* infections among young people, particularly in adolescent girls, and can improve the overall health of adolescents in Africa.

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Appendix

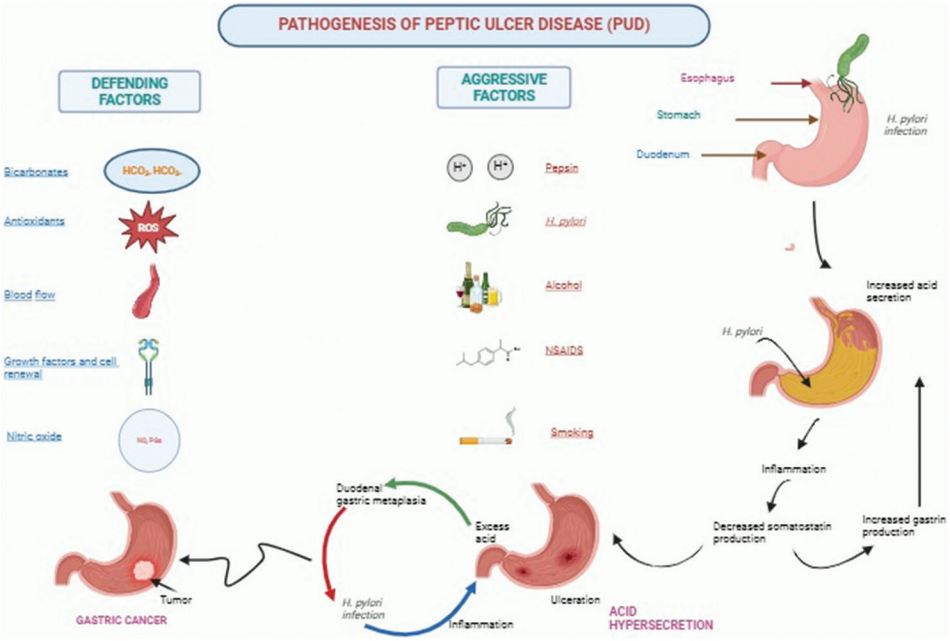

Figure A1. Pathogenesis of peptic ulcer disease involves both aggressive and defensive factors

Table A1. Symptoms of peptic ulcer disease (PUD) among adolescents in Africa, as reported in studies published between 2013 and 2024

Symptoms	Description
Dyspepsia	Dyspepsia may result in gastroscopy, which typically indicates *H. pylori** infection.[8]
Gastroesophageal reflux symptoms	Experiencing persistent heartburn and acid reflux for the past 3 months.[45]
Abdominal pain	Stomach discomfort or pain can be challenging to manage.[45]
Irritable bowel syndrome	Abdominal pain or discomfort at any site, combined with reported disturbances in bowel habits.[45]
Atypical PUD symptoms	Gastrointestinal symptoms such as pain or discomfort, which are common in PUD, may occur.[45]
Epigastric pain or discomfort is common	Abdominal pain and a sense of fullness after eating symptoms of uncomplicated PUD.[46]

Note: **H. pylori: Helicobacter pylori*

Table A2. Prevalence of *Helicobacter pylori* infection among adolescents in Africa, as reported in studies published between 2013 and 2024

Country	Prevalence (%)	Age (years)	References
Ghana	14.20	7 – 16	35
Cameroon	4.28	<20	47
Uganda	5.10	<18	13
Sudan	8.40	<18	14
Tanzania	20.6	>10	48

ACCSCIENCE PUBLISHING

Microbes & Immunity

REVIEW ARTICLE

Comparison of immune response parameters between homologous and heterologous COVID-19 vaccines: A scoping review

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Abstract

It has been over 4 years since the emergence of the coronavirus disease 2019 (COVID-19) pandemic. This highly contagious respiratory infection has endangered the health of millions and significantly impacted healthcare systems and economies. Vaccines are believed to confer immunity against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative agent of COVID-19, reducing both the severity of infection and the spread of the virus. Within a short period, various COVID-19 vaccines were developed and extensively tested before being approved by the WHO for distribution and administration. Now, due to concerns about emerging new strains of the virus and limited vaccine availability, a heterologous vaccine strategy is being deployed. Therefore, this paper aims to conduct a scoping review of existing evidence to compare the immunogenicity of heterologous vaccines with homologous vaccines and determine which confers better immunity against COVID-19. A literature search was conducted across three electronic databases (Ovid MEDLINE, PubMed, and Scopus). The retrieved studies were screened for relevance and eligibility using the online platform Covidence. A total of 31 articles were shortlisted for data extraction and analysis. Among these, 21 were observational studies, and 10 were clinical trials. The analysis demonstrated that participants who received heterologous vaccination regimens generated higher levels of IgG antibodies against the spike protein of SARS-CoV-2, antibodies targeting the receptor-binding domain, and T-cell responses compared to those who received homologous vaccination regimens. Furthermore, heterologous vaccination produced higher titers of neutralizing antibodies against several variants of concern (VOC), including Alpha, Beta, Gamma, Delta, and Omicron. No severe vaccine-related adverse events were reported in these studies, and common local and systemic side effects were manageable. Overall, heterologous vaccination regimes induced strong humoral and cellular immunity, comparable to homologous vaccination regimes, with stronger neutralizing antibody activity against VOCs.

Keywords: COVID-19; SARS-CoV-2; Heterologous vaccines; Homologous vaccines; Immunogenicity

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1. Introduction

On December 31, 2019, several hospitals in Wuhan, China, reported increasing clusters of patients suffering from acute atypical pneumonia of unknown etiology. The causative pathogen was later identified to be a novel coronavirus that was named severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). The World Health Organization (WHO) termed the following respiratory illness as coronavirus disease 2019 (COVID-19) and subsequently declared it a global pandemic in March 2020 due to the outbreak of the virus that affected approximately 200 countries worldwide. As of May 2024, data from WHO indicate a cumulative number of 775 million confirmed cases of COVID-19, with 7 million deaths.[1]

The SARS-CoV-2 is a coronavirus containing positive-stranded RNA. Its spike proteins, located on its surface, bind to angiotensin-converting enzyme 2 through receptor-binding domains (RBD) in these proteins, facilitating entry into host cells.[2] The virus spreads through human-human transmission, primarily when mucosal surfaces (nose, mouth, and eyes) are exposed to air droplets or fomites containing the virus. The severity of infection can range from mild to moderate to severe, with common symptoms including fever, cough, shortness of breath, headache, lethargy, anosmia, and ageusia.[2]

The COVID-19 pandemic is undeniably a major public health crisis, posing significant threats to both individual health and global healthcare systems and economies. Over the past 5 years, various strategies have been implemented by governments and health authorities to mitigate the spread of the virus, including social distancing, mandatory mask-wearing, quarantine of infected individuals and their close contacts, widespread lockdowns, and international travel bans. In addition, numerous companies have invested heavily in vaccine development since the start of the pandemic, driven by the strong belief that vaccines are preventative medicine that can effectively reduce the rate of infections and decrease morbidity and mortality from COVID-19.[3] Various vaccines have been approved by the WHO for inclusion in global COVID-19 vaccination programs or for emergency use, with their efficacy and safety profiles well-established through evidence from extensive clinical trials.[4] These vaccines include Pfizer/BioNTech (BNT 162b2), Moderna (mRNA-1273), Oxford/AstraZeneca (ChAdOx1-S), Johnson and Johnson (Ad 26.COV2-S), Sinopharm (BBIP-CorV), Sinovac (CoronaVac), Covaxin (BBV152), and Novavax (NVX-CoV2373).[4]

Although homologous vaccinations remain the traditional approach of vaccination, the use of heterologous vaccinations, which involve administering a prime and booster vaccine developed from different platforms, has been widely adopted in many countries. According to interim guidance from the WHO, the rationale for implementing heterologous vaccines was due to concerns of inadequate vaccine supply and delay of the administration of booster doses, especially in lower-income countries. More importantly, heterologous vaccines confer better immunity against COVID-19 and enhance vaccine efficacy.[5] Therefore, the objective of this paper is to conduct a scoping review of the existing scientific evidence to compare the efficacy of homologous and heterologous vaccination strategies, as well as demonstrate that heterologous vaccinations are more efficacious and confer better immunity against COVID-19.

2. Methodology

This scoping review was conducted in accordance with Arksey and O'Malley's five-stage framework.

Arksey, H. and O'Malley, L., 2005. Scoping studies: toward a methodological framework. International journal of social research methodology, 8(1), pp.19-32.

2.1. Research question

The research question for the study was, "Do heterologous vaccines provide better immunity against COVID-19 when compared to homologous vaccines?" The PICO framework was used to define the research question as well as develop alternate terms for the literature search (Table 1).

2.2. Identification of relevant studies

Three databases (Ovid MEDLINE, PubMed, and Scopus) were used to source relevant studies. During the literature search, comprehensive keywords including "heterologous vaccines," "homologous vaccines," "COVID-19," "SARS-CoV-2," and "immunogenicity" were employed, along with MeSh terms and Boolean operators ("OR" and "AND") to combine keywords and refine the search terms. Given the extensive research on COVID-19, the search initially yielded an extensive number of articles. To narrow the results, the search was limited to studies published from 2020 – 2024 and in English, focusing on clinical trials or observational studies.

Table 1. PICO template used to develop search terms

PICO terms	Description
P: Patient/Problem	COVID-19
I: Intervention	Heterologous vaccination
C: Comparison	Homologous vaccination
O: Outcome	Stronger anti-vaccine immune response

2.3. Study selection

The identified articles were imported into Endnote X9 software to perform a primary duplicate screening. The remaining articles were then exported to Covidence (https://www.covidence.org), an online software tailored for systematic reviews that facilitates de-duplication and screening. Articles were screened for eligibility based on inclusion and exclusion criteria in a two-stage process: (i) Title and abstract screening, followed by (ii) full-text review. The inclusion criteria were original research articles from clinical trials or observational studies comparing heterologous versus homologous COVID-19 vaccination regimes, focusing on the immunogenicity or efficacy of these regimes in healthy adults aged ≥18 years old. Publications that were review articles (systematic, scoping, or narrative) or case reports articles, examining only homologous COVID-19 vaccination, studies involving subjects with pre-existing medical conditions or pediatric populations, and animal studies were excluded using the search options of the database. The screening process involved a total of three reviewers; two researchers independently reviewed each paper, and any disagreements were resolved by the study supervisor. The Preferred Reporting Items for Systematic Reviews and Meta (PRISMA) chart was used to illustrate the screening and study selection process.

3. Results

The literature search across the databases yielded 317 articles, which were imported into the Endnote X9 software. Following an initial duplicate screening, 44 articles were identified as duplicates and removed. The remaining 273 articles were uploaded into Covidence, where its de-duplication function identified and removed an additional six duplicates, leaving 267 articles (Figure 1). Following title and abstract screening, 175 articles were excluded as irrelevant to the interest of this study. Among the remaining 92 articles subjected to full-text review, 65 articles were excluded for the following reasons: eight studies were systematic reviews or case reports; two were animal studies; four were irrelevant to this study; three studies assessed the wrong outcomes; four studies involved pediatric populations; two involved patient populations with pre-existing medical conditions; 19 studies used wrong comparators; 17 studies had inappropriate interventions; one study was an ongoing clinical trial; two studies were study protocols; and three more were duplicates (Figure 1). Ultimately, 27 articles met the inclusion criteria and were shortlisted for data extraction and analysis in this scoping review. In addition, four studies were included through a manual search on Google Scholar, totaling the included studies to 31 articles. These 31 studies compared the immunogenicity of heterologous vaccines against homologous vaccines by measuring several parameters, namely, antibodies to the spike protein (IgG) of SARS-CoV-2; antibodies to the RBD; neutralizing antibodies; and T-cell response to the spike protein. Immunological data obtained from each study are summarized in Table 2.

3.1. Characteristics of included studies

The research methodology classified most of the identified studies as observational studies, with the remaining ones being clinical trials (Figure 2A). Geographically, more than half of the studies were conducted in European countries such as Germany, the United Kingdom, Spain, Italy, France, Denmark, and Sweden. Around one-third of the studies involve Asian continents such as China, Hong Kong, Thailand, Singapore, and India, while the remaining studies were done in the United States, Brazil, and Lebanon (Figure 2B).

A total of 128 vaccination strategies were reported in the 31 short-listed research articles, which included 61 heterologous vaccination groups (48%), 56 homologous vaccination groups (44%), and 11 control groups (8%) (Figure 3). Among the 61 heterologous vaccination strategies, the most popular combination investigated was the AstraZeneca/Pfizer (ChAd/BNT) (13/61), where subjects received the AstraZeneca (ChAd) as the first vaccine, followed by the Pfizer (BNT) as the second vaccine (Figure 4). Three studies used the AstraZeneca/Moderna (ChAd/mRNA1273) combination; two studies used the Pfizer/Moderna (BNT/mRNA1273) combination, while only one study used the Moderna/Pfizer (mRNA1273/BNT) combination (Figure 4).

3.2. Comparison of immune response parameters

The majority of the studies that compared homologous and heterologous vaccination regimes reported higher anti-spike protein IgG and anti-spike protein IgA antibodies (Table 3). Similar findings were observed with antibodies to the RBD and T-cell responses (Table 3).

3.2.1. Antibodies to the spike protein

In general, participants who received a heterologous vaccine regimen exhibited a marked rise in spike IgG levels from baseline to 14 days after receiving the second dose booster and had higher titers compared to those who received a homologous regimen. The Com-COV study, a randomized controlled trial (RCT) conducted by Liu *et al.*,[22] revealed a higher spike in the IgG antibody levels ($P < 0.05$) in the heterologous ChAd/BNT vaccine group compared to the homologous ChAd/ChAd group with antibody levels of 12,906 EU/mL and 1,392 EU/mL, respectively. The computed geometric mean ratio (GMR)

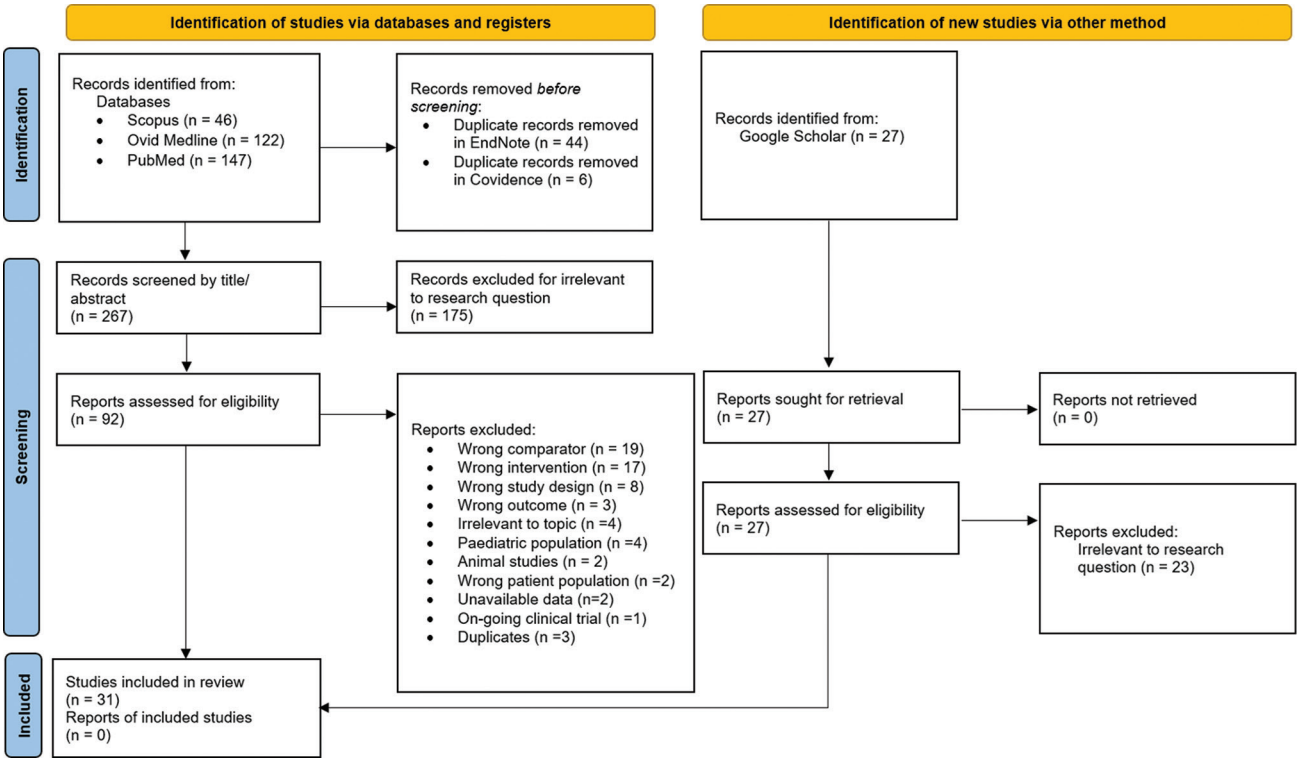

Figure 1. Preferred Reporting Items for Systematic Reviews and Meta chart demonstrating the details of screening and selecting articles

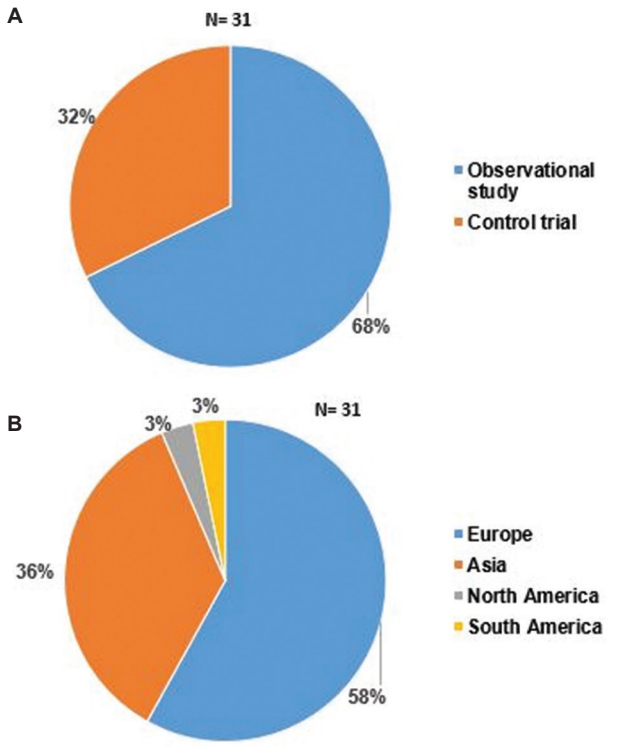

Figure 2. Analysis of the shortlisted research articles. (A) Type of studies included in the scoping review and (B) regions of studies included in the scoping review

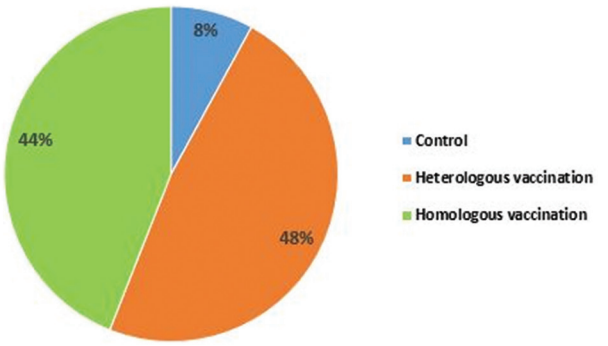

Figure 3. Overview of the different vaccination strategies used for the first and second doses of the COVID-19 vaccination

was 9.2, which suggests there was no significant difference in the protection conferred by the heterologous vaccination regime compared to the homologous vaccination regime.[22] A margin of GMR >0.63 was used to determine the non-inferiority of the heterologous group.[22] On the other hand, participants who received heterologous BNT/ChAd had lower spike IgG antibody levels compared to participants vaccinated with homologous BNT/BNT (7,133 EU/mL to 14,080 EU/mL) with a GMR of 0.51.[22] It is important to highlight that when a comparison was made between heterologous ChAd/BNT with homologous BNT/BNT groups, the resulting spike IgG levels were comparable

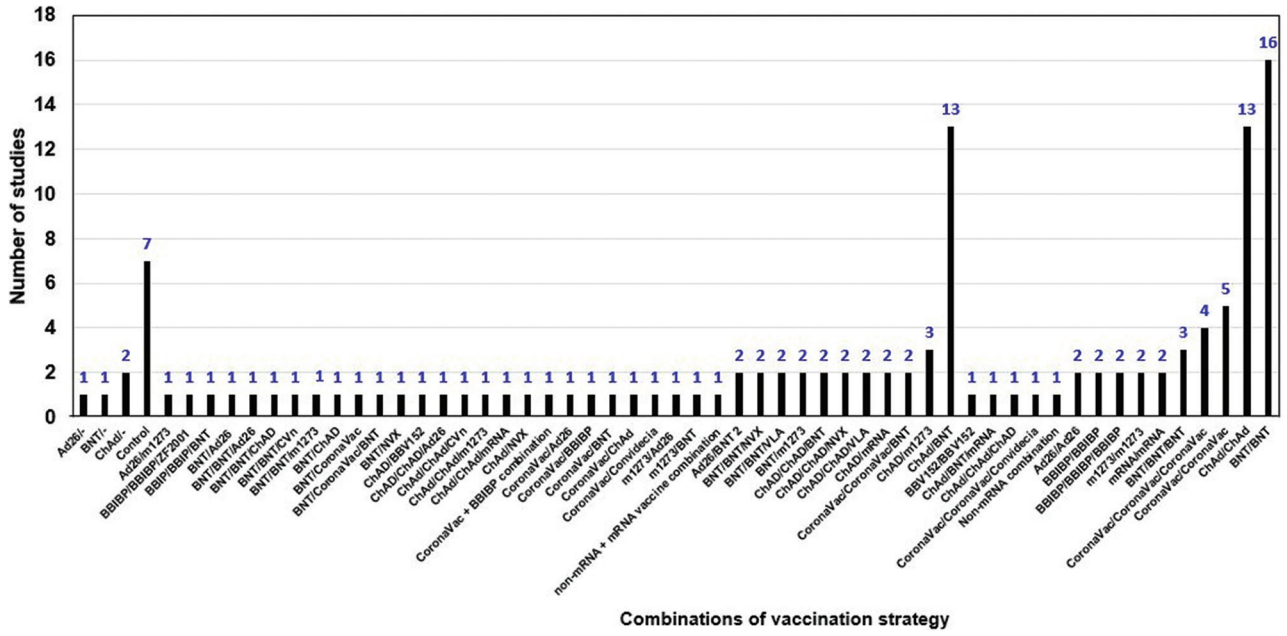

Figure 4. The number of vaccination strategies investigated. The vaccine name mentioned before the "/" is the first vaccine dose given (primary vaccination), and the vaccine name mentioned after is the second dose (booster vaccination). In each combination, the vaccine named first was given as the first dose and the vaccine named after was administered as the second dose

to one another (12,906 EU/mL and 14,080 EU/mL, respectively).[22]

In an observational study, Rose *et al.*,[26] reported a higher spike in IgG levels in participants who received the heterologous ChAd/BNT compared to homologous ChAd/ChAd (783 BAU/mL to 123 BAU/mL). However, the spike IgG antibody levels were higher in the homologous BNT/BNT group at 1,334 BAU/mL.[26] In the Com-COV 2 study conducted by Stuart *et al.*,[28] groups who received heterologous ChAd/mRNA-1273 and ChAd/NVX had higher spike IgG levels ($P < 0.05$) compared to the homologous ChAd/ChAd group with antibody levels of 20,114 ELU/mL, 5,597 ELU/mL and 1971 ELU/mL, respectively (Table 3). The heterologous ChAd/mRNA-1273 and ChAd/NVX groups also showed non-inferiority to the homologous group with a GMR of 10.2 and 2.8, respectively.[28] Among the groups who received heterologous BNT vaccines, the BNT/mRNA-1273 arm had the highest spike IgG levels at 22,978 ELU/ml and was higher compared to the homologous BNT/BNT arm.[28] The heterologous BNT/NVX had lower spike IgG antibody levels at 8,874 ELU/ml.[28] A RCT conducted in Brazil evaluated the immunogenicity of heterologous vaccines in participants receiving an inactivated vaccine prime with a mRNA boost or viral vector boost.[12] The anti-spike IgG levels for heterologous CoronaVac/BNT were highest with a geometric fold rise of 152 from baseline, while participants receiving CoronaVac/Ad 26 and CoronaVac/ChAd had a moderate geometric fold rise of 77 and 90, respectively.[12] All heterologous vaccination groups had a significantly higher increase in spike IgG levels compared to the homologous CoronaVac/CoronaVac group.

3.2.2. Antibodies to the RBD

Similar to the spike in IgG levels, the heterologous vaccination regimens generally induced high titers of RBD antibodies compared to homologous vaccinations (Table 3). In an RCT conducted in Brazil, Borobia *et al.*,[11] observed a significant rise in RBD antibody titer in participants who received heterologous ChAd/BNT from 71.46 BAU/mL to 7,756.68 BAU/mL 14 days after the booster dose.[11] Similarly, in an observational study by Firinu *et al.*,[13] participants who received the heterologous ChAd/BNT (497.6 AU/mL) showed a higher RBD antibody titer compared to the homologous ChAd (40.015 AU/mL) and homologous BNT (253.75 AU/mL) groups.[13] A Swedish study involving ChAd-primed individuals receiving an mRNA-1273 booster showed remarkably high RBD antibody levels (41680 BAU/mL) compared to individuals vaccinated with homologous ChAd (1224 BAU/mL).[25] Another study that investigated vaccine combinations of ChAd with BBV152 showed that the heterologous ChAd/BBV152 combination elicited higher RBD antibody levels (1,866 BAU/mL) when compared to the homologous BBV152 (710 BAU/mL) but was lower than the homologous ChAd regime (2,260 BAU/mL) regime.[18]

Table 2. Summary of studies included in this review

No	References	Country	Study design	Vaccine (dose)	Median age (range)	Groups	Number of subjects	Intervention	Interval between doses
1	Ai *et al.*,[6] 2022	China	Cohort study	BBIBP (0.5 mL)	45 (34 – 54)	Group 1 (homologous)	7	CoronaVac/ CoronaVac	2 – 4 weeks
				ZF2001 (0.5 mL)		Group 2 (homologous)	10	BBIBP/BBIBP	2 weeks
				CoronaVac (0.5 mL)		Group 3 (homologous booster)	10	BBIBP/BBIBP/ BBIBP	2 – 4 weeks; | 4 – 8 months for 3rd dose
						Group 4 (heterologous)	10	BBIBP/BBIBP/ ZF2001	
2	Atmar *et al.*,[7] 2022	United States	Clinical trial	mRNA-1273 (0.5 mL)	57 (24 – 81)	Group 1 (heterologous)	53	Ad26/m1273	12 weeks
				Ad26 (0.5 mL)	53 (24 – 76) 55 (22 – 85)	Group 2 (homologous)	51	m1273/m1273	
						Group 3 (heterologous)	50	BNT/m1273	
				BNT (0.3 mL)	50 (24 – 77)	Group 4 (homologous)	50	Ad26/Ad26	
					50 (20 – 75)	Group 5 (heterologous)	49	m1273/Ad26	
					50 (20 – 76)	Group 6 (heterologous)	51	BNT/Ad26	
					48 (22 – 74)	Group 7 (heterologous)	53	Ad26/BNT	
					54 y (23 – 75)	Group 8 (heterologous)	51	m1273/BNT	
					50 (19 – 80)	Group 9 (homologous)	50	BNT/BNT	
3	Barros-Martins *et al.*,8 2021	Germany	Prospective cohort study	ChAd (0.5 mL)	41 (21 – 64)	Group 1 (homologous)	32	ChAd/ChAd	2 – 3 months
				BNT (0.3 mL)	39 (22 – 61)	Group 2 (heterologous)	55	ChAd/BNT	
4	Behrens *et al.*,[9] 2021	Germany	Prospective study	ChAd (0.5 mL)	41 (24 – 64)	Group 1 (homologous)	12	ChAd/ChAd	Mean: 73.5 days
				BNT (0.3 mL)	46 (27 – 56)	Group 2 (heterologous)	11	ChAd/BNT	
5	Benning *et al.*,[10] 2021	Germany	Prospective single-center study	ChAd (0.5 mL)	55 (33 – 60)	Group 1 (homologous)	17	ChAd/ChAd	82 days
				BNT (0.3 mL)	30 (24 – 45)	Group 2 (heterologous)	35	ChAd/BNT	83 days
					45 (33 – 56)	Group 3 (homologous)	82	BNT/BNT	20 days
6	Borobia *et al.*,[11] 2021	Spain	RCT	ChAd (0.5 mL)	44 (18 – 49)	Group 1 (heterologous)	450	ChAd/BNT	8 – 12 weeks
			(Phase 2, Multicenter, Open-label)	BNT (0.3 mL)		Group 2 (control)	226	ChAd/-	
7	Clemens *et al.*,[12] 2021	Brazil	RCT (Phase 4,	CoronaVac (0.5 mL)	59 (22 – 98)	Group 1 (heterologous)	294	CoronaVac/Ad26	182 days

(Cont'd...)

Table 2. ***(Continued)***

No	References	Country	Study design	Vaccine (dose)	Median age (range)	Groups	Number of subjects	Intervention	Interval between doses
			Non-inferiority, Single blind)	Ad26 (0.5 mL)	61 (21 – 95)	Group 2 (heterologous)	333	CoronaVac/BNT	
				ChAd (0.5 mL)	60 (21 – 96)	Group 3 (heterologous)	296	CoronaVac/ChAd	
				BNT (0.3 mL)	58 (21 – 95)	Group 4 (homologous)	281	CoronaVac/ CoronaVac	
8	Firinu *et al.*,[13] 2021	Italy	Observational study	BNT (0.3 mL)	33 (IQR 5.2)	Group 1 (homologous)	50	BNT/BNT	3 weeks
				ChAd (0.5 mL)	34 (IQR 18)	Group 2 (homologous)	36	ChAd/ChAd	8 – 12 weeks
					25.2 (IQR 18.8)	Group 3 (heterologous)	49	ChAd/BNT	8 – 12 weeks
9	Glöckner *et al.*,[14] 2021	Germany	Observational study	BNT (0. 3 mL	45 (IQR 30 – 53)	Group 1 (homologous)	22	BNT/BNT	3 weeks
				ChAd (0.5 mL)	36 (IQR 32 – 44)	Group 2 (heterologous)	21	ChAd/m1273 or BNT	12 weeks
				mRNA-1273 (0.5 mL)					
10	Gram *et al.*,[15] 2021	Denmark	Cohort study	ChAd (0.5 mL)	45 (IQR 33 – 55)	Group 1 (control)	7809	ChAd/-	8 – 12 weeks
				BNT (0. 3 mL)	46 (IQR 34 – 55)	Group 2 (heterologous)	88050	ChAd/BNT	
				mRNA-1273 (0.5 mL)	46 (IQR 34 – 55)	Group 3 (heterologous)	48501	ChAd/m1273	
11	Groß *et al.*,[16] 2022	Germany	Cohort study	ChAd (0.5 mL)	30.5 (25 – 46)	Group 1 (heterologous)	26	ChAd/BNT	8 weeks
				BNT (0.3 mL)	41 (25 – 65)	Group 2 (homologous)	14	BNT/BNT	3 weeks
12	Hillus *et al.*,[17] 2021	Germany	Prospective cohort study	ChAd (0.5 mL)	34 (29 – 43)	Group 1 (homologous)	174	BNT/BNT	3 weeks
				BNT (0.3 mL)	51 (33 – 59)	Group 2 (homologous)	38	ChAd/ChAd	10 – 12 weeks
					37 (29 – 51)	Group 3 (heterologous)	104	ChAd/BNT	
13	Kant *et al.*,[18] 2021	India	Observational study	ChAd (0.5 mL)	65.5 (IQR 62 – 69)	Group 1 (homologous)	40	ChAd/ChAd	6 weeks
				BBV152 (0.5 mL)	56 (IQR 45.5 – 63)	Group 2 (homologous)	40	BBV152/BBV152	4 weeks
					62 (IQR 54.25 – 69.75)	Group 3 (heterologous)	18	ChAd/BBV152	6 weeks
14	Khong *et al.*,[19] 2022	Hong Kong	Prospective cohort study	CoronaVac (0.5 mL)	53 (26 – 76)	Group 1 (homologous)	15	BNT/BNT/BNT	Received first 2 vaccinations for>6 months
				BNT (0.3 mL)	47 (22 – 58)	Group 2 (heterologous)	5	BNT/CoronaVac/ BNT	

(Cont'd...)

Table 2. ***(Continued)***

No	References	Country	Study design	Vaccine (dose)	Median age (range)	Groups	Number of subjects	Intervention	Interval between doses
					58 (31 – 64)	Group 3 (homologous)	9	CoronaVac/ CoronaVac/ CoronaVac	
					58.5 (27 – 70)	Group 4 (heterologous)	8	CoronaVac/ CoronaVac/BNT	
15	Khoo *et al.*,[20] 2022	Singapore	Observational study	Ad26 (0.5 mL)	39.2 (25 – 69)	Group 1 (control)	13	Ad26/-	
				BNT (0.3 mL)	51.2 (25 – 75)	Group 2 (homologous)	28	Ad26/Ad26	41 – 71 days
					42.8 (32 – 53)	Group 3 (control)	16	BNT/-	
					42 (23 – 62)	Group 4 (homologous)	44	BNT/BNT	21 – 104 days
					45.5 (25 – 70)	Group 5 (heterologous)	14	Ad26/BNT	11 – 180 days
16	Li *et al.*,[21] 2022	China	RCT	CoronaVac (0.5 mL)	47.0 (IQR 40.3 – 51.0)	Group 1 (A) (homologous) 3 dose regimen cohort	100	CoronaVac/ CoronaVac/ Convidecia	3 – 6 months for 3rd dose
				Convidecia (0.5 mL)	47.0 (IQR 41.0 – 52.0)	Group 2 (B) (homologous) 3 dose regimen cohort	100	CoronaVac/ CoronaVac/ CoronaVac	
					47.0 (IQR 35.0 – 51.0)	Group 3 (C) (heterologous) 2 dose regimen cohort	50	CoronaVac/ Convidecia	14 – 21 days
					43.5 (IQR 38.5 – 49.3)	Group 4 (D) (homologous) 2 dose regimen cohort	50	CoronaVac/ CoronaVac	
17	Liu *et al.*,[22] 2021	United Kingdom	RCT	ChAd (0.5 mL)	57.6 (50.1 – 69.1)	Group 1 (homologous)	90	ChAd/ChAd	8 – 12 weeks
				BNT (0.3 mL)	57.6 (50.3 – 68.1)	Group 2 (heterologous)	90	ChAd/BNT	8 – 12 weeks
					57.7 (50.2 – 69.3)	Group 3 (homologous)	93	BNT/BNT	3 weeks
					56.1 (50.5 – 68.9)	Group 4 (heterologous)	90	BNT/ChAd	3 weeks
18	Moghnieh *et al.*,[23] 2021	Lebanon	Prospective cohort study	BNT (0.3 mL)	56 (IQR 41 – 75)	Group 1 (homologous)	50	BNT/BNT (Covid naïve)	2 weeks
				BBIP (0.5 mL)	37 (IQR 29 – 61)	Group 2 (homologous)	25	BNT/BNT (COVID recovered)	2 weeks
					52 (IQR 47 – 63)	Group 3 (heterologous)	50	BBIP/BBIP/BNT	3rd dose interval: 3 months

(Cont'd...)

Table 2. ***(Continued)***

No	References	Country	Study design	Vaccine (dose)	Median age (range)	Groups	Number of subjects	Intervention	Interval between doses
19	Munro *et al.*,[24] 2021	United Kingdom	RCT	ChAd (0.5 mL)	68.1 (IQR 55.1 – 75.9)	Group A (prime with ChAd/ ChAd)	109	Control	3rd dose interval: 84 days
				BNT (0.3 mL)	67.8 (IQR 52.2 – 75.7)		111	ChAd (homologous)	
				Ad26 (0.5 mL)	65.3 (IQR 52.6 – 74.1)		115	NVX (heterologous)	
				mRNA-1273 (0.5 mL)	65.8 (IQR 49.9 – 75.6)		108	NVX half (heterologous)	
				VLA (0.5 mL and 0.25 mL)					
					62.4 (IQR 49.4 – 78.5)	Group A (prime with BNT/ BNT)	118	Control	
				Cvn (0.6 mL)	61.9 (IQR 46.5 – 76.3)		109	ChAd (heterologous)	
				MenACWY (0.5 mL) Control	62.7 (IQR 48.0 – 75.5)		114	NVX (heterologous)	
					62.2 (IQR 49.9 – 77.3)		112	NVX half (heterologous)	
					72.6 (IQR 57.6 – 77.2)	Group B (prime with ChAd/ChAd)	106	Control	
					71.4 (IQR 53.8 – 77.0)		107	BNT (heterologous)	
					71.8 (IQR 51.2 – 76.5)		109	VLA (heterologous)	
					71.0 (IQR 51.2 – 75.9)		111	VLA half (heterologous)	
					71.9 (IQR 51·0 – 76.4)		108	Ad26 (heterologous)	
					63.5 (IQR 50·4 – 78·3)	Group B (prime with BNT/ BNT)	109	Control	
					64·2 (IQR 49·8 – 77.4)		110	BNT (homologous)	
					61.2 (IQR 46.2 – 77.7)		110	VLA (heterologous)	
					62.0 (IQR 51.8 – 76.2)		110	VLA half (heterologous)	
					61.6 (IQR 49.2 – 78.3)		106	Ad26 (heterologous)	
					70.3 (IQR 54.4 – 75.1)	Group C (prime with ChAd/ ChAd)	114	Control	
					71.0 (IQR 55.8 – 75.3)		117	BNT half (heterologous)	
					70.2 (IQR 53.0 – 75.3)		112	m1273 (heterologous)	
					70.3 (IQR 54.8 – 75.1)		119	CVn (heterologous)	

(Cont'd...)

Table 2. ***(Continued)***

No	References	Country	Study design	Vaccine (dose)	Median age (range)	Groups	Number of subjects	Intervention	Interval between doses
					66.8 (IQR 51.9 – 78.0)	Group C (prime with BNT/BNT)	112	Control	
					64.4 (IQR 47.7 – 78.2)		110	BNT half (homologous)	
					65.0 (IQR 50.3 – 75.5)		111	m1273 (heterologous)	
					63.4 (IQR 47.3 – 76.6)		106	CVn (heterologous)	
20	Normark *et al.*,[25] 2021	Sweden	Observational study	ChAd (0.5 mL)	46 (28 – 62)	Group 1 (homologous)	37	ChAd/ChAd	9 – 12 weeks
				mRNA-1273 (0.5 mL)	40 (23 – 59)	Group 2 (heterologous)	51	ChAd/m1273	
21	Rose *et al.*,[26] 2022	Germany	Observational study	ChAd (0.5 mL)	27 (18 – 56)	Group 1 (heterologous)	40	ChAd/BNT	10 – 12 weeks
				BNT (0.3 mL)	41 (23 – 61)	Group 2 (homologous)	9	ChAd/ChAd	10 – 12 weeks
					35 (23 – 51)	Group 3 (homologous)	8	BNT/BNT	2 weeks
22	Schmidt *et al.*,[27] 2021	Germany	Observational study	ChAd (0.5 mL)	48.6	Group 1 (homologous)	55	ChAd/ChAd	8 – 12 weeks
				BNT (0.3mL)	40.8	Group 2 (heterologous)	96	ChAd/mRNA (either BNT or m-1273)	8 – 12 weeks
				mRNA-1273 (0.5 mL)	44.7	Group 3 (homologous)	62	mRNA/mRNA	3 weeks
23	Stuart *et al.*,[28] 2022	United Kingdom	RCT	ChAd (0.5 mL)	64.4 (50.1 – 74.2)	Group 1 (homologous)	180	ChAd/ChAd	8 – 12 weeks
				BNT (0.3 mL)	64.1 (50.2 – 74.4)	Group 2 (heterologous)	181	ChAd/m1273	
				mRNA-1273 (0.5 mL)	64.2 (50.1 – 74.6)	Group 3 (heterologous)	179	ChAd/NVX	
				NVX (0.5 mL)	62.3 (50.4 – 77.1)	Group 4 (homologous)	175	BNT/BNT	
					62.4 (50.0 – 77.7)	Group 5 (heterologous)	177	BNT/m1273	
					62.7 (50.2 – 78.1)	Group 6 (heterologous)	180	BNT/NVX	
24	Tenbusch *et al.*,[29] 2021	Germany	Observational study	ChAd (0.5 mL)	38.7 (20 – 65)	Group 1 (homologous)	537	BNT/BNT	2 weeks
				BNT (0. 3mL)	57 (31 – 64)	Group 2 (homologous)	66	ChAd/ChAd	9 – 12 weeks
					49.6 (18 – 65)	Group 3 (heterologous)	482	ChAd/BNT	9 – 12 weeks
25	Vallée *et al.*,[30] 2021	France	Retrospective, cross-sectional monocenter study	ChAd (0.5 mL)	37 (IQR 24 – 50)	Group 1 (heterologous)	130	ChAd/BNT	12 weeks
				BNT (0. 3mL)	32 (IQR 21 – 43)	Group 2 (homologous)	67	BNT/BNT	4 weeks

(Cont'd...)

Table 2. ***(Continued)***

No	References	Country	Study design	Vaccine (dose)	Median age (range)	Groups	Number of subjects	Intervention	Interval between doses
26	Zhang *et al.*,[31] 2022	Hong Kong	Open trial	CoronaVac (0.5 mL) BNT (0.3 mL)	44.5 (IQR 36 – 50.5)	Group 1 (heterologous)	42	BNT/CoronaVac	4 weeks
					49 (IQR 39.5 – 54.5)	Group 2 (homologous)	41	CoronaVac/ CoronaVac	
					47 (IQR 33 – 51.75)	Group 3 (homologous)	40	BNT/BNT	
27	Sila *et al.*,[32] 2024	Thailand	Prospective cohort study	CoronaVac ChAd	Not available	Group 1 (non-vaccinated control)	23	Control	Not available
				BNT		Group 2 (homologous)	14	Non-mRNA (CoronaVac, ChAd)	
				mRNA-1273		Group 3 (homologous)	5	mRNA (BNT, m1273)	
						Group 4 (heterologous)	10	non-mRNA combined with mRNA vaccine	
28	Xu *et al.*,[33] 2023	China	Case-case study	CoronaVac	Not available	Group 1 (homologous)	40	CoronaVac/ CoronaVac	Not available
				BBIBP-CorV		Group 2 (homologous)	20	BBIBP/BBIBP	
						Group 3 (heterologous)	7	CoronaVac/ BBIBP	
						Group 4 (homologous)	8	CoronaVac/ CoronaVac/ CoronaVac	
						Group 5 (homologous)	7	BBIBP/BBIBP/ BBIBP	
						Group 6 (heterologous)	2	CoronaVac+ BBIBP	
29	Mok *et al.*,[34] 2022	China	RCT	CoronaVac	51.50 (44.25 – 57)	Group 1 (homologous)	40	CoronaVac/ CoronaVac/ CoronaVac	Not available
				BNT	50.00 (IQR 45.25 – 57)	Group 2 (heterologous)	40	CoronaVac/ CoronaVac/BNT	
30	Gerhards *et al.*,[35] 2023	Germany	Cohort study	ChAd	39.64 (24.83 – 54.45)	Group 1 (homologous)	26	ChAd/ChAd	Not available
				BNT		Group 2 (heterologous)	53	ChAd/BNT	
				mRNA-1273		Group 3 (homologous)	4	BNT/BNT	
						Group 4 (homologous)	1	m1273/m1273	
						Group 5 (homologous)	47	ChAd/BNT/ mRNA	
						Group 6 (heterologous)	24	ChAd/ChAd/ mRNA	

(Cont'd...)

Table 2. ***(Continued)***

No	References	Country	Study design	Vaccine (dose)	Median age (range)	Groups	Number of subjects	Intervention	Interval between doses
31	Intapiboon *et al.*,[36] 2021	Thailand	RCT	BNT 0.3 mL, IM (Sinovac)	39.9 (18 – 61)	Group 1 (heterologous)	30	CoronaVac/ CoronaVac/BNT	Interval of 1st and 2nd dose: 21 days. Interval of 3rd dose: 73 days
				BNT 0.15 mL IM)		Group 2 (heterologous)	30	CoronaVac/ CoronaVac/BNT	
				BNT 0.06 mL, ID		Group 3 (heterologous)	31	CoronaVac/ CoronaVac/BNT	
				CoronaVac (0.5 mL)		Group 4 (homologous)	30	CoronaVac/ CoronaVac	

Abbreviations: IQR: Interquartile range; RCT: Randomized controlled trial.

Table 3. Comparing immune response parameters obtained from homologous versus heterologous COVID-19 vaccination regime

Immune response parameters	Total studies	Number of studies showing higher immune parameters with *heterologous COVID-19 vaccination regime (*n* [%])
Anti-spike IgG antibodies	24	19 (79)
Anti-Spike IgA antibodies	3	3 (100)
Anti-receptor binding domain antibodies	10	8 (80)
T-cell responses	3	3 (100)

Note: *Heterologous: Different COVID-19 vaccines used in the first and second vaccination schedules.

An observational study in Germany reported higher anti-RBD/S1 antibody levels ($P < 0.05$) in a heterologous group (ChAd/BNT: 9,378.50 U/mL) compared to a homologous group (ChAd/ChAd: 826.30 U/mL).[35]

3.2.3. T-cell responses

In general, the heterologous vaccinations demonstrated greater T-cell response to the spike protein compared to homologous vaccinations (Table 3). A prospective cohort study conducted in Germany by Hillus *et al.*,[17] demonstrated that participants vaccinated with heterologous ChAd/BNT had higher spike T-cells against the SARS-CoV-2 virus with a value of 4762 mIU/mL compared to homologous ChAd and homologous BNT vaccinations (1061 mIU/mL, 2,026 mIU/mL, respectively).[17] In the Com-COV and Com-COV 2 RCT, groups who received heterologous ChAd with BNT, mRNA-1273 or NVX all demonstrated spike T cells levels that were higher than homologous ChAd and homologous BNT vaccinations (ChAd/BNT: 184 SFC per million PBMC, ChAd/mRNA-1273: 148 SFC per million PBMC, ChAd/NVX: 190 SFC per million PBMC).[22,28] Another study that observed the effect of heterologous Ad 26 COV 2/BNT vaccination also showed higher frequency of spike T cells level compared to its corresponding homologous vaccination group (347.5 – 152 SFC/10^6 PBMC).[20]

3.2.4. Neutralizing antibodies

The studies measured neutralizing antibody titers for pseudo-type viruses and the SARS-CoV-2 variants of concern (VOC), including alpha, beta, delta, and the Omicron variant. Overall, receiving a heterologous vaccine regimen produced higher neutralizing antibody titers and had more potent neutralizing activity against VOC compared to homologous vaccination (Table 4).

In the Com-COV study by Liu *et al.*,[22] heterologous ChAd/BNT vaccination revealed a higher neutralizing antibody titer than homologous ChAd/ChAd (antibody titer: 515 to 61) with a GMR of 8.5, demonstrating non-inferiority-of-heterologous-vaccination against homologous vaccination.[22] In contrast, participants vaccinated with heterologous BNT/ChAd had lower neutralizing antibodies compared to the group receiving homologous BNT/BNT (antibody titer: 383 to 574).[22] In addition, two other studies reported higher neutralizing antibodies, in terms of percentage of inhibition, for heterologous ChAd/BNT (96.80 – 100%) and homologous BNT/BNT (97 – 100%) compared to homologous ChAd/ChAd (93.50 – 98%).[10,26] In the Com-COV 2 study, both heterologous ChAd/mRNA-1273 and ChAd/NVX vaccinations were not inferior to homologous ChAd/ChAd, with a GMR of 10 and 3.4 while exhibiting robust neutralizing antibody titers of 1358 and 473, respectively.[28] Likewise, heterologous vaccination with BNT/mRNA-123 and BNT/NVX showed non-inferiority to homologous BNT/BNT vaccination with a GMR of 1.4 and 0.9, respectively, with neutralizing antibody titers of 1260 and 787.[28] A clinical trial conducted by Atmar *et al.*[7] demonstrated that heterologous vaccination with Ad 26/mRNA-1273 and Ad 26/BNT showed higher

Table 4. Comparing the neutralizing antibody levels from homologous versus heterologous COVID-19 vaccination regimes among the different variants of concern of COVID-19 virus

Variants	Total studies	Number of studies showing higher neutralizing antibody levels with *heterologous COVID-19 vaccination regime (*n* [%])
Wild-type	22	17 (77)
VOC-Alpha	6	4 (67)
VOC-Beta	11	9 (81)
VOC-Delta	11	9 (81)
Omicron	5	3 (60)

Note: *Heterologous: Different COVID-19 vaccines used in the first and second vaccination schedules.
Abbreviation: VOC: Variant of concern.

neutralizing antibody titers of 676 and 344, which was higher than homologous Ad 26/Ad 26 vaccination.[7] Clemens *et al.* elucidated that heterologous vaccination with CoronaVac/Ad 26, CoronaVac/BNT, and CoronaVac/ChAd all produced high neutralizing antibody titers compared to homologous CoronaVac vaccination (211.1) with CoronaVac/BNT vaccination having the greatest geometric mean fold rise of 175.5.[12] Another observational study showed that participants who received heterologous BNT/CoronaVac had lower surrogate neutralizing antibody levels compared to homologous BNT/BNT (37.1 to 70.6) vaccinations but higher compared to homologous CoronaVac vaccinations (5.5).[31]

Against the VOC, all heterologous vaccinations effectively neutralized Alpha, Beta, Gamma, and Delta variants with high neutralizing antibody titers when compared to homologous vaccination regimens. Among the heterologous regimens, ChAd/BNT and ChAd/BBV-152 produced substantially higher neutralizing activity against alpha and beta variants compared to homologous vaccination regimens (ChAd/BNT: Alpha: 212.5, Beta: 48.5; ChAd/ChAd: Alpha: 212.5, Beta: 48.5; BNT/BNT: Alpha: 369.2, Beta: 72.4; ChAd/BBV152: Alpha: 396.1, Beta: 15; BBV152/BV152: Alpha: 112.4, Beta: 52.09, Delta: 54.37).[8,16,17,19,27] Heterologous ChAd/mRNA-1273 vaccination and BNT/mRNA-1273 produced greater neutralizing antibody titers of 672 and 863, respectively, against the Delta.[28]

3.3. Comparison of immunogenicity in heterologous third dose regimen

A total of eight studies investigated the immunogenicity of a third booster dose in participants vaccinated with two doses of COVID-19 vaccines, reporting on 14 combinations of heterologous prime-boost vaccinations. A prospective cohort study conducted in Hong Kong observed that heterologous vaccination groups (BNT-CoronaVac-BNT and CoronaVac-CoronaVac-BNT) elicited higher neutralizing antibodies (geometric mean titer (GMT) of B-C-B: wild type: 106, Beta variant: 106, Delta: 139, Omicron: 10; C-C-B: wild type: 207, Beta: 87.2, Delta: 160, Omicron: 23.8) against wild type, Beta, Delta, and Omicron variants than the homologous vaccination group (GMT of C-C-C: wild type: 34.3, Beta variant: 18.5, Delta: 20, Omicron: 5) that received three dosages of the CoronaVac vaccine.[19] In contrast, the homologous group that received three dosages of BNT vaccine showed the highest immunogenicity (GMT of B-B-B: wild type: 306, Beta variant: 175, Delta: 184, Omicron: 27.6) compared to the heterologous groups. These findings are further supported by a RCT in Hong Kong, whereby the heterologous vaccinated group (CoronaVac-CoronaVac-BNT) displayed significantly higher percent inhibition of sVNT against the β, γ, and δ variants (β: 92.29%; γ: 92.51%; δ: 95.33%) than the homologous CoronaVac-boosted group (β: 38.79%; γ: 32.22%; δ: 48.87%).[34]

Another study that analyzed the immunogenicity of the same vaccine platform, observed that participants primed with two doses of CoronaVac who received a full intramuscular dose of BNT booster had higher RBD antibody titers and neutralizing antibodies against the Delta variant compared to the other groups that received BNT booster at half dose through intramuscular or a fractional dose (1/5 dose) through intradermal. All heterologous BNT-boosted groups were reported to have significantly higher immunogenicity compared to the non-boosted homologous (CoronaVac-CoronaVac) group.[36]

Compared with a different vaccine platform, participants primed with 2 doses of CoronaVac who received a heterologous Convidecia booster showed a better immunogenic profile compared to the homologous three-dose CoronaVac group, with a higher level of RBD-specific IgG GMT and IFN-γ+ spot counts (heterologous group: 3090.1, 65 SFC per 10^6 PBMCs) (homologous group: 369.0, 60 per 10^6 PBMCs). Similarly, the heterologous group displayed significantly higher neutralizing antibody GMTs against wild-type and Delta variants compared to the homologous group (heterologous group: wild type: 150.3, Delta: 55.0; heterologous group: wild type: 35.3, Delta: 6.6).[21]

The COV-BOOST RCT by Munro *et al.*,[24] observed the immunogenic profile of an extensive list of vaccine platforms as a third dose booster. Results demonstrated that groups who were primed with two doses of ChAd and received an mRNA booster had the highest titers of spike

IgG, neutralizing antibodies, and spike T cells against the SARS-CoV-2 virus when compared to the homologous ChAd booster group. For the ChAd/ChAd/BNT group, values were 20517 ELU/ml for spike IgG antibody levels, 1621 for neutralizing antibodies, and 115.5 SFC per 10^6 PBMC for spike T cells. The ChAd/ChAd/mRNA-1273 groups had values of 3,111 ELU/ml, 2,368 and 128.9 SFC per 10^6 PBMC, respectively. For the other heterologous vaccine booster groups, including NVX, VLA, Ad 26, and CVn, the spike IgG, spike T cells, and neutralizing antibodies produced were generally higher compared to the homologous ChAd booster group.[24] On the other hand, participants who were primed with 2 doses of BNT and received a heterologous NVX, VLA, or Ad 26 vaccine all had lower immunogenicity in terms of spike IgG, spike T-cell, and neutralizing antibody titers when compared to participants who received a homologous BNT booster. The exception was seen in the heterologous BNT/BNT/mRNA-1273 group that showed higher spike IgG, neutralizing antibodies, and spike T cells of 33,768 ELU/mL, 2,019, and 112 SFC per 10^6 PBMC, respectively.[24] A study conducted in China by Ai *et al.*,[6] showed that heterologous vaccinated participants who had received two doses of an inactivated vaccine (BBIBP-CorV) and a protein subunit (Zifivax) booster produced higher neutralizing antibodies against SARS-CoV-2 VOC, including Beta, Delta, and Omicron, with GMTs of 789.60, 1501, and 95.86, respectively, compared to the homologous BBIBP-CorV booster group (GMT of homologous group: Beta variant: 215.7, Delta: 250.8, Omicron: 48.73) at 14 days post booster vaccination.[6] A similar finding was reported from a case-case study in China where heterologous vaccination of BBIBP-CorV and CoronaVac displayed higher vaccine efficacy against pneumonia and severe disease (79.5%) compared to the homologous vaccination groups (CoronaVac: 61.8%; BBIBP-CorV: 70.1%).[33]

Lastly, a study investigated the immunogenicity of mRNA-based vaccines as boosters in subjects who previously received heterologous ChAd/BNT and homologous ChAd/ChAd vaccination. No significant difference was observed in anti-RBD/S1 antibody level between ChAd/BNT/mRNA group and ChAd/ChAd/mRNA group (12,852 U/mL versus 10,582 U/mL).[35]

4. Discussion

The primary objective of this scoping review was to collate and summarize scientific evidence available for heterologous and homologous vaccinations by comparing their immunogenic profiles and discerning if heterologous vaccinations provide better immunity against the SARS-CoV-2 virus. The evidence from the included studies showed that heterologous vaccination regimens generally elicit a strong immune response against the SARS-CoV-2 virus in terms of spike IgG antibodies, RBD antibodies, neutralizing antibodies, and spike T cells, compared to homologous vaccination regimens. Depending on the vaccine type, heterologous vaccination with a viral vector/mRNA vaccine, such as ChAd/BNT, exhibited robust immunogenicity, but when the order of vaccines was reversed (mRNA/viral vector), the immune response was less potent. Similarly, heterologous regimens that involve priming with an inactivated vaccine and receiving a viral vector or mRNA vaccine boost, such as CoronaVac/ChAd or CoronaVac/BNT, both demonstrated strong immune responses, with the mRNA vaccine booster being superior. Even heterologous regimens that involve BNT/mRNA-1273 seem to elicit a stronger humoral and cellular response despite being produced by different companies and sharing the same vaccine platform. Similar results were derived from an animal study that involved heterologous vaccination of mice with an RNA vaccine and a viral vector vaccine. The outcomes were strong cellular immunity and high neutralizing antibody titers, therefore providing the prospect of implementing heterologous vaccination regimens in humans.[37]

The underlying mechanism of heterologous vaccinations remains ambiguous; however, established evidence of the immunological mechanism of different vaccine types could explain this phenomenon. Firstly, mRNA vaccines can produce high titers of neutralizing and RBD antibodies but have lower CD8+ T cell response, while viral vector vaccines produce potent T cell response and have polyfunctional antibodies to mediate neutralizing antibodies. Inactivated vaccines, on the other hand, generally induce humoral immunity and produce neutralizing antibodies.[38,39] This demonstrates that vaccines developed from different platforms provide protection against the SARS-CoV-2 virus through divergent immunological pathways, and therefore, receiving a heterologous vaccination would allow benefits to be reaped from both sides.

Vaccine effectiveness and efficacy were demonstrated in two studies. Gram *et al.* demonstrated the effectiveness of heterologous vaccination of ChAd and mRNA (BNT or mRNA-1273) vaccination (66 – 88%) to be higher than a single dose of ChAd (–47 – 44%).[15] Xu *et al.*[33] reported superior vaccine efficacy against pneumonia and severe disease from heterologous vaccination of BBIBP-CorV and CoronaVac (79.5%) compared to the homologous vaccination cohorts (CoronaVac: 61.8%; BBIBP-CorV: 70.1%).[33] Regardless of the combination of vaccines administered, both studies have reported higher VE from heterologous vaccination compared to homologous vaccination, supporting the deployment of a heterologous

vaccination regime. Despite there is hesitancy regarding how immunogenicity of heterologous vaccines correlates to vaccine effectiveness against SARS-CoV-2 infections when administered in the real-world environment, a study by Khoury *et al.*[40] elucidated that utilization of neutralizing antibody titers was a good predictor of immunity against symptomatic COVID-19 infection. In terms of the reactogenicity of heterologous vaccines, common local and systemic side effects were experienced and generally tolerable. In addition, there were no significant vaccine related adverse events reported by the studies.

The studies included in this scoping review had several limitations. Firstly, the number of participants included was inconsistent among the studies and, therefore, would affect the precision of results when comparing between groups. With regards to measuring immunogenicity, the units of measurement were not congruent among the studies, and there were difficulties in determining an accurate unit conversion due to possible differences in immunoassays or methods of measurement used between studies. In addition, the interval between prime and booster doses was incongruent between studies.

The review process, while synthesizing the following scoping review, was subject to several limitations. With regards to study design, most of the included studies were observational studies (cohort studies, cross-sectional studies), and according to the hierarchy of evidence, the evidence quality is ranked lower compared to RCTs. In addition, direct comparisons of data from the studies may not give the most precise estimate of the effect and would require a meta-analysis to be carried out.

5. Conclusion

In conclusion, this scoping review concludes that heterologous vaccination regimens generate a higher humoral and cellular immune response against SARS-CoV-2 compared to homologous regimens. The heterologous vaccinations appear to exhibit stronger neutralizing antibody activity against VOC, including alpha, beta, gamma, delta, and Omicron. However, it is important to note that there are similarities and differences between the spike protein epitopes in the different variants of COVID-19 and that several variants may be circulating at the same time. The reactogenicity profile of heterologous vaccination shows tolerable local and systemic side effects with no significant adverse events. Integration of heterologous vaccinations into COVID-19 vaccination strategies could strengthen immunity, particularly against the emerging variants, alleviate concerns about vaccine shortages, and achieve herd immunity.

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Conflict of interest

The authors declare that they have no competing interests.

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ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

Interplays between host pattern-recognition receptors and pathogen ligands in immunogenic cell death

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Abstract

The strategic induction of cell death serves as a crucial immune defense mechanism for the eradication of pathogen infections within host cells. Investigating the molecular mechanisms underlying immunogenic cell pathways has significantly enhanced our understanding of the host's immunity. This review provides a comprehensive overview of the immunogenic cell death mechanisms triggered by pathogen infections, focusing on the critical role of pattern recognition receptors. In response to infections, host cells dictate a variety of cell death pathways, including apoptosis, pyroptosis, necrosis, and lysosomal cell death, which are essential for amplifying immune responses and controlling pathogen dissemination. Key components of these mechanisms are host cellular receptors that recognize pathogen-associated ligands. These receptors activate downstream signaling cascades, leading to the expression of immunoregulatory genes and the production of antimicrobial cytokines and chemokines. Particularly, the inflammasome, a multi-protein complex, plays a pivotal role in these responses by processing pro-inflammatory cytokines and inducing pyroptotic cell death. Pathogens, in turn, have evolved strategies to manipulate these cell death pathways, either by inhibiting them to facilitate their replication or by triggering them to evade host defenses. A deeper understanding of immunogenic cell death is crucial for developing novel immunotherapies, advancing infectious disease and cancer treatment, and revealing the complex interactions between dying cells and the immune system. This review aims to provide systematic summarization as well as recent proceedings regarding the dynamic interplay between host immune mechanisms and pathogen strategies, highlighting the intricate co-evolution of microbial virulence and host immunity.

Keywords: Cell death; Ligands; Receptors; Inflammasome; Pathogens; Apoptosis; Pyroptosis; Lysosomes

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1. Introduction

Immunogenic cell death induced by infections caused by pathogens plays a major role in host immune responses to eradicate evading bacteria or viruses.[1] Investigation of signaling pathways involved in host innate immunity has revealed the rich and diverse mechanisms that govern the sensing of immune cells to various ligands, particularly pathogen-associated molecular patterns (PAMPs).[2] Upon the recognition of PAMPs, the host germ line-encoded pattern recognition receptors (PRRs) dictate host antimicrobial responses as well as proinflammatory reactions.[3] Subsequently, PRRs located at the cell surface or intracellularly activate a series of downstream signaling cascades, involving ligands, receptors, adaptor molecules, kinases, and transcription factors.[4] The activation of these signal transduction pathways commands the host to express a wide array of immunoregulatory genes, resulting in the synthesis of cytokines and chemokines that recruit other activated immune cells to eliminate the invading pathogen.[5] While the execution of the innate immune response is accomplished by the actions of phagocytes and antigen-presenting cells, the orchestration of adaptive immunity is facilitated by specialized immune cells.[6] This review mainly discusses recent proceedings regarding pathogen-mediated receptor signaling and cell death in innate immune cells, with an emphasis on macrophages and dendritic cells.

Host antimicrobial responses following the PRR-mediated signaling include proinflammatory reactions and immunogenic cell death.[3,7] When inflammation and other innate immune responses fail to combat the infection, infected cells opt to initiate diverse pathways that lead to immunogenic cell death.[1] These diverse forms of cell death play crucial roles in amplifying various downstream immune responses, restricting pathogen dissemination, and eliminating infections.[1] Apoptosis, pyroptosis, necrosis, and lysosomal cell death, representing the predominant and extensively investigated types of cell death triggered by pathogen infections,[8,9] are discussed in this review.

Investigating the myriad manifestations of immunogenic cell death instigated by pathogen infection is pivotal across several scientific domains.[10] Such research provides pivotal insights into the pathophysiology of infectious diseases, facilitating the formulation of bespoke therapeutic and prophylactic approaches.[11] In addition, enhanced comprehension of immunogenic cell death mechanisms is instrumental in refining immunization strategies and therapeutic modalities to bolster endogenous immune defenses against pathogenic assaults.[12] This review encapsulates the forefront of discoveries in delineating PAMPs-recognizing host receptors that trigger the activation of inflammasomes and alternative signal transduction pathways, which culminates in an array of immunogenic cell death phenotypes.

2. Host receptors for pathogen component recognition

The dynamic interaction between PAMPs and PRRs empowers the host to distinguish self-entities from foreign pathogens and to efficiently deter pathogenic invasions. Despite the immense diversity in the microbial constitution, the host is nonetheless able to distinguish them through a small number of receptors using mechanisms that are strikingly similar yet significantly distinct.[13] Within the PRR family, members include Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs), and C-type lectin receptors (CLRs).[2] The activation of PRRs typically leads to the assembly of the inflammasome complex, a crucial sensor and mediator that subsequently triggers the activation of downstream inflammatory signalings.[14] The forefront of research focused on deciphering the complex interactions between hosts and pathogens, identifying novel PAMP-PRR interactions as well as the intricate mechanisms of recognition and the subsequent signaling cascades[3] (Figure 1). For instance, the primate-specific protein, NLR family pyrin domain-containing protein 11 (NLRP11) has been newly identified as a PRR for cytosolic lipopolysaccharide (LPS), necessary for activating the caspase-4 inflammasome in human macrophages during infection by Gram-negative bacteria.[15]

2.1. Role of Toll-like receptors in immune surveillance

Among all PRRs, members of the TLRs family have received the most attention from researchers in the past decades.[16] TLRs were initially identified due to their homology with the *Drosophila melanogaster* Toll protein, which acts as an immune guarder in the defense against fungal infections.[17] The observation that *Drosophila melanogaster* lacking Toll protein is susceptible to fungal infection contributed to the discovery of the importance of Toll protein in other species.[18] TLRs are expressed on cell membranes of diverse antigen-presenting cells, including macrophages and dendritic cells.[19] Although ubiquitously expressed in many scenarios, specific TLR expression can be inducible and exclusive to pathogen infections.[20]

The TLR is composed of an extracellular domain containing the leucine-rich repeat (LRR) motif, and a Toll/interleukin-1 (receptor [TIR] homology domain

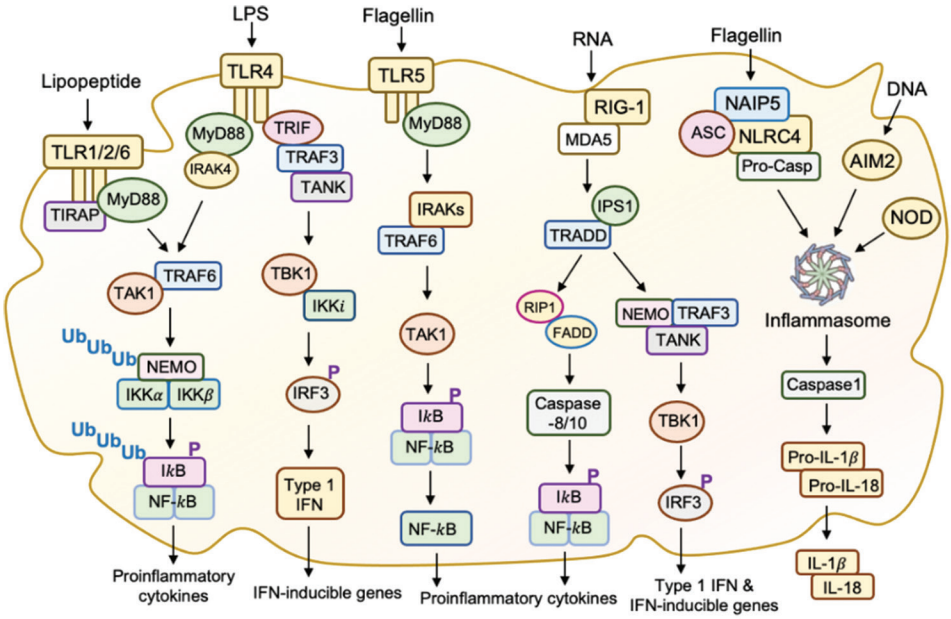

Figure 1. The complex network of signaling pathways involved in the innate immune response. The major role of various pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-1), and NOD-like receptors (NLRs) is detecting pathogen-associated molecular patterns (PAMPs) such as lipopeptides, flagellin, RNA, and DNA, respectively. Upon activation, these receptors initiate a cascade involving the interactions between the receptors with corresponding adaptor proteins (*e.g.*, MyD88, TRIF, and TRADD), kinases (*e.g.*, IRAKs, TAK1, and TBK1), and other molecules (*e.g.*, NEMO, IKK complex, and IRF3). The activation of these enzymes catalyzes the post-translational modification of downstream effector proteins that ultimately lead to the transcriptional activation of nuclear factor kappa B (NF-κB) and IRF3, which then translocate into the nucleus to regulate gene expressions. This results in the production of proinflammatory cytokines and type I interferons (IFN-I), which are crucial for the inflammatory response and the establishment of an antiviral state. The assembly of the inflammasome, a multiprotein oligomer that activates caspase-1, leads to the processing and secretion of proinflammatory cytokines interleukin (IL)-1β and IL-18. In addition, ubiquitination (Ub) and phosphorylation (P) play critical roles in mediating the sequential activation of the molecules in these pathways, emphasizing the tight regulation of signaling required for an appropriate immune response. Image provided by the author.

which facilitates intracytoplasmic signaling. On binding to pathogenic ligands, TLRs undergo oligomerization, triggering the onset of intracellular signal transmission.[20] In humans, 10 TLRs have been identified to date, and these can be categorized into various subfamilies according to the PAMPs they recognize as well as their subcellular localizations. TLR1/TLR2/TLR4/TLR5/TLR6/TLR10 are distributed on the surface of immune cells, whereas TLR3/TLR7/TLR8/TLR9 are mostly found on intracellular organelles, including endosomes and lysosomes.[20] TLRs on the cell surface can only recognize portions of bacteria, whereas TLRs on the endo-lysosomal membranes can bind to components of the host cell, thereby activating a wider variety of outcomes.[21] Among these TLRs, TLR5, and TLR4 are the most extensively studied TLRs with regard to their involvement in sensing canonical components from bacterial infections.[3] Specific single nucleotide polymorphisms (SNPs) in TLR genes are associated with increased or decreased risks of conditions such as cancer, diabetes, and infectious diseases across different populations.[22,23] These genetic variations may impact experimental outcomes in cell lines used for research, underscoring the importance of screening for TLR SNPs in relevant studies.[23,24]

TLR5 is able to detect flagellin, the subunit constituting the filament of bacterial flagella.[25] This structural element facilitates bacterial locomotion toward propitious environments and aids in the evasion of host immune defenses.[26] Upon TLR5 binding with flagellin, MyD88 is recruited to TRAF6, subsequently activating TAK1. This activation results in the phosphorylation of nuclear factor kappa B (NF-κB) and its subsequent translocation to the nucleus.[27] TLR5's detection of flagellin is an essential part of the innate immune responses to bacterial infections, especially those caused by motile bacteria such as *Salmonella* Typhimurium and *Pseudomonas aeruginosa*.[27]

TLR4 is another crucial component of the host's innate immunity, responsible for sensing LPS of Gram-negative bacteria.[28] LPS is a complex molecule composed of a lipid element that attaches to the outer membrane and a polysaccharide portion that stretches outward.[28] On the one hand, LPS is recognized by TLR4 located on the cell membrane, further activating a MyD88-TAK1-NF-κB

signaling cascade. On the other hand, LPS is detected by endosome-localized TLR4, activating TRIF-mediated IRF3/7 phosphorylation.[29] The activation of both NF-κB and IRF3/7 promotes the expression of type I interferons (IFN-I) as well as pro-inflammatory cytokines, ultimately recruiting and activating other immune cells to eradicate infections.[29]

2.2. Recognition of nucleic acids by RIG-I-like receptors

Beyond the recognition of outer membrane components of pathogens, the host innate immune system also monitors the inner portions of these intruding bacteria that are released into host cells during infections,[30] including their nucleic acids RNA and DNA.[31] RLRs are responsible for detecting exogenous RNA from both viral and bacterial infections, such as their double-stranded RNA (dsRNA), 5'-triphosphate RNA, and short dsRNA.[11] For instance, RIG-I recognizes RNA fragments produced by *Listeria monocytogenes* and *Mycobacterium tuberculosis*, subsequently triggering the activation of the inflammasome and facilitating IFN-β production.[32] It is worth mentioning that dsRNAs are not exclusively recognized by RLRs, but also by other receptor types such as TLR3.[33,34] This led to the discovery of newly defined TLR3 agonists as an effective adjuvant for vaccines, especially against intracellular pathogens.[35]

Exogenous cytosolic DNA derived from bacteria is also sensed by the host's receptors. These receptors include the DNA-dependent activator of IFN-regulatory factors (DAI), the cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway, TLR9 and absent in melanoma 2 (AIM2).[2] (1) DAI, the first identified cytoplasmic DNA sensor, exhibits a high affinity for DNA, thereby facilitating a robust immune response upon binding.[36] (2) Furthermore, the cGAS-STING pathway operates through a distinct mechanism.[37] Upon detecting the presence of foreign DNA, cGAS generates the secondary messenger cyclic GMP-AMP (cGAMP), which subsequently interacts with STING, instigating the production of IFN-I. (3) The TLR9 receptor specifically recognizes unmethylated CpG motifs, which are prevalent in bacterial DNA.[38] (4) AIM2 is adept at recognizing cytosolic dsDNA from bacterial infections.[39] Upon dsDNA binding, AIM2 partners with the adaptor apoptosis-associated Speck-like protein containing a CARD (ASC) and procaspase-1, leading to the activation of caspase-1 and the release of pro-inflammatory cytokines interleukin (IL)-1β and IL-18.[39] Similar to TLR, SNPs in TLR gene are also linked with disease susceptibility, including viral infections and autoimmune diseases.[12] Studies in novel mouse models expressing human-equivalent SNPs have provided insights into how these variants influence immune responses, including antiviral immunity and autoimmunity.[40] Understanding the specific roles of these RLRs in immune cells is crucial for developing targeted therapies that balance protection against infections with minimizing autoimmune damage.[41]

2.3. NOD-like receptors as intracellular sensors

NLRs are a class of intracellular PRRs that have piqued the interest of researchers because of their essential roles in recognizing bacteria that replicate intracellularly.[42] Being evolutionarily conserved proteins found in both vertebrates and invertebrates, NLRs are characterized by an N-terminal caspase recruitment domain (CARD), a central NOD domain, and a C-terminal LRR domain. They are activated by a range of PAMPs, including flagellin, LPS, and components derived from bacterial peptidoglycan.[43] Members of the NLR family include neuronal apoptosis inhibitory protein (NAIP), NOD1/2, NLRC3/4/5, and NLRP1/3/6/12:[44] (1) NAIP5, a member of the NAIP family, was initially characterized for its capacity to detect flagellin originating from *Legionella pneumophila*.[45] Macrophages derived from mice with multiple polymorphisms in the *Naip5* gene display increased susceptibility to *L. pneumophila* infections.[46] (2) NOD1 and NOD2 are specialized in the detection of numerous components from pathogenic bacteria.[47] NOD1 senses diaminopimelic acid from Gram-negative bacteria, such as *Shigella flexneri* and *P. aeruginosa*.[48] NOD2 recognizes muramyl dipeptide found in both Gram-positive and -negative bacterial species, including *M. tuberculosis* and *Listeria monocytogenes.*[49] (3) Besides, the lethal toxin produced by *Bacillus anthracis* is recognized by NLRP1b, culminating in its cleavage and subsequent activation of inflammasomes.[50] (4) Additional ligands detected by NLRs encompass bacterial peptidoglycan and diminished levels of cytosolic ATP.[42]

Upon activation, NLRs undergo a conformational change, allowing them to oligomerize through CARD-CARD interactions.[47] This connection further recruits an adaptor protein and a procaspase, assembling the multimeric protein complex, inflammasome, whose activation culminates in the secretion of pro-inflammatory cytokines IL-1β and IL-18 or the induction of pyroptotic cell death.[51] Collectively, these receptor systems provide a comprehensive defense mechanism, allowing the host to detect and respond to invading pathogens effectively. As key players in initiating and regulating these cell death pathways, NLRs have been increasingly investigated as important targets for both protective immunity and controlling excessive inflammation, such as the cytokine storm seen in severe COVID-19 cases.[52] Despite extensive research, further studies are needed to understand NLRs' roles in drug resistance and adaptive immunity.[53,54]

2.4. Mechanism of inflammasome activation

The inflammasome is a multi-protein complex assembled on detection of PAMPs by PRRs, in the process of orchestrating host defenses. It typically constitutes a nucleotide-binding domain and LRR-containing protein, the adaptor protein ASC, and pro-caspases.[55] Two signals are required for the inflammasome to be activated. The first signal is priming, which involves the recognition of PAMPs by membrane-bound or cytoplasmic PRRs. This causes the upregulation of pro-IL-1 and pro-IL-18.[14] The second signal is provided by the inflammasome complex itself, which triggers the oligomerization of NLRs and the recruitment of ASC and pro-caspase-1.[14] This complex then undergoes a conformational change that results in the activation of caspase-1 and the processing of pro-IL-1β and pro-IL-18 into their mature biologically active forms.[56,57] The coordinated interplay of these sequential processes ultimately facilitates the activation of immune responses and the efficient elimination of invasive pathogens.[58,59]

Many varieties of inflammasomes have been identified so far; each is activated by specific stimuli and composed of diverse members of the NLR family and caspase effectors:[55] (1) The NLRP3 inflammasome, comprising the receptor NLRP3, adaptor protein ASC, and pro-caspase-1, represents the most extensively analyzed inflammasome assembly.[60] Its activation can be triggered by a broad spectrum of PAMPs, including bacterial RNA, DNA viruses, and fungi.[61,62] The priming for canonical NLRP3 inflammasome activation is mediated by caspase-8 whereas non-canonical NLRP3 inflammasome activation requires the binding of caspase-11 and cytosolic LPS.[62] Recent studies have elucidated the pivotal role of the NLRP3 inflammasome in mediating the proinflammatory response characteristic of chronic liver diseases, including ALD and NAFLD.[63] Its central involvement in the pathogenesis of these conditions highlights the potential for therapeutic interventions aimed at modulating inflammasome components or the cytokines they generate.[64] (2) Another important inflammasome is the NAIP-NLRC4 inflammasome, which can be activated by cytosolic bacterial flagellin and needle proteins, inner rod proteins of the type III secretion system (T3SS) of pathogenic bacteria.[65] Interestingly, proteins involved in cellular metabolic pathways, which are subject to caspase-1-mediated cleavage during infection with *Salmonella* Typhimurium, also instigate the activation of the NLR family CARD domain-containing protein 4 (NLRC4) inflammasome.[66] (3) AIM2 inflammasome[67] plays a critical role in sensing cytosolic dsDNA from invading bacteria.[39] Structural analyses reveal that upon binding to dsDNA, AIM2 oligomerizes and recruits ASC, which then recruits pro-caspase-1 to form the inflammasome complex.[68] Certain bacteria encode effectors that allow them to escape detection by the AIM2 inflammasome. *L. pneumophila*, for instance, employs SdhA to maintain the integrity of the bacterial replicative vacuole, preventing the leakage of DNA into the cytoplasm.[69] (4) Finally, recent investigations have unveiled a unique mechanism employed by the pyrin inflammasome to identify bacterial infections.[70] In this context, pyrin recognizes the bacterial-induced modifications of Rho GTPases, such as the glycosylation by *Clostridium difficile* toxins TcdA/B[71] and the mono ADP-ribosylation by the C3 exoenzyme from *Clostridium botulinum*.[72] Additional bacteria-induced modifications, including adenylation and deamidation, also serve as initiating stimuli for the activation of the pyrin inflammasome.[55]

The activation of inflammasomes subsequent to the detection of PAMPs by PRRs culminates in an enhanced immune response through the proteolytic processing and subsequent release of pro-inflammatory cytokines, notably IL-1β and IL-18.[73] These cytokines play a crucial role in orchestrating the inflammatory cascade, inducing pyrexia, and mobilizing immune cells to the site of infection.[74] While this inflammatory response is indispensable for the containment and eradication of pathogens, it necessitates precise regulation to mitigate the risk of hyperinflammation, which may precipitate tissue injury,[75] autoinflammation,[76] or systemic sepsis.[77] Moreover, in instances where the pathogenic onslaught surpasses the host's defensive capabilities, infected cells may resort to immunogenic cell death mechanisms as a fail-safe to impede further microbial propagation.[78] The intricate interplay between infection-triggered immunogenic cell death and host-pathogen dynamics warrants a detailed exploration,[79] which will be elucidated in the subsequent discourse.[80]

3. Immunogenic cell death triggered by the pathogen infections

One effective strategy of host immune defense in response to the infection of pathogens is the induction of cell death,[1] an event that will eliminate the niche for pathogen propagation.[81] Investigation of host responses including cell death triggered by pathogens has led to the identification of PRRs and novel immune mechanisms.[2] The dynamic interaction between PAMPs and host germline-encoded pattern-recognition receptors empowers the host to distinguish self-entities from foreign pathogens and to efficiently eradicate pathogens.[81] Despite the immense diversity in the microbial constitution, the host is nonetheless able to distinguish them through a small

number of receptors using mechanisms that are strikingly similar yet significantly distinct.[13]

3.1. Highly regulated cell death: Apoptosis

Apoptosis is a highly regulated form of cell death commonly observed in normal development and during pathogen infections.[82] This process is a multifaceted orchestration involving a series of messengers and enzymes, including members of the caspase family and mitochondrial-associated proteins without discharging cellular components into the extracellular milieu.[83] Hence, apoptosis is considered to proceed without eliciting an inflammatory response. Therefore, it is considered immunologically silent,[1] distinct from the inflammatory cell death pyroptosis.[84] Characteristics of infected cells undergoing apoptosis include DNA fragmentation, nuclear condensation, cytoplasmic blebbing, cell shrinkage, and the formation of apoptotic bodies.[85] Induction of apoptosis facilitates the removal of infected cells, thus preventing the spread of pathogens into deep tissues.

The induction of apoptosis by infection occurs through multiple distinct pathways: (1) caspases are proteases that are activated in a cascade manner upon specific apoptotic stimuli, such as LPS and Fas ligand (FasL).[86] More precisely, the interaction between FasL derived from *Helicobacter pylori* and its cognate Fas receptor (CD95)[87] and tumor necrosis factor receptor 1 (TNFR1), triggers the assembly of death-inducing signaling complex (DISC), resulting in the activation of caspases.[12] (2) Infection-induced apoptosis can also occur through the impairment of mitochondrial integrity, accompanied by the release of pro-apoptotic factors. For example, the toxin EspC secreted by *M. tuberculosis* triggers the permeabilization of the outer mitochondrial membrane (MOMP), allowing the release of cytochrome c, calcium ions (Ca^{2+}), and other apoptogenic factors into the cytoplasm.[88] Cytochrome c then activates caspase-9, which initiates the intrinsic apoptotic pathway.[89] (3) Recent studies showed that infections by *L. pneumophila* lead to extensive apoptosis in specialized phagocytes, such as dendritic cells.[90] From a molecular perspective, infections by these pathogens tip the balance between the pro-apoptotic and anti-apoptotic constituents of the Bcl-2 protein family, leading to the initiation of the MOMP and subsequent activation of caspase-3-mediated apoptosis.[90] Intriguingly, infections of *L. pneumophila* in permissive macrophages did not exhibit obvious apoptosis, suggesting that *L. pneumophila* possesses mechanisms to prevent infected macrophages from apoptotic cell death. This hypothesis gained experimental support when it was observed that infections by *L. pneumophila* strains lacking *sdhA* or *sidF* elicited enhanced induction of apoptosis in macrophages.[91] *SidF* appears to function by inhibiting the activity of a pro-death member of the Bcl2 protein family,[92] whereas ShdA functions by maintaining the integrity of the bacterial phagosome[93] (Figure 2).

In light of the immune-defense functions of apoptosis, pathogens have evolved a wide array of strategies to counteract and inhibit apoptosis, thereby ensuring their successful replication within host cells: (1) some bacterial species synthesize proteins that specifically engage with and proteolytically cleave vital elements of the host's apoptotic pathways. An illustration of this is the AIP56 toxin secreted by *Photobacterium damselae* subsp. *piscicida*, which catalyzes the cleavage of NF-κB p65, consequently inhibiting the NF-κB-dependent transcription of pro-inflammatory genes.[94] (2) Certain bacterial pathogens prevent apoptosis by modulating autophagy.[95,96] For example, *Salmonella* Typhimurium can switch the fate of host cells by triggering autophagy and preventing infected cells from undergoing apoptosis.[97] This is achieved by the leakage of amino acids from the pores formed by its T3SS1, which activates acute starvation stress, triggering the eIF2α/ATF4-mediated autophagy pathway.[98] (3) Many bacteria can inhibit the initiation of apoptosis by inducing the transcription of anti-apoptotic genes. For example, *L. pneumophila* infection induces the activation of the MAP kinase pathway in a Dot/Icm-dependent manner, resulting in increased expression of anti-apoptotic proteins.[99] (4) Effector proteins secreted by some bacteria directly hijack constitutes of apoptotic pathways. As an illustration, *L. pneumophila* effector SidF selectively antagonizes the activities of two pro-apoptotic Bcl2 members, thereby impeding the apoptosis of infected cells.[92] In summary, bacterial pathogens employ complex approaches with multiple effectors to manipulate host apoptosis pathways to counteract elimination caused by cell death. These balancing acts between apoptosis induction and inhibition highlight the evolutionary mechanisms pathogens adapt to thrive within hosts.

Investigating the mechanisms of apoptosis in response to pathogen infections holds substantial clinical relevance, as it elucidates the intricacies of the host's anti-bacterial immune response and identifies potential therapeutic targets for other diseases, including inflammatory bowel disease[100] and cancers.[101] Recent studies suggest that chronic infections can precipitate sustained inflammation, in part due to the suppression of apoptosis in immune cells.[102] Modulating apoptotic pathways in these cells can attenuate inflammation and facilitate the resolution of chronic infections. Moreover, the dysregulation of apoptosis is a defining characteristic of cancer.[103] By deciphering the ways in which pathogens modulate apoptotic processes,

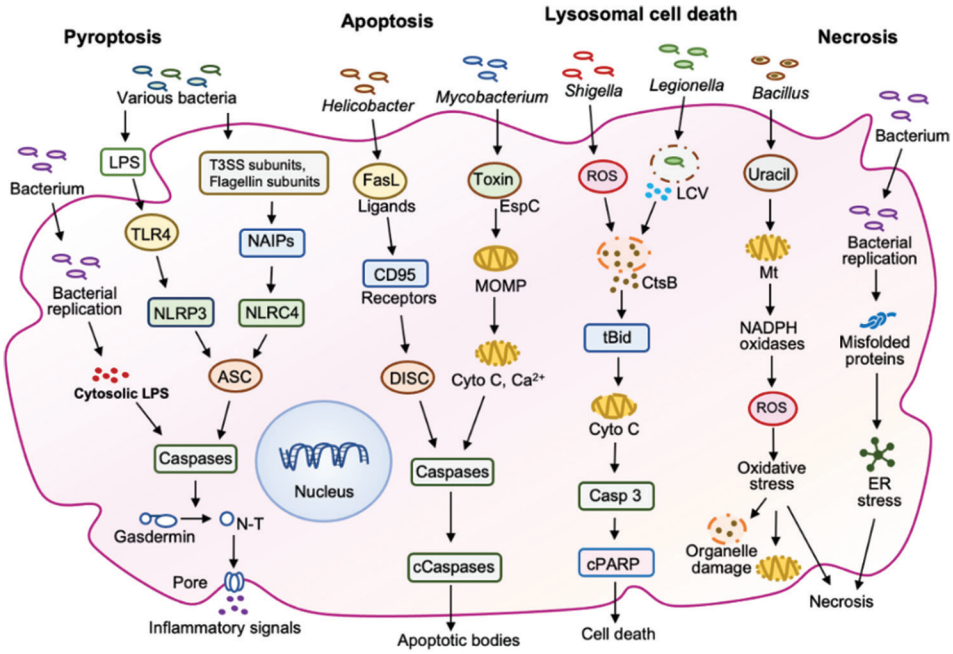

Figure 2. Mechanisms of pathogen-induced immunogenic cell death: pyroptosis, apoptosis, lysosomal cell death, and necrosis. (1) Pyroptosis is initiated by bacterial components such as lipopolysaccharide (LPS), which are recognized by TLR4, triggering the activation of NLR family pyrin domain-containing protein 3 (NLRP3), or NLR family CARD domain-containing protein 4 (NLRC4) inflammasomes. These inflammasomes then facilitate the processing of pro-caspases into active caspases, which cleave members of the Gasdermin family. The N-terminus of Gasdermin is inserted into the cell membrane, forming pores that allow the release of inflammatory signals. (2) Apoptosis is depicted as being induced by several bacterial strategies, including the activation of death receptors. This leads to the formation of the death-inducing signaling complex (DISC), the release of cytochrome c from mitochondria, and the activation of caspases that result in apoptotic body formation and cell death. (3) Lysosomal cell death is triggered by reactive oxygen species (ROS) and involves lysosomal membrane permeabilization (LMP), along with various other stimuli. This process leads to the release of cathepsins, which activate CtsB. CtsB cleaves Bid, resulting in the release of tBid, which inserts into the mitochondrial membrane, leading to cytochrome c release and subsequent caspase activation. (4) Necrosis is illustrated as being caused by factors such as uracil from bacteria that lead to mitochondrial (Mt) dysfunction and oxidative stress through nicotinamide adenine dinucleotide phosphate (NADPH) oxidases. This results in ROS production, organelle damage, and ultimately, necrotic cell death characterized by a loss of membrane integrity and uncontrolled release of cell contents. Image provided by the author.

researchers have recently developed promising therapies aimed at restoring normal apoptosis in cancer cells.[104]

3.2. The inflammatory cell death: Pyroptosis

Pyroptosis is a highly inflammatory form of programmed cell death involved in the host's defenses against microbial infections. It is initiated by the activation of the inflammasome that senses PAMPs or damage-associated molecular patterns (DAMPs) derived from the invading pathogens or damaged host cells.[105] Activation of inflammasomes leads to the activation of caspases, which cleave cytokine precursors and/or members of Gasdermin family[106] to release the N-terminal portion of these proteins to form pores in the plasma membrane, leading to cell swelling, osmotic imbalances and the leakage of cellular contents.[107] These ultimately result in the lysis of infected cells and the release of inflammatory signals.[108] Pyroptosis, due to its role in host defenses against microbial infections, presents a potential therapeutic target for treating infectious diseases and inflammatory conditions.[109] Recent studies have shown that by modulating inflammasome activation or Gasdermin-mediated pore formation, clinicians can enhance pathogen clearance or reduce excessive inflammation.[110]

Pyroptosis is mediated through various intricate mechanisms and pathways.[102] (1) A selection of inflammasomes, including NLRP3, AIM2, pyrin, and NLRC4, orchestrate the activation of caspase-1.[39] For example, upon recognition of flagellin from *L. pneumophila* by NAIP5,[111] the NLRC4 inflammasome recruits and activates caspase-1, which then cleaves GSDMD. (2) Alternatively, caspase 4/5/11 directly senses cytosolic bacterial LPS and activates itself to cleave and activate GSDMD.[112] In both scenarios, pro-IL-1β and pro-IL-18 are cleaved by caspase-1, leading to the release of mature cytokines and intracellular DAMPs, thereby amplifying the inflammatory responses against the pathogen invasions.[105] (3) Similarly, Gasdermin E (GSDME) undergoes specific cleavage by caspase 3, with

the resultant N-terminal fragment instigating pyroptosis as a countermeasure against bacterial infection.[113] Interestingly, the caspase-3-GSDME axis may also be activated by granzyme B (GZMB) within lung alveolar epithelial cells infected by the H7N9 virus, resulting in an overwhelming cytokine response and pyroptosis.[114] (4) Furthermore, cytotoxic T lymphocytes and natural killer cells possess the capability to secrete serine proteases granzymes, targeting infected cells or cancer cells. Granzyme A (GZMA) released from these cytotoxic lymphocytes cleaves Gasdermin B (GSDMB) within targeted cells. The N-terminal domain of GSDMB forms pores on membranes, leading to pyroptosis.[115] (5) In a separate mechanism, the AIM2 inflammasome detects and binds to cytosolic dsDNA signatures from invading bacteria,[39] thereby inducing AIM2 oligomerization, which in turn recruits the adaptor ASC. This complex then recruits pro-caspase-1, paving the way for inflammasome assembly and the induction of pyroptosis.[68]

Intriguingly, the proteolytically cleaved form of GSDMD possesses the ability to directly lyse bacteria by assembling pores in the bacterial cell membrane.[116] More specifically, this happens when its N-terminal fragment binds with cardiolipin, a phospholipid localized in the cell membranes of bacterial species such as *Staphylococcus aureus* and *Bacillus megaterium*.[117]

To counter the damage caused by pyroptosis, bacterial pathogens have evolved effective strategies to inhibit the activation of pyroptosis within infected host cells:[118] (1) Among these, *Yersinia pestis* capitalizes on the functionalities of its effectors YopK and YopM. While YopK inhibits the recognition of its T3SS by the NLRC4 inflammasome,[119] YopM inhibits the activation of the pyrin inflammasome.[120] This dual action ultimately hinders caspase-1 activation, effectively suppressing the initiation of pyroptosis. (2) *L. pneumophila*, for instance, employs its effector SdhA to maintain the structural integrity of its replicative vacuole, thus preventing the leakage of DNA into the host cytoplasm,[69] which will avoid pyroptosis caused by AIM2 activation[69] and by IFN-I induction.[121] (3) Furthermore, *S. flexneri*, implicated in bacillary dysentery, inhibits LPS-induced pyroptosis through its effector OspC3.[122] OspC3 catalyzes arginine ADP-riboxanation on caspase-4/-11, halting the proteolytic processing of GSDMD and subsequent pyroptosis.[123] Similar to *Shigella* OspC3, the effector CopC, secreted by *Chromobacterium violaceum*, also possesses ADP-riboxanase activity.[124] Once specifically interacting with host calmodulin (CaM), CopC mediates arginine ADP-riboxanation of apoptotic caspases encompassing caspase-7/-8/-9.[125] Collectively, bacterial pathogens deploy a myriad of effectors to intricately modulate and impede host pyroptosis, highlighting the perpetual evolutionary interplay between microbial virulence and host immunity.

3.3. The last line of defense: Necrosis

Necrosis is another type of cell death induced by pathogen infections,[126] characterized by rupture of the plasma membrane, nuclear swelling, and release of cellular contents into the extracellular space,[127] resulting in inflammation independent of caspases.[128] During pathogen infections, several mechanisms can lead to necrosis: (1) Certain pathogens produce toxins or enzymes that directly damage host cells and lead to necrosis. For example, the alpha-toxin of *S. aureus* causes the formation of pores in the plasma membrane, resulting in cell swelling and lysis.[129] (2) Besides, the replication of bacteria in host cells leads to altered homeostasis and the accumulation of misfolded proteins, resulting in endoplasmic reticulum (ER) stress and subsequent necrosis.[130] (3) Moreover, pathogen infections can induce necrosis through the production of reactive oxygen species (ROS).[131] For example, the uracil released by the *Bacillus thuringiensis* promotes mitochondrial dysfunction and the activation of NADPH oxidases, which then leads to the production of ROS.[132] ROS subsequently induces oxidative stress and damages cellular components, resulting in necrosis.[133] (4) Finally, cellular Ca^{2+} dysregulation during infections also triggers necrosis in infected cells, the disruption of Ca^{2+} homeostasis leads to an influx of Ca^{2+} into the cytoplasm.[134] Excessive cytoplasmic Ca^{2+} levels can activate various enzymes that perturb cellular processes and ultimately lead to necrotic cell death.[135]

The consequences of pathogen-induced necrosis are usually detrimental to both the infecting organisms and the hosts. Cellular contents released upon cellular damage or inflammation can lead to the activation of autoimmune responses and the amplification of inflammation, thereby exacerbating tissue destruction as well as broader systemic effects.

3.4. Lysosomal cell death triggered by microbes

Lysosomes are membrane-bound organelles containing various hydrolytic enzymes involved in intracellular degradation and cell recycling.[136] During bacterial infections, lysosomes can exhibit both beneficial and detrimental effects on infected cells depending on the magnitude of lysosomal perturbations.[128] Concurrently, lysosomes contribute to host defenses by fusing with phagosomes to degrade the engulfed bacteria.[137] This process aids in the eradication of intracellular pathogens and prompts immune reactions. However, microbial infections can compromise lysosomal membrane integrity,

a phenomenon referred to as lysosomal membrane permeabilization (LMP).[138] The occurrence of LMP is accompanied by the release of cathepsins from the lysosomal lumen, which leads to the cleavage of Bid to generate tBid.[139] tBid then forms pores in mitochondria to trigger the release of cytochrome c (Cyto c), which activates the classic apoptotic pathway that ultimately leads to caspase-3 activation and cell death[9,140] (Figure 3).

Bacterial infections can trigger lysosomal cell death through multiple mechanisms:[141] (1) Toxins or pore-forming proteins secreted by some bacteria directly target lysosomal membranes, leading to LMP, such as the nigericin from *Streptomyces hygroscopicus* and the pyocyanin from *P. aeruginosa*.[9] (2) In some context, sensing of bacterial ligands by host receptors leads to lysosomal destabilization. The effector VepA secreted by *Vibrio parahaemolyticus* interacts with H^+-ATPase and such binding disrupts the integrity of lysosomal membranes and induces LMP.[142] (3)

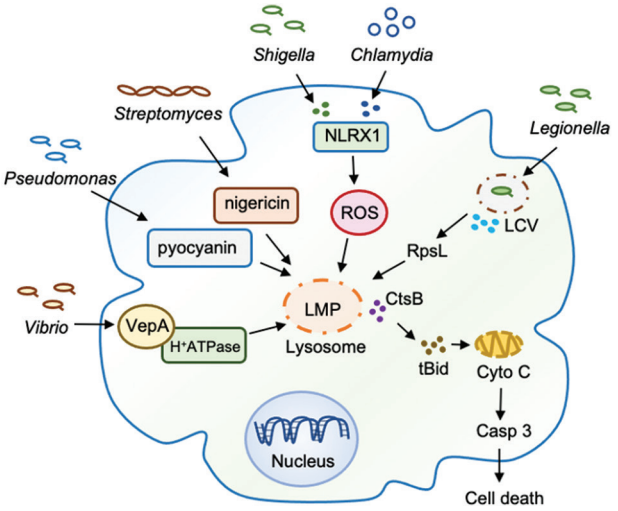

Figure 3. Approaches employed by pathogens in lysosomal cell death. Bacteria induce cell death by causing lysosomal damage, which triggers a subsequent cellular pathway leading to apoptosis. Various bacterial species release toxins and other ligands that directly or indirectly compromise the integrity of lysosomal membranes. *Pseudomonas* produces pyocyanin, which contributes to the generation of reactive oxygen species (ROS) within the cell. *Vibrio* species release VepA, which inhibits the H^+ ATPase function. *Streptomyces* contributes nigericin as part of its pathogenicity mechanism. Pathogens such as *Shigella* and *Chlamydia* are shown to manipulate host cell functions, involving the recognition of cellular damage by the NLR family member X1 (NLRX1) receptor, which then triggers the production of ROS. The increase in ROS leads to lysosomal membrane permeabilization, releasing cathepsins (CtsB) into the cytosol. *Legionella* RpsL, an antibiotic resistance-associated protein, induces cellular stress and has recently been implicated in lysosomal membrane damage. This lysosomal damage results in the release of cathepsins, which subsequently activate Bid. Activated Bid facilitates the release of cytochrome c (Cyto C) from the mitochondria. The presence of Cyto C then initiates the activation of caspase 3, leading to apoptosis. Image provided by the author.

In addition, the accumulation of ROS produced during intracellular bacterial infections causes oxidative stress and damages to lysosomal membrane proteins. For instance, infections by *Shigella*[143] or *Chlamydia* are detected by NLR family member X1 (NLRX1), which localizes to mitochondria and induces the production of ROS,[144] resulting in subsequent lysosomal cell death. (4) An earlier study discovered that infections by *L. pneumophila* strains harboring wild-type *rpsL* such as Lp02$rpsL_{WT}$ induce extensive lysosome damage and apoptosis in mouse bone marrow-derived macrophages, resulting in the termination of bacterial replication.[138] Although the mechanism of this unique infection-induced cell death remains unknown, lysosomes appear to be involved.[145] Cellular events upstream of lysosomal membrane permeabilization await further investigations.[145]

4. Conclusion and perspectives

In light of their critical roles in the innate immune response and inflammation, the PRRs have been explored as potential drug targets for a variety of diseases in the past two decades, including bacterial and viral infectious diseases, autoimmune disorders, and cancers.[146] For instance, given the critical role of NLRs in inflammasome formation and regulation of IL-1β and IL-18, NLRP3 has been targeted for the development of anti-inflammatory drugs. Inhibitors of the NLRP3 inflammasome are being applied to relieve excessive inflammation, such as gout, type 2 diabetes, and atherosclerosis.[62] Furthermore, agonists of TLR7 and TLR9 have been explored for their potential to enhance antitumor immunity by promoting the activation of dendritic cells and B cells.[26] In addition, a recent study demonstrated that nanoparticle-encapsulated TLR9 agonists effectively activate plasmacytoid dendritic cells to secrete factors that enhance antigen presentation by myeloid dendritic cells, underscoring the importance of targeting both dendritic cell types in cancer vaccine immunotherapy.[147] The advancement of pharmacotherapeutics that target PRRs signifies a promising frontier in the field of immunotherapy.[148] Future research endeavors might focus on elucidating novel host receptors and delineating their mechanisms of recognition.

The intricate interplay between host immune defenses and pathogen evasion strategies outlined in this review emphasizes the pivotal role of immunogenic cell death in controlling pathogen infections.[112] Central to this complex dance is the activation of host receptor-mediated signaling pathways. Equally important is the downstream cell death, which enables hosts to detect and respond effectively to invading pathogens.[112] Such immune responses often include the activation of the inflammasome complex, which further augments cell death

and amplifies inflammation.[62] The distinct mechanisms of immunogenic cell death not only facilitate the elimination of infected cells but also significantly contribute to training subsequent adaptive immune responses. Recent studies have identified novel immunogenic cell death pathways, for instance, ferroptosis, which is linked to redox biology, metabolism, and various diseases.[149] Despite an incomplete understanding of its regulatory networks, significant progress has been made in developing pharmacological tools to target ferroptosis in infectious disease treatment and prevention.[150,151] Deeper dissection into the molecules participating in these signaling awaits further investigation. Yet, pathogens exhibit remarkable adaptability through the evolution of complex strategies to circumvent host immune defenses.[4] This is achieved either by direct interference with pivotal elements within immunogenic cell death pathways or by impeding the functionality of critical components necessary for the induction of inflammation.[2] The primary objective of these microorganisms is to alter host cellular environments to enhance their own survival and proliferation.[152] Consequently, elucidating the molecular mechanisms underlying these interactions offers significant potential for developing novel approaches to combat infectious diseases.

Immunogenic cell death in response to pathogenic invasion is a double-edged sword.[153] On the one hand, cell death represents a defense mechanism that efficiently clears infected cells to limit pathogen spread.[154] On the other hand, excess inflammation causes damage to the mucosal barrier and tissues, an opportunity that can be exploited by infectious microbes.[128] In addition, certain pathogens have developed strategies to inhibit cell death, thereby enhancing their survival within the host. This tug-of-war between cell death induction and inhibition mirrors the evolutionary battle between host defenses and microbial virulence.[70] Although apoptosis is the most extensively studied type of cell death, the roles of pyroptosis and necrosis in immune responses demonstrate the host's ability to utilize diverse cell death mechanisms to combat infections.[83] Recent research has uncovered PANoptosis, a unique form of inflammatory programmed cell death regulated by PANoptosome complexes, which integrate elements from various cell death pathways.[155] PANoptosis has distinct biological effects not explained by other pathways alone, but a better understanding of the activation and regulation of PANoptosis has shown great potential for its implications in disease and future therapeutic strategies.[156] Further investigation is encouraged to elucidate hitherto unidentified pathways of cell death signaling. The identification of novel molecules implicated in immunogenic cell death is anticipated to provide a valuable repository of targets with therapeutic potential.

New insights into these dynamic host-pathogen interactions are unfolding, shedding light on immune defense mechanisms and pathogen evasion tactics.[157] It becomes evident that the host's ability to initiate various forms of cell death is a critical factor in the outcome of infections. The ongoing discovery of novel receptors, signaling pathways, and microbial evasion tactics deepens our understanding of the immune system's complexity and adaptability.[158] Future research should delve deeper into these complex molecular mechanisms, potentially revealing novel therapeutic targets for emerging infectious diseases.[159] Understanding how pathogens modulate immunogenic cell death pathways might pave the way to the development of innovative immune response strategies, improving the efficacy of treatments against various infectious agents.[160] Overall, further studying the diverse mechanisms of immunogenic cell death triggered by pathogen infections is a multidisciplinary endeavor that has far-reaching implications for medicine, public health, and our understanding of human biology.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

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ACCSCIENCE PUBLISHING

Microbes & Immunity

PERSPECTIVE ARTICLE

Is vagus nerve-mediated regulation of immunity an etiological target for therapeutic intervention in endometriosis?

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Abstract

Endometriosis is a complex chronic neuro-inflammatory disorder, affecting roughly 10% of reproductive-age women. It is characterized by the presence of endometrial-like tissue outside the uterus, which induces a chronic inflammatory reaction. This disease can present a wide range of symptoms, including chronic pain and infertility. Despite extensive research, the exact pathogenesis of endometriosis remains incompletely understood. New strategies and paradigms on pathogenesis and treatment are needed. Schematic factors contributing to the development of endometriosis lesions include genetic, hormonal, and immunological factors. Although genetics may contribute to the epidemiologically suggested heritability of endometriosis, epigenetics has gained an increasing consideration in research. Remarkably, microbiota dysbiosis, acting as a catalyst for the main acknowledged epigenetic etiologies (locally produced estradiol, pro-inflammatory cytokines, and hypoxic stress) demands further attention. Indeed, over the past 10 years, it has become clear that the vagus nerve, the fastest component of the microbiota-gut-brain axis, can efficiently control inflammation through the cholinergic anti-inflammatory pathway. Therefore, stimulation of the vagus nerve could be a good candidate for modulating the severity of endometriosis. The detrimental consequences of microbiome dysbiosis and the estrobolome activity on the initiation of the disease as well as counterpart dysfunctions in the central nervous system will be focused on, both supporting a key role of the vagus nerve since the early stage of endometriosis. Consequently, the rationale for using non-invasive vagus nerve stimulation will be discussed, introducing a fruitful shift of paradigm in this still enigmatic disease.

Keywords: Endometriosis; Pathophysiology; Epigenetics; Immunity; Microbiota-gut-brain axis; Non-invasive vagus nerve stimulation

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1. Introduction

Endometriosis, a very common but complex chronic disorder affecting young women worldwide,[1] is classically defined as the presence of endometrial-like glands and stroma outside the uterine cavity, leading to chronic pelvic pain and infertility. With the advent of a validated non-invasive saliva-based diagnostic microRNA signature,[2-4] histopathological confirmation may not soon remain essential for the diagnosis of endometriosis. Thus, an earlier diagnosis is likely to open new avenues to improve the prognosis and the quality of life of the patients, provided an etiological and equally non-invasive treatment can be rapidly initiated.

At the beginning of 2024, a group of international experts called for a full revision of the pathogenesis and pathophysiology of endometriosis.[5] This reassessment is a rare opportunity to question an upstream unifying rationale underpinning this seemingly heterogeneous chronic disease. This review aims to pave the way for an innovative, scientifically proven therapeutic option: Non-invasive vagus nerve stimulation (VNS).

Having a family member with endometriosis noticeably increases a woman's chances of developing it as well.[6] A 2023 meta-analysis,[7] including 60,674 cases and 701,926 controls, identified 42 genome-wide significant loci comprising 49 distinct association signals with endometriosis. A significant genetic correlation between endometriosis and 11 pain conditions (including migraine), as well as inflammatory conditions was shown in this meta-analysis. Moreover, multitrait genetic analyses identified substantial sharing of variants associated with endometriosis and migraine. Nevertheless, the identified genetic signals only explained up to 5.01% of endometriosis variance and regulated not only expression but also methylation (hence epigenetic mechanisms) of genes in endometrium and blood.[7] Besides, three programmed cell death-related genes have recently been identified as key biomarkers of endometriosis, through machine learning and Mendelian randomization.[8] Actually, the results revealed marked upregulation of the expression of TNFSF12 and PDK2 in endometriotic samples, coupled with a significant downregulation of the expression of AP3M1, emphasizing, once more, the importance of the epigenetic mechanisms in this disease.

Thus, the main determinants of endometriosis (and main therapeutic targets to focus on) are likely to be epigenetic ones, resulting in altered expression of genetic material, independent of the modification of the genetic sequence itself.[9-11] Three driving microenvironmental cues modulating the expression of genes for the development of endometriosis have been identified: Locally produced steroid hormones, pro-inflammatory cytokines, and hypoxic stress.[11,12] Remarkably, the gut microbiota is a major regulator of circulating estrogens (through the estrobolome,[13] the collection of genes of the gut microbiota responsible for estrogen metabolism, in particular, the β-glucuronidase gene coding for an enzyme that deconjugates estrogens into their active forms),[14] immune response[15,16] and stress[17] (including hypoxic stress)[18] as well. Therefore, gut microbiota dysbiosis, acting as a catalyst of the main epigenetic cues, appears as the most interesting therapeutic target to focus on in endometriosis.

After reviewing the detrimental consequences of microbiome dysbiosis on endometriosis pathogenesis, we will underscore brain dysfunctions underpinning endometriosis pathophysiology before focusing on vagus nerve dysfunction, a pivotal, yet underappreciated, target for endometriosis progression. Consequently, non-invasive VNS appears as an innovative therapy, naturally connecting the central and peripheral nervous systems[19] and gathering the necessary conditions to provide a safe, global, and long-lasting maintenance of homeostasis regarding endometriosis.

2. Targeting microbiota dysbiosis as a potential strategy to prevent endometriosis

During homeostasis, a balance between the microbiota and the immune system maintains immune quiescence. Dysbiosis is defined as the perturbances to microbiota resulting from alterations in the bacteria, immune system, or local environment.

The issue of the involvement of microbiota dysbiosis and the estrobolome in endometriosis has been reviewed lately, confirming their importance in the physiopathology of the disease.[20] Altered microbiota have been reported in the genital tract of infertile patients with chronic endometritis or endometrial polyps[21] and in women with histology-proven stage 3/4 endometriosis.[22] A complete absence of *Atopobium* in the vaginal and cervical microbiota of the case group, as well as an increase of *Gardnerella*, *Streptococcus*, *Escherichia*, *Shigella*, and *Ureaplasma*, in the cervical microbiota of the endometriosis group were found. Besides, an enrichment of *Shigella*/*Escherichia* was found in the stool microbiome of the endometriosis group.[22] Peritoneal microbiota is also modified in endometriosis,[23,24] and this dysbiosis probably accounts for local inflammation and pelvic pain.[25]

Noteworthily, a growing body of recent evidence also suggests the existence of gut dysbiosis (notably gut dysbiosis-derived β-glucuronidase, *i.e.*, the estrobolome), promoting the development of endometriosis,[26-29] underscoring a potential similar role of microbiota in endometriosis and

irritable bowel syndrome (IBS) conditions[30] and even inflammatory bowel disease (IBD). IBS is a common functional bowel disorder (abdominal pain and distension with an altered bowel movement), whereas IBD refers to inflammation in the gastrointestinal tract, traditionally categorized into ulcerative colitis and Crohn's disease. Actually, IBD, more than IBS (because of the absence of histologic lesions), shares a similar pathophysiology with endometriosis. A positive association between endometriosis and IBD has been confirmed in a systematic review.[31] Unfortunately, a meta-analysis on this topic is currently not possible due to the heterogeneity of the groups and because information on the temporal sequence of endometriosis and IBD is not available in several studies. A large-scale genome-wide association study has confirmed an increased risk of developing IBD after endometriosis, but not *vice versa*.[32]

Finally, two Mendelian randomization studies (assessed by two different teams) using huge consortium databases on gut microbiota (MibioGen, including 18,340 cases from 24 cohorts, mainly from Europe) and endometriosis (FinnGen, including data from 77,257 European participants) supported the causal relationship between gut microbiota and endometriosis without bidirectional causal effects.[33,34] More precisely, some families (*Prevotellaceae*, genus *Anaerotruncus*, genus *Olsenella*, genus *Oscillospira*) and order Bacillales were identified as risk factors for endometriosis, while others (Melainabacteria and genus *Eubacterium ruminantium* group) were protective factors.[33] Therefore, it seems that gut microbiota modification can trigger the onset of endometriosis, but any gut microbiota dysbiosis cannot promote endometriosis. Subsequently, gut microbiota dysbiosis that favors endometriosis is likely to also favor IBD, depending on the concomitance of other risk factors. Indeed, similarly, gut microbiota dysbiosis, especially a decrease in the abundance and diversity of specific genera (reduction in *Faecalibacterium prausnitzii*; *Alistipes*, *Collinsella*, and *Ruminococcaceae*), has been suggested as a trigger for IBD-initiating events.[35] Similarly, the onset of IBD is likely to be more strongly influenced by environmental factors, especially gut microbiota, than by genetic factors.[36]

Besides, gut dysbiosis triggers inflammation through recruitment and/or activation of immune cells,[37] as well as through modulation of the vasoactive intestinal peptide (VIP) signalling.[38,39] Because of the altered composition of the intestinal microbiota, a significant number of Gram-negative bacteria translocate and infiltrate outside the intestinal cavity, resulting in the destruction of intestinal tight junctions and the reduction of tight junction protein 2 (ZO-2) expression,[40] leading to the infiltration of a significant amount of Gram-negative bacteria outside the intestine.[41] According to Harada *et al.*[41] lipopolysaccharide can activate the macrophage TLR4 in innate immunity, leading to the production of significant levels of tumor necrosis factor alpha and interleukin 8 and the development of an inflammatory environment.[42] Otherwise, VIP is a non-cholinergic non-adrenergic neurotransmitter mainly expressed in the nerve terminals of the digestive tract, the genitourinary tract, the adrenal glands, and the central nervous system,[43] playing a key role in controlling the balance of pro- and anti-inflammatory cytokines[44] and in angiogenesis,[45] notably through alternative splicing.[46] VIP expression is upregulated in women with endometriosis and chronic pelvic pain,[47] concomitantly with inflammation, and the increase in nerve fiber density within ectopic endometrial tissue.[48] Moreover, dysfunction of VIP signaling could be involved in genital barrier disruption,[49] allowing endometriotic cell migration, as well as impacting gut[50,51] and brain barrier permeability,[52] supporting the recent insight that endometriosis is "no longer a pelvic disease."[53]

3. Brain and vagus nerve dysfunction in endometriosis

In addition to peripheral alterations provoked by endometriosis, such as peritoneal inflammation and angiogenesis, central repercussions, such as stress, pain, anxiety, depression,[54] and even bipolar and panic disorder[55] have been described, supported by experimental studies. Alteration in gene expression and electrophysiology in distinct brain regions,[56] upregulation of the expression of glial markers (GFAP and IBA-1) as well as morphological changes in glial cells in the spinal cord,[57,58] the hippocampus and the hypothalamus[59] were found in mice with endometriosis. Moreover, in a murine model, endometriosis lesions were shown to develop in the central nervous system, as endometriosis-derived cells were able to migrate and engraft to the brain.[60] Several teams have suggested chronic stress as a central, top-down mechanism exacerbating endometriosis by triggering the dysregulation of the hypothalamic-pituitary-adrenal axis, ending up with a release of inflammatory mediators in the circulatory system.[61,62] Endometriosis-linked central stress could also influence the desynchronization of both the Hypothalamic-pituitary-gonadal axis and the circadian system,[61] underpinning the occurrence of several comorbidities. Indeed, night shift work has been significantly associated with an increased risk of endometriosis as well as an increased risk of estrogen-influence diseases (namely breast cancer and adverse coronary events) and menstrual disruption.[63]

Whether endometriosis results from a top-down neuroinflammation[61] or a bottom-up activation of microglia by peripheral inflammatory mediators[59] remains an elusive question. Regarding the current validated level

of knowledge in the pathophysiology of endometriosis, this distinction is rather ambitious, since endometriotic lesion can grow very slowly and the diagnosis is often delayed. Nevertheless, as the bidirectional microbiota-gut-brain axis is known to involve the vagus nerve[19,37,64-66] and as severe endometriosis leads to a reduced vagal tone in women,[67] the rationale for using non-invasive VNS in endometriosis is very much appealing.

4. Rationale for using VNS in endometriosis

In a nutshell, gut dysbiosis and estrobolome activity seem to be essential to initiate endometriosis.[20,33,34,68] Although preliminary, antibiotic and probiotic treatments have demonstrated efficacy in treating endometriosis,[13] modulating the gut and/or genital microbiota by other means, potentially including non-invasive VNS, has been suggested as a novel therapeutic strategy to improve outcomes in patients with chronic endometriosis.[29,69,70] Indeed, minimally or non-invasive VNS is already known to mitigate gut dysbiosis[71] and is currently advocated for managing both IBS[72] and IBD.[73-76]

VNS appears particularly promising to help delay or even prevent severe endometriosis, since in a mouse model, vagotomy has been shown to promote the progression of endometriosis, whereas VNS could relieve it.[67] Actually, besides mitigation of gut dysbiosis and estrobolome activity, non-invasive VNS is likely to be helpful to several therapeutic mechanisms: (i) decreasing local and systemic inflammation (by stimulating alpha 7 nicotinic receptors (α7nAChR),[77,78] involved in the cholinergic anti-inflammatory pathway,[79-84] which are significantly reduced in endometriotic lesions);[85] (ii) counteracting VIP-induced increase of intestinal and brain barrier permeability;[86-88] (iii) decreasing the central symptoms of endometriosis, *i.e.*, stress, pain, anxiety, and depression;[89-92] (iv) protecting from hypoxia;[93,94] (v) acting through epigenetic regulatory mechanisms (histone deacetylation, micro-RNA and methylation of DNA),[11,95-98] and (vi) finally modulating the downstream MAPK or NF-κB pathways signaling pathways, which are known to be involved in endometriosis.[98-100]

Traditionally, VNS was achieved through surgical implantation. In 2017, however, non-invasive approaches that involve stimulating the cervical vagus nerve and the auricular branch of the vagus nerve were approved by the U.S. Food and Drug Administration (FDA) for cluster headache and abdominal pain, respectively. Since then, numerous new indications have been cleared by the FDA, and remarkably for the treatment and the prevention of migraine attacks[101,102] (this is of highest importance since multitrait genetic analyses identified substantial sharing of variants associated with endometriosis and migraine)[7] as well as in case of threatening inflammation with Emergency Use Authorization from the FDA in July of 2020 for patients with known or suspected coronavirus disease 2019 (COVID-19).[103] We are seeing a paradigm shift in our understanding of how disease is modulated by infection and/or inflammation across numerous disorders from the use of electroceuticals in the treatment of IBD[75] and rheumatoid arthritis[104] to the treatment of cytokine storm associated with COVID-19.[105]

The benefits of non-invasive VNS are potentially plethora for women with endometriosis. First and foremost, non-invasive VNS can provide a significant reduction of side effects, compared to the actual drugs (from non-steroidal anti-inflammatory drugs whose anticipated side effects are relatively mild to progestins whose prolonged use has been linked with a malignant transformation of ovarian endometrioma).[106] Indeed, non-invasive VNS has proven to be very well tolerated[107] and is likely to be more ethical for young ladies than dienogest.[108,109]

Second, most current drugs merely alleviate symptoms without reversing the progression of endometriosis. Guo[110] even stated in 2014 that "no blockbuster drug for endometriosis seems to be on the horizon yet", probably because interdisciplinary clinical research, fully funded by non-industrial sources, is lacking.[111] The same author has even called for a paradigm shift in drug research and development in endometriosis lately.[112] Remarkably, non-invasive VNS has all the requisites to become an all-in-one tool (both etiological and symptomatic) in endometriosis (that is the main aim of this article). Indeed, non-invasive VNS has already been successfully used in different types of chronic pain[113] (chronic pain being an interdisciplinary clinical research field by essence), has shown promising results not only in chronic pelvic pain,[114] but also in a wide array of comorbidities of endometriosis (FDA-approved for preventing migraine attacks, stress, anxiety, and depression), and has the potential to reverse the several pathophysiologic mechanisms involved in endometriosis. Non-invasive VNS does not modify one but a variety of factors, both peripherally and centrally, and has demonstrated an expanded scope and value for holistic therapy.[19] This ability relies mainly not only on the widespread innervation of the vagus nerve but also on its ability to shift the body and brain from a sympathetic to a parasympathetic dominance. Indeed, another therapeutical approach (a fluid therapy comprising adenosine, lidocaine, and magnesium) allowing a similar shift from sympathetic to parasympathetic tone has been intitled "Revolution in sepsis: a symptoms-based to a systems-based approach?" as it enables to "maintain

cardiovascular-endothelial glycocalyx coupling, reduce inflammation, correct coagulopathy, and maintain tissue O_2 supply" all by itself.[115]

Last but not least, non-invasive VNS, which is easier and more sustainable than pharmacological (hormonal[116] or anti-inflammatory[117]) options or microbiota transplants,[118] could even prevent the spontaneous occurrence of cancers linked to endometriosis. Indeed, ovarian cancer is the most important associated cancer, wherein a direct clonal relationship between endometriosis and cancer has been made.[119] A recent large cohort from Utah (including 78,893 women with endometriosis and those without endometriosis, in a 1:5 ratio) confirms a marked increase of ovarian cancer risk (multiplied by 4.2 in average, but up to 7.48, depending on the histological type of cancer) in women with ovarian and/or deep infiltrating endometriosis.[120] Moreover, although research has not found a direct link between endometriosis and breast cancer, so far, women with hormone-sensitive breast cancers should not be subjected to hormonal regulation of their endometriosis. On the contrary, an increased vagal tone, notably induced through non-invasive VNS, is correlated with a better prognosis in breast cancer[120] as well as in cancer in general.[108,109] Non-invasive VNS thus appears as a promising candidate for primary as well as secondary prevention of endometriosis. Thus, non-invasive VNS could be a potential lifelong innovative therapeutic solution for endometriosis, since the early phase of symptom manifestation, as it improves compliance.[121]

5. Conclusion

Merging both top-down and bottom-up mechanisms, vagus nerve-mediated regulation of immunity emerges as an etiological therapeutic intervention in endometriosis, offering patients a convenient therapeutic strategy to improve their quality of life as well as their prognosis. It is necessary to conduct clinical trials assessing the efficiency and tolerability of the very early use of this disruptive approach to tackle, particularly, pain, inflammation, and even the onset of endometriosis.

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Conflict of interest

Claire-Marie Rangon and Peter S. Staats are the Guest Editors of this special issue but were not in any way involved in the editorial and peer-review process conducted for this paper, directly or indirectly. Peter S. Staats is the founder of the ElectroCore company which sells nVNS device called gammaCore. Separately, other authors declared that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

Occurrence and seroprevalence of infectious viral, bacterial, and protozoal diseases among patients attending the Ore General Hospital in southwestern Nigeria

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Abstract

Infectious diseases caused by viruses, bacteria, protozoans, and fungi continue to pose significant challenges globally, with transmission routes including person-to-person contact, animal vectors, and environmental exposure. Despite global efforts to control these diseases, limited studies and resource constraints in Ore, Nigeria, have led to increasing prevalence, highlighting the need for targeted public health interventions. This study aimed to determine the distribution of infectious diseases among patients attending General Hospital Ore, Odigbo, Nigeria. Serum samples from consenting patients were assayed for the presence of malaria parasites, human immunodeficiency virus (HIV), hepatitis B surface antigen (HBsAg), *Salmonella* infection, *Helicobacter pylori* infection, high vaginal swab (HVS) results, and urinary tract infections (UTIs). The subjects included 1900 males (38.8%) and 3000 females (61.2%). The overall prevalence rates were as follows: HIV (40%), malaria (35%), typhoid (37.5%), HBsAg (20%), *H. pylori* (6.3%), HVS (45%), and UTIs (10%). The high rates of infectious diseases observed in this study, compared to rates reported elsewhere, suggest the need to strengthen public health measures and infection prevention strategies in the area. In addition, routine screening for these diseases and early passive or active immunization for vaccine-preventable diseases are essential to further reduce the burden of these infections.

Keywords: Infectious diseases; Distribution; Ondo State; Human immunodeficiency virus; Hepatitis B surface antigen; Urinary tract infections

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1. Introduction

Globally, viruses, bacteria, protozoans, and fungi are a common group of microbes implicated in infectious diseases affecting both humans and animals.[1] In humans, the transmission of infectious diseases can occur through person-to-person contact, insects or animals to humans, direct contact with the infectious agent in the environment, or ingestion of contaminated food or water.[1] The carriers of infectious agents may be houseflies, mosquitoes, animals, or men. Once inside the human host, these pathogens multiply in numbers and manipulate the normal functioning of body tissues, causing diseases with varying signs and symptoms depending on the specific organism involved.[2]

Infectious disease constituted the most serious health issue in the world until the beginning of the 20th century, devastating a significant proportion of the population in many European cities and developed countries.[3] Malaria is caused by a protozoal parasite belonging to the genus *Plasmodium*.[4] In 2020, Nigeria accounted for approximately 27% of malaria cases globally, ranking first among six countries that together represent more than half of all malaria cases.[5] These countries – Democratic Republic of the Congo, Uganda, Mozambique, Cote d'Ivoire, Angola, and Niger – had malaria prevalence rates of 12%, 5%, 4%, 4%, 3%, and 3%, respectively, and are considered endemic for malaria.[5]

Typhoid fever is an acute illness characterized by fever and caused by infection with the enteric *Salmonella typhi* and occasionally *Salmonella paratyphi.* The contamination of food or water by feces from a human carrier increases the chances of infection as well as the environmental dissemination through water and food-related routes.[6,7] Typhoid fever remains a public health problem across the globe, with an estimated 14 million new cases annually and 136,000 mortality in 2017.[6] The disease burden is particularly high in developing countries with poor or no sanitation facilities.[7]

Human immunodeficiency virus (HIV), a single-stranded diploid RNA virus, belongs to the *Retroviridae* family.[8] Countries in sub-Saharan Africa generally have the highest burden of HIV and AIDS compared to other regions of the world.[9] Hepatitis B virus (HBV), an enveloped virus, has a partially double-stranded circular DNA genome. It is a unique DNA virus that occurs in people of all ages with the same incident throughout the year.[10] According to a World Health Report in 2023, over two billion people globally are living with HBV, of which approximately 350 million are chronic carriers. Each year, about 600,000 patients die from HBV-related liver complications such as liver cirrhosis and hepatocellular carcinoma.[11] In West Africa, the prevalence of HBV in the general population is estimated at 8%, while in the other regions (Central, Eastern, and Southern Africa), the estimated prevalence is between 5% and 7%.[12] In sub-Saharan Africa with a significant burden, more than 45,000 HBV infections were transmitted annually between 2000 and 2011 through contaminated or unsafe transfusion.[13]

Helicobacter pylori is a predominant etiology of upper gastrointestinal disease, which includes heartburn, dyspepsia ulcer diseases, and gastroesophageal reflux disease. Worldwide, *H. pylor*i infection is the most common infectious pathogen, affecting more than 50% of the human population. In addition, it is responsible for 90% of duodenal ulcers and 70% of benign gastric ulcers.[14] High vaginal swab (HVS) infection of the genitor-urinary tracts and reproductive tracts is a frequent problem that affects women's sexual health. Women of reproductive age are present in most of the cases, and conditions such as vaginal discharge are common. Sexually transmitted infection (STI) and bacterial vaginitis are conditions often warranting HVS samples.[15] Urinary tract infection (UTI) refers to any infection involving any part of the urinary tract, such as the urethra, kidneys, ureters, and bladder. The urinary tract divides into two compartments: the upper (ureters and kidney) and the lower (urethra and bladder).[16]

Various misconceptions, in addition to drug resistance, insecticide resistance, misdiagnosis ignorance, multiple sexual partners, and poverty, are responsible for the failure of control programs on infectious diseases.[17] Worldwide, significant efforts are being made to understand these diseases and determine effective control measures. However, in Ore, Nigeria, these infectious diseases have not been adequately studied, and their prevalence has been increasing among the inhabitants of this locality due to poverty and the unavailability of specialists and resources.[18] The increase in the burden of infectious diseases caused by the selected pathogens in different population settings warrants the current epidemiological investigation.[19] In view of the dearth of data on the epidemiology of infectious diseases in the area, we investigated the distribution of infectious diseases among the people of Odigbo to generate information that can inform policy formulation on disease prevention and infection control strategies in the area.

2. Methods

2.1. Study area

The study was carried out in Ore, located in the Odigbo local government area of Ondo State, Nigeria. Ore is located at a latitude of 7°6'0.0181' N (7.100005) and a longitude of 4°5030.30984' E (4.841694). Ondo State shares borders with Kogi and Ekiti States to the north, Ogun and Oyo states in the west, Edo State in the east, and the Atlantic Ocean in the south. The total area of Ondo State is 14,788,723 km^2, with an estimated population of 3,441,024, making it the second-largest state in Nigeria. Predominantly, the inhabitants are subsistence farmers, with trading and fishing as secondary occupations. Ondo State experiences a tropical climate characterized by two distinct seasons: the rainy season (April – October) and the dry season (November – March). The temperatures typically range from 21°C to 29°C, and humidity levels are relatively high. Annual rainfall varies with estimates of 1150 mm and 2000 mm in the northern and southern areas, respectively. The state boasts lush vegetation, featuring a high forest zone (rain forest) in the south and sub-savannah forest in the northern regions.

2.2. Study period and population

This is a cross-sectional study in which consenting males and females aged 0 – 70 years were randomly recruited into the study between January and July 2019. A total of 4900 patients attending the Ore General Hospital were enrolled during this period. The Ore General Hospital, comprising both inpatient and outpatient departments, operates daily and serves as a referral hospital in Ondo State. The inclusion criteria included patients attending the selected hospital who consented to the study and were present in the hospital on the sampling day. Patients not attending the Ore General Hospital and who declined to participate in the study were excluded from the study. Patients aged ≥18 years provided both oral and written informed consent. For patients who were aged between 0 and 17 years, oral consent was obtained from their parents or legal guardians.

2.3. Sample collection and sample preparation

Blood samples (4 mL) were obtained from each subject using a syringe and needle through venipuncture and collected in sterile ethylene diamine tetraacetic acid bottles. A 2 mL aliquot of each sample was centrifuged at 5000 rpm for about 5 min to separate sera from the whole blood. Both the sera and the remaining 2 mL of whole blood were stored at 2 – 8°C for up to 2 days before assay.

2.4. Assay for malaria parasite

The SD bioline malaria antigen *Plasmodium falciparum* test cassette (Bio SD Inc., USA) was used for the detection of the histidine-rich protein II (HRP-II) antigen of malaria *P. falciparum* in human whole blood. The test is immunochromatographic and qualitatively detects the HRP II of *P. falciparum* in a human sample. It is an *in vitro* diagnostic technique with a diagnostic specificity of 99.5% and a sensitivity of 99.7%. The test procedure and interpretation were according to the manufacturer's instructions. All kit components and specimens were allowed to warm up to room temperature before testing. The test device was removed from the foil pouch and it was placed on a flat, dry surface. The fingertip was cleaned and prickled with a lancet. Approximately 5 μL of whole blood aliquot of each sample was drawn into the round sample well, and four drops of assay diluent were added. The result was read within 15 – 30 min. A positive result was indicated by two visible lines on both the test and control regions.

2.5. Assay for antibodies to HIV

The Chembio HIV ½ STAT-PAK test (Chembio Diagnostic System, USA) was used for the detection of antibodies to HIV-1/–2 in human whole blood. The test procedures and interpretation were according to the manufacturer's instructions. Briefly, the 5 μL of each serum prepared above was dispensed into the sample pad. The running buffer bottle was inverted, and three drops were added to each sample well. A positive result was indicated by two visible lines on both the test and control regions after 10 min.

2.6. Assay for hepatitis B surface antigen (HBsAg)

HBsAg rapid test strips were used for the detection of HBsAg (Guangzhou Wondfo Biotechnical Co. Ltd., China). The test is a rapid chromatographic immune assay that

qualitatively detects HBsAg in human blood. It is an *in vitro* diagnostic technique with a sensitivity and specificity of 92.2% and 99.3%, respectively. The test was performed and interpreted according to the manufacturer's specifications. Briefly, one drop (approximately 25 µL) of the whole blood aliquot sample was drawn into the specimen pad of the test strip, and one drop of buffer (approximately 40 µL) was added. A positive result was indicated by two visible lines on both the test and control regions between 10 and 15 min.

2.7. Assay for typhoid

The study employed the Widal Kit (Rapid Labs Diagnostic, United Kingdom) for the Widal agglutination test. Briefly, eight drops of each serum prepared above were dispensed in each of the eight circles on the test card. To each circle, a drop of polyvalent *Salmonella O* (*somatic antigen*) and *H* (*flagellar antigen*) *antiserum* was added. Using a disposable stirrer, the content of each circle was stirred to mix and spread over the entire ring of the circle in the test card. A mechanical rotator was used to rock the test card for 4 min, and agglutination was recorded thereafter at various ratios (1:20, 1:40, 1:80, 1:160, and 1:320), depending on the concentration of the agglutination reaction. Any serum with antibody titer >1/40 for *Salmonella* specimen somatic (O) or (H) antigen was considered positive for *Salmonella* infection. However, individual serum with a titer <1/40 was considered negative for *Salmonella* infection.

2.8. Assay for *H. pylori*

The procedure for the Wampole *H. pylori* assay (Cortez Diagnostic, China) was performed at room temperature. The test sera obtained after the separation above were diluted, as well as the cutoff calibrator and control sera 1:21 (e.g., 10 µL + 200 µL) in serum diluent. Six control/cutoff calibrators' determination was allowed (a reagent blank, a negative control, a positive control, and a cutoff calibrator run in triplicate). Patients are run in singlicate. The unused strips were returned properly to the pouch with desiccant. An adequate wash solution was prepared for the run (dilution of 1 part concentrate + 19 parts deionized water). All calibrators' controls and specimens were tested at the same time and run in duplicate.

2.9. Assay for UTIs

A urine specimen was collected through midstream or in-and-out catheter. This was collected before antibiotic treatment was started. The urine sample, refrigerated, was submitted to the laboratory within 24 h of collection. After urine culture, a bacterial count greater than or equal to 103 CFU/L with typical signs and symptoms compatible with UTI was considered significant. The presence of more than two organisms is not significant and indicates probable contamination.

2.10. Statistical analysis

The data generated in this study was analyzed using the Statistical Package for the Social Sciences software version 17.0 for Windows. In addition, one-way analysis of variance was employed where appropriate to determine the level of statistical significance. A $P < 0.05$ was considered significant.

3. Results

3.1. Distribution of infectious diseases among patients attending the Ore General Hospital

HIV accounted for a prevalence of 40% (400/1,000), malaria was 35% (700/2,000), HBsAg was 20% (80/400), *H. pylori* was 6.3% (20/300), HVS was 45% (90/200), and UTI was 10% (60/600) (Table 1).

3.2. Age distribution of some infectious diseases in Ore

Ages 31 – 40 years showed higher infection rates with HIV (45.5%) and HBV (25%) than the other age categories (Table 2). Malaria was more prevalent among ages 0 – 10 years (40%), while ages 61 – 70 years had the least prevalence (30%). Typhoid bacteria and UTI accounted for 43.8% and 12.5%, respectively, in ages 11 – 20 years, while ages 0 – 10 years with 0% prevalence were the least. Except for malaria, patients that were aged 0 – 10 years generally recorded the least prevalence of infectious diseases, and the difference was significant ($P < 0.05$) (Table 2).

3.3. Sex distribution of infectious diseases in Ore

The prevalence of HIV was 25% in males and 50% in females. Statistically, the difference was significant ($P < 0.05$). Malaria positives were 26.7% in males and 40%

Table 1. Distribution of infectious diseases among patients attending General Hospital Ore, Ondo State, Nigeria

Diseases	Number screened	Number positive	Prevalence (%)
Human immunodeficiency virus	1,000	400	40
Malaria	2,000	700	35
Typhoid	400	150	37.5
Hepatitis B virus	400	80	20
Helicobacter pylori	300	20	6.3
High vaginal swab	200	90	45
Urinary tract infections	600	60	10
Total	4,900	1,500	30.6

Table 2. Age distribution of some infectious diseases in Ore, Ondo State, Nigeria

Age range (years)	Human immunodeficiency virus			Malaria			Typhoid			Hepatitis B surface antigen			*Helicobacter pylori*			High vaginal swab			Urinary tract infections		
	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.
0 – 10	40	0	0	250	100	40	40	18	20	0	0	0	0	0	0	2	0	0	30	0	0
11 – 20	120	50	41.7	340	120	35.3	80	35	43.8	25	2	8	10	0	0	30	14	46.7	90	7	7.8
21 – 30	200	80	40	400	60	37.5	70	25	35.8	50	8	16	40	3	7.5	40	20	50	120	15	12.5
31 – 40	220	100	45.5	300	100	33.4	70	25	35.8	100	25	25	50	3	6	50	25	50	100	8	8
41 – 50	200	80	40	280	90	32.2	60	20	33.4	85	20	23.6	8	6	7.5	35	15	42.9	100	10	10
51 – 60	140	60	42.9	230	80	34.8	50	15	30	80	15	18.8	70	4	5.8	20	10	50	95	10	10.6
61 – 70	80	80	37.5	200	60	30	30	12	40	60	10	16.7	50	4	8	23	6	26.1	70	10	14.3
Total	1000	400	40	2000	700	35	400	150	37.5	400	80	20	300	20	6.3	200	90	45	600	60	10

Abbreviation: Prev.: Prevalence.

in females. The rate of typhoid fever in the females was twice as high as the rate in their male counterparts (20% vs. 40%), while an equal proportion was infected by HBV (20% each). *H. pylori* prevalence was 5.4% (8/150) in males against 8% (12/150) of the females. UTI was lower in the males (8%) compared to the females (11.5%). However, the observed difference was not significant ($P > 0.05$) (Table 3).

3.4. Distribution of infectious diseases based on occupation

Civil servants with an HIV prevalence of 50% (100/200) and a UTI rate of 20% had the predominance of HIV and UTIs compared to the other occupational categories. Similarly, students in the study had malaria predominance (43.5%), the clergy had a predominance of typhoid fever (62.5%), artisans had hepatitis B surface antigenemia predominance (40%), while subjects involved in trading had *H. pylori* predominance of infection (8.4%) relative to the other occupational groups (Table 4).

3.5. Distribution of infectious disease based on education status

Holders of primary school certificates had a higher *H. pylori* of 7.5% (3/40) compared to those with secondary and tertiary qualifications. Those with senior secondary school certificates generally had a higher prevalence of HIV (45%), malaria (38.5%), typhoid infection (35.8%), and HBV (20%) infections compared to the other educational groups (Table 5). Statistically, the observed difference was significant ($P < 0.05$). Those with tertiary institutions had more UTIs (12.5%, 10/80) compared to the others in the group (Table 5).

3.6. Distribution of infectious diseases based on marital status

The divorced had a higher prevalence of HIV (62.5%), followed by the married and widowed (50% each) and singles (10%), respectively. A statistically significant difference was observed between marital status and the occurrence of HIV in patients ($P < 0.05$). Similarly, the divorced had significantly more malaria (50%) than the singles 35.8%, married (30%), and widowed (25%) patients. Further, typhoid fever was more prevalent among the divorced patients (45%) compared to the singles (40%) and married (37.5%) patients. In the singles, the prevalence of HBsAg was 20% (16/80), which is higher than the 15% and 5% rates observed among divorced and married patients. In general, the prevalence of *H. pylori* was fairly close, as it was 5% among singles, 6% among the married, and 6.7% in divorced patients. Statistically, the difference was not significant ($P > 0.05$). UTI was lowest among the singles (1.2%) and highest among the divorced (33.7%) (Table 6).

Table 3. Sex distribution of infectious diseases in Ore, Ondo State, Nigeria

SEX	HIV		MALARIA		Typhoid		HBsAg		*Helicobacter pylori*		HVS		UMCS	
	n	*n* (%)	*n*	*n* (%)	*n*	*n* (%)	*n*	*n* (%)	*n*	*n* (%)	*n*	*n* (%)	*n*	*n* (%)
Male	400	100 (25)	750	200 (26.7)	150	60 (40)	150	30 (20)	150	8 (5.4)	-	-	250	20 (8)
Female	600	300 (50)	1,250	500 (40)	250	90 (36)	250	50 (20)	150	12 (8)	200	90 (45)	350	40 (11.5)
Total	1,000	400 (75)	2,000	700 (40)	400	150 (37.5)	400	80 (40)	300	20 (6.3)	200	90 (45)	600	60 (10)

Note: N=total number tested; n=number of positive samples.
Abbreviations: HIV: Human immunodeficiency; HVS: High vaginal swab; UMCS: Urine microscopy, culture, and sensitivity tests; HBsAg: hepatitis B surface antigen.

3.7. Distribution of infectious diseases based on religion

Christians had an HIV prevalence of 43.4%, followed by Muslims (40%) and the traditional group (30%). The malaria prevalence was fairly close: 35.8% among Christians, 35% among Muslims, and 33.4% among those who practiced traditional religion. Typhoid infection was more prevalent among the Muslims (44.5%) compared to the Christians (31.3%) and traditional groups (33.4%). HBV was more prevalent among traditional people (25%), followed by Muslims (22.5%), while Christians had the least (15.7%). Overall, none of the religions significantly influenced HIV, typhoid fever, HBV, *H. pylori* UTI, or malarial infection ($P > 0.05$) (Table 7).

3.8. Distribution of some infectious diseases based on parturition

Participants who recorded >7 births had a higher prevalence of HIV (58.4%), while those with 3 – 4 births had the lowest prevalence (35.8%) of HIV. Malaria was more common in those with >7 births (60%), while those with 1 – 2 births had the least (53.4%). Typhoid fever was higher among those with 3 – 4 births (40%) but least recorded among those with 1 – 2 births (31.3%). Furthermore, HBsAg dominated among those with 5 births and above while those with 1 – 2 births recorded the lowest antigenemia rate. *H. pylori* detection rates varied from 5% to 7.5% across the groups, with higher birth rates associated with increased prevalence of infection. The urine microscopy revealed a fairly close prevalence range (12.3% among those with 1 – 2 births and 15% each among those with 5 – 6 and >7 births) (Table 8).

4. Discussion

The present study revealed that the overall prevalence rate of HIV, malaria, typhoid, HBsAg, *H. pylori,* HVS, and UTI is 40% (400/1,000), 35% (700/2,000), 37.5% (150/400), 20% (80/400), 6.3% (20/300), 45% (90/200), and 10% (60/600), respectively. These rates were significantly different ($P < 0.05$) between the infectious diseases, thus highlighting their endemic nature in the area. Previous studies in different parts of Nigeria and outside Nigeria have reported varying prevalence rates among selected groups.[9,20] Differences in sociodemographic risk factors, period of study, diagnostic screening modality, and duration of study could be the reason for the prevalence rate disparity.

The HIV infection rate of 40% in this study is significantly higher than the 0.3% reported by Abraham *et al.*[21] in Kogi State and the 5.0% national prevalence previously reported by the Federal Ministry of Health.[22] Furthermore, the infection rate of 40% reported for HIV in this study is lower than the 77% reported by Landoh *et al.*,[20] who carried out their research in Togo, which focused primarily on stable heterosexual couples, as well as the 17% observed among those engaged in casual sex. The higher prevalence obtained in this study compared to the national sentinel seroprevalence rate may be explained by the fact that the study was carried out in a sub-region of Ondo State, which is reportedly endemic for HIV.

The malaria infection rate of 35% in the study is lower than the findings of Ukaegbu *et al.*[23] in Jos, Plateau State, Nigeria, who observed 54.00% of malaria infection. However, the infection rate of 35% reported for malaria in this study is higher than 14.7% reported by the Ibashe community in Ikorodu, Lagos State by Aina *et al.*[24] and 22 – 40% reported by Omatola and Okolo,[25] and Okolo *et al.*[4] in Anyigba town, Nigeria. The difference in prevalence rates between the various studies could be due, in part, to the differences in seasons and prevalent risk factors for disease acquisition in different geographical settings. As a precaution, the use of insecticide-treated net (ITN) and other protective measures to further reduce the exposure of the individual to mosquito bites is advocated.

The typhoid fever rate of 37.5% in this study is higher than the findings of Ukaegbu *et al.*[23] in Jos Plateau State, Nigeria, who observed 22.67 – 25.67% of typhoid infection. However, the infection rate of 37.5% reported for typhoid in this study is lower than the finding of Okolo *et al.*[4] in

Table 4. Distribution of infectious diseases based on occupation in Ore, Ondo State, Nigeria

Occupation	Human immunodeficiency virus			Malaria			Typhoid			Hepatitis B surface antigen			*Helicobacter pylori*			High vaginal swab			Urinary tract infections		
	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.
Civil servant	200	100	50	280	100	35.8	60	30	50	80	16	20	80	6	7.5	40	20	50	40	8	20
Student	200	50	25	460	200	43.5	120	40	33.4	60	6	10	30	1	3.4	30	10	33.4	200	16	8
Trading	260	120	46.2	400	120	30	100	30	30	120	22	18.4	60	5	8.4	50	25	50	180	14	7.8
Clergy	85	30	25	300	80	26.7	32	20	62.5	100	20	20	70	4	5.8	20	5	25	80	10	10
Artisans	220	100	45.5	460	150	32.6	80	25	31.1	40	16	40	60	4	6.7	60	30	50	100	10	10
Total	965	400	41.5	1900	650	34.3	392	145	36.9	400	80	20	300	20	6.3	200	90	45	600	60	10

Abbreviation: Prev.: Prevalence.

Table 5. Distribution of infectious disease in relation to educational qualifications of participants in Ore, Ondo State, Nigeria

Education	Human immunodeficiency virus			Malaria			Typhoid			Hepatitis B virus			*Helicobacter pylori*			High vaginal swab			Urine Mcs		
	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.
Primary	200	80	40	600	200	33.3	120	40	33.3	100	20	20	40	3	7.5	60	20	33.3	180	16	8.9
Secondary	400	180	45	65 0	250	38.5	140	50	35.7	200	40	20	60	4	6.7	80	30	37.5	260	20	7.7
Tertiary	300	90	30	400	140	35	60	20	33.3	60	10	16.7	110	8	7.3	40	20	50	80	10	12.5
Total	900	350	115	1,650	590	37.8	320	110	34.4	360	70	19.5	210	15	7.2	180	70	38.9	520	46	8.9

Note: *P*-value=0.000. Abbreviation: Prev.: Prevalence.
Abbreviation: Urine MCS: Urine microscopy, culture, and sensitivity tests.

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Table 6. Marital status distribution of some infectious diseases in Ore, Ondo State, Nigeria

Marital status	Human immunodeficiency virus			Malaria			Typhoid			Hepatitis B surface antigen			*Helicobacter pylori*			High vaginal swab			Urinary tract infections		
	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.
Single	300	30	10	560	200	35.8	100	40	40	80	16	20	20	1	5	40	20	50	180	2	1.1
Married	400	200	50	1,000	300	30	160	60	37.5	180	40	22.3	100	6	6	70	40	57.2	300	30	10
Divorced	80	50	62.5	200	100	50	40	18	45	40	6	15	60	4	6.7	50	20	40	30	10	33.4
Widow	160	80	50	160	80	50	80	20	25	80	14	17.5	100	8	8	40	10	25	60	13	21.7
Widower	60	40	66.7	80	20	25	20	12	60	20	4	20	20	2	10	0	0	0	30	5	16.7
Total	1,000	400	40	2,000	700	35	400	150	37.5	400	80	20	300	20	6.3	200	90	45	600	60	10

Note: One-way analysis of variance: P=0.002.
Abbreviation: Prev.: Prevalence.

Table 7. Distribution of infectious diseases in relation to religion of subjects in Ore, Ondo State, Nigeria

Religion	Human immunodeficiency virus			Malaria			Typhoid			Hepatitis B virus			*Helicobacter pylori*			High vaginal swab			Urine Mcs		
	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.
Christian	300	130	43.3	700	250	35.7	160	50	31.3	160	25	15.6	120	6	5	60	30	50	260	20	7.7
Muslim	600	240	40	1,000	350	35	180	80	44.4	200	45	22.5	130	10	7.7	100	50	50	300	30	10
Tradition	100	30	30	300	100	33.3	60	20	33.3	40	10	25	50	4	8	40	10	25	40	10	25
Total	1,000	400	113.3	2,000	700	104	400	150	109	400	80	63.1	300	20	20.7	200	90	125	600	60	42.7

Abbreviations: Prev.: Prevalence; Urine MCS: Urine microscopy, culture, and sensitivity tests.

Table 8. Distribution of infectious diseases based on parturition in Ore, Ondo State, Nigeria

Parturition	Human immunodeficiency virus			Malaria			Typhoid			Hepatitis B surface antigen			*Helicobacter pylori*			High vaginal swab			Urinary tract infections		
	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.	No. screened	No. (+ve)	% Prev.
1–2	180	80	44.5	300	160	53.4	80	25	31.3	100	10	10	40	2	5	80	30	37.5	180	22	12.3
3–4	280	100	35.8	400	220	55	100	40	40	60	16	26.7	60	3	5	10	6	60	120	18	15
5–6	200	85	42.5	280	160	57.2	80	30	37.5	100	20	20	100	8	8	50	22	44	80	12	15
>7	120	70	58.4	200	120	60	40	15	37.5	60	18	30	80	6	7.5	20	12	60	40	6	15
Total	1,000	400	40	2,000	700	35	400	150	37.5	400	80	20	300	20	6.3	200	90	45	600	60	10

Note: One-way analysis of variance: P=0.00.
Abbreviation: Prev.: Prevalence.

Kogi State, Nigeria, who observed a 47% prevalence of typhoid infection. The variation in the results could be attributed to differences in the environmental conditions of the studied populations, such as poor hygiene that leads to fecal contamination and a lack of access to clean drinking water, as previously observed.[23] Evidence from this study indicates that the community is highly vulnerable to typhoid infection disease, principally due to contamination of the source of water to the community (River Arun in Idanre), which could lead to the infection of other individuals and deaths of the infected ones.

The HBV infection rate of 20% in this study is, however, higher than the 3.5 – 8.0% reported among HIV-positive patients in Kogi State[26,27] and the 7.5% reported by Okolo and Omatola,[28] among apparently healthy individuals in North Central Nigeria. Furthermore, the infection rate of 20% reported for HBsAg in this study is lower than the findings of Mbaawuaga *et al.*,[29] who observed 30% of HBV positivity in Lagos, Southern Nigeria. The differences in this study could be attributed to differences in population selection.[29] People with this infection should be treated effectively to prevent further spread of HBsAg infection, which may lead to serious short- and long-term health implications for the populace.

The high *H. pylori* infection rate of 6.3% observed in this study may be because of the unavailability of safe and treated drinking water in Ore, Ondo State. 45% of HVS in this study is comparable with the findings of Aggarwal *et al.*[30] who observed 48.50% among women of reproductive age in rural areas of Haryana, India. Our prevalence rate is far lower than the 78% positivity rate earlier reported.[31] The variation in prevalence rates could be explained by the behavioral and host factors differences in the different populations sampled. As previously observed, Roberta *et al.*[32] reported that the prevalence of bacterial vaginosis increased significantly in patients who practiced regular douching. This finding corroborates the results from this study, in which participants who were douched with either water or antiseptics/soaps to ease the symptoms associated with bacterial vaginosis showed a high predisposition to *H. pylori* infection. According to Lawrence *et al.*,[31] the presence of vaginal microbial flora, including members of the *Lactobacilli* family, helps maintain the pH of the vagina and further prevents the overgrowth of potential pathogens. Therefore, there is a need for comprehensive healthcare education for women of reproductive ages, including the use of barrier methods and routine checks of their vaginal health for early detection of HVS.

The UTI infection rate of 10% in this study is in agreement with the findings of Kaye and Sobel,[33] who showed that 10% of women in the US manifest with one or more episodes of symptomatic UTIs each year.

Importantly, urine microscopy can help to differentiate etiologies of acute kidney injury, uncover the necessity for kidney biopsy, and guide decisions toward a definitive therapy. The studies of Perazella *et al.*[34] have shown that urine microscopy could better differentiate acute tubular necrosis conditions from perennial azotemia. Besides, the findings of urine microscopy have been correlated with renal outcomes.[35]

From Table 6, the prevalence rate of HIV infection (66.7%) in relation to widowers is significantly higher than any other group in marital status in the table ($P < 0.05$). This observation is similar to the finding of Landoh *et al.*,[20] who observed 77% of the infections that appear mostly in stable heterosexual couples in Togo. The differences in prevalence in these studies could be attributed to differences in population selection.[20] The reason for the high infection rates among divorced and widowed individuals may be due to the multiple sexual partners. Individuals are encouraged to avoid sexual contact with infected persons, which is the cornerstone of HIV prevention. The use of barrier methods such as a sheath or condom during intercourse may further reduce the transmission.

The malarial prevalence rate (50%) in this study is similar to the findings of Ukaegbu *et al.*[23] in Jos Plateau State, Nigeria, but higher than the 28.8% reported by Mofolorunsho *et al.*[36] in Lokoja, Nigeria. The prevalence rate of typhoid infection (60%) in relation to widowers is higher than in any other marital group. This is similar to the finding of Buckle *et al.*,[37] who reported 69% typhoid fever prevalence in 21 regions from a pooled study across the world in 2010. The 60% prevalence of typhoid infection in relation to widowers in this study is, however, lower than the finding of Malisa and Nyaki,[38] who reported a typhoid fever prevalence rate of 95% in areas endemic to typhoid fever in the Singida region of Tanzania. It is lower than the findings of Ukaegbu *et al.*[23] in Jos Plateau State, Nigeria, who observed 24.67 – 25.67% typhoid infection. The variation in the result could be attributed to differences in the environmental conditions at the time of study.

The high prevalence of HBV infection (22.3%) among married persons is similar to the findings of Alao *et al.*,[39] who reported a 25.6% rate among elderly people. This is also similar to previous studies, which observed that HBsAg and HCV prevalence rates increase among young age groups, which have been previously reported in studies elsewhere in Nigeria and outside Nigeria. Buseri *et al.*[40] reported the HBV prevalence rate to be highest among the age range of 18 – 27 years. This shows that HBV is an epidemic in the studied population among married people in Ore.

The prevalence rate of *H. pylori* infection in relation to marital status shows that widowers have the highest prevalence rate of 10% when compared to any other marital group. *H. pylori* has been shown to demonstrate a remarkable genetic and phenotypic diversity, multiple transmission paths, high prevalence, and dramatic increase in antibiotic resistance. Thus, there is a need for focused attention on disease epidemiology, prevention strategies, updated antibiotic guidelines, therapeutic options, and the development of effective vaccines.

The prevalence rate of HVS in relation to marital status in this study shows that married women have the highest prevalence rate of 57.2% when compared to other marital groups. However, this is lower than what the World Health Organization estimated: 75% to 80% of new cases of sexually transmitted diseases are in developing countries. 74% observed by Udenze *et al.*[41] among female students in Abia State, Nigeria, who had both symptomatic and asymptomatic lower genital infection. The reason may be that dwellers of Ore are not conscious of the menace of developing HVS infection. It may also be due to the difference in sample size. Nevertheless, the rate of UTI observed in this study is considered high, and a plausible reason may be due to unprotected sexual intercourse with the infected patient, and it may be due to the study period, sample size, and the diagnostic procedure used. The implications of these findings can emanate from kidney failure, diabetes mellitus, etc.

The high prevalence (58.4%) of HIV observed in those with >7 births is lower than the 3.4% rate reported by Adelekan *et al.*[42] during the 2012 National HIV/AIDS and Reproductive Health Survey. This finding could be attributed to the fact that some people in Ore indulged in unprotected sexual activity, contact with contaminated blood/blood products, and use of contaminated body-piercing instruments that may lead to greater infection with HIV. The prevalence rate of malaria infection in relation to parturition shows that those having seven or more children had the highest infection rate of 60% in this study. The presence of bushy environments in the area and the unavailability of ITNs could have contributed to the disease burden. Importantly, the use of ITN and other protective measures, such as the provision of screens to doors and windows and the wearing of long-sleeved clothing to reduce the exposure of the individual to mosquito bites, are advocated. The prevalence rate of HBsAg infection in relation to parturition shows that those with seven or more children have the highest rate of 30% when compared to any other parity group. This finding concurred with what was earlier observed by Nbaawuaga *et al.*[29] on the distribution of HBV infection in Nigeria, including south-south, where he observed a 30% prevalent rate in Lagos, Southern Nigeria, for HBV-related liver cirrhosis and hepatocellular carcinoma.

The current study has certain limitations. First, not all 4900 patients were screened for all the infectious agents under investigation due to resource constraints, leading to testing being restricted to the suspected condition that prompted the hospital visit and the specific screening requested by the physician. This limitation hinders the generalizability of our findings to the true burden of infectious diseases in the area. Second, the study relied solely on serological-based diagnoses, which are unable to detect occult infections. Future studies utilizing more sensitive molecular techniques could better identify both symptomatic and asymptomatic infections caused by microbial agents in the area. Notwithstanding, the current study provides valuable baseline epidemiological information regarding infectious diseases in the area, serving as a foundation for future studies.

5. Conclusion

This study revealed an endemic level of globally important infectious diseases among patients attending the general hospital in Ore-Odigbo, Ondo State, Nigeria. The overall prevalence rates of HIV, malaria, typhoid, HBV, *H. pylori,* HVS, and UTI were 40%, 35%, 37.5%, 20%, 6.3%, 45%, and 10%, respectively. Efficient use of ITN, clearing bushy environments, and proper waste disposal may further reduce the malaria burden in the area. Providing a reliable source of quality water could also help decrease the prevalence of endemic typhoid. In addition, promoting behavioral changes – including fidelity among married partners and sexual abstinence among young adults – could further reduce the burden of STIs in the area.

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Conflict of interest

The authors declare that they have no competing interests.

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Ethical approval for the study was obtained from the State Ministry of Health in accordance with the Helsinki Code of Conduct for biomedical research involving human subjects.

Consent for publication

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All the data used in the study are included in the manuscript.

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ORIGINAL RESEARCH ARTICLE

Hydrogen alleviates non-alcoholic fatty liver disease in mice by regulating intestinal flora

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Abstract

Despite being the most common form of chronic liver disease, there are still no approved drugs for the treatment of non-alcoholic fatty liver disease (NAFLD). The aim of this study was to elucidate the therapeutic effects and possible mechanisms of hydrogen (H_2) inhalation in mice with NAFLD. Male C57BL/6 mice (6 weeks old) were fed either a 60% fat diet (high-fat diet [HFD]) or a 10% fat diet (normal diet) for 11 weeks. Then, H_2 was administered to random HFD-fed mice for another 11 weeks before they were euthanized. Biochemical analysis of serum samples, histological analysis of liver and ileum samples, 16S rRNA sequencing analysis of stool samples, and analysis of the expression levels of related factors by enzyme-linked immunosorbent assay were conducted to determine the effect of H_2 intervention on NAFLD. H_2 inhalation alleviated hyperglycemia and impaired glucose tolerance; decreased the serum concentrations of triglycerides, cholesterol, alanine aminotransferase, aspartate aminotransferase, lipopolysaccharide, and tumor necrosis factor-alpha; and ameliorated liver injury by a HFD, although no weight loss was observed. Interestingly, H_2 inhalation increased the relative abundance of *Akkermansia muciniphila* and decreased the *Firmicutes*-to-*Bacteroidia* ratio in the intestinal tract of NAFLD mice. These data indicate that H_2 alleviated the symptoms of NAFLD by increasing the abundance of *A. muciniphila* in the intestine. Thus, H_2 may be a new potential treatment strategy for patients with NAFLD.

Keywords: Hydrogen; Non-alcoholic fatty liver disease; Intestinal flora; High-fat diet

1. Introduction

Non-alcoholic fatty liver disease (NAFLD), which is caused by unknown factors other than alcohol and other factors of liver injury, refers to a clinicopathological syndrome that is characterized by excessive deposition of fat in liver cells.[1] NAFLD is one of the most common liver diseases. With the increasing prevalence, NAFLD has become the

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predominant cause of morbidity and mortality among liver-related diseases,[2] particularly due to the significantly growing prevalence of diabetes-related metabolic diseases and cardiovascular disease in NAFLD patients.[3] Currently, no approved drugs that are specific for NAFLD are available.[4]

Intestinal microorganisms are involved in the development of NAFLD through the gut-liver axis. An imbalance in the intestinal flora causes changes in intestinal permeability, resulting in the release of metabolites produced by the intestinal flora into the blood, thus activating the inflammatory pathway and releasing pro-inflammatory factors (*e.g.*, tumor necrosis factor-alpha [TNF-α] and interleukin [IL]-6) into the liver. Elevated serum concentrations of TNF-α in NAFLD patients correlate with the severity of liver tissue destruction.[5] Moreover, previous studies have shown that the intestinal bacterium *Akkermansia muciniphila* is negatively associated with metabolic disorders such as obesity, diabetes, and inflammation.[6] Oral administration of *A. muciniphila* was found to increase the expression of intestinal tight junction proteins such as zonula occludens-1 and occludin in the intestines of mice with NAFLD.[7] Therefore, *A. muciniphila* is thought to reduce endotoxemia levels and alleviate local inflammation by reducing intestinal permeability and strengthening the intestinal barrier.[8,9]

Hydrogen (H_2) is a novel method for treating inflammatory diseases.[10] Recent studies have shown that H_2 plays a promising role in antioxidation,[11] weight reduction[12,] and Kawasaki disease treatment.[13] H_2 can also alleviate NAFLD-related symptoms.[14] Although a few reports have suggested that the development of NAFLD is related to changes in intestinal microflora, whether H_2 alleviates NAFLD patients' symptoms through alterations in certain intestinal microflora remains unclear.[15,16] To explore the therapeutic effect and mechanism of H_2 in NAFLD, in our study, we investigated changes in the gut microbiota in NAFLD model mice treated with 30% H_2. This study provides a theoretical basis for the treatment of NAFLD with H_2.

2. Materials and methods

2.1. Materials and instrument

Male C57BL/6 mice (aged 6 weeks; SCXK (su) 2018 – 0008) were procured from GemPharma Tech LLC (China). Materials and instruments used in this study are listed as follows: High-fat diet (HFD; 60% fat diet, XTHF60, XIETONG Biology, Nanjing, China); AMS-H-01 H_2/oxygen nebulizer (Asclepius, Shanghai, China); glucometer (GA-6, Sanocare, Shenzhen, China); hematoxylin-eosin (H&E) solution (E607318-0200, Sangon Biotech, Shanghai, China); Oil Red O solution (C0157S, Beyotime Technology, Shanghai, China); Mouse Lipopolysaccharides (LPSs), enzyme-linked immunosorbent assay (ELISA) kit (CSB-E13066m, Cusabio, Wuhan, China); Mouse TNF-α, TNF-α ELISA Kit (CSB-E04741m, Cusabio, Wuhan, China); SpectraMax M5 Reader (Molecular Devices, USA); genomic DNA extraction kit for soil and fecal samples (CW2091S, Novogene, Tianjing, China); GeneJET Gel Recovery Kit (K0691, Thermo Scientific, USA); TruSeq DNA polymerase chain reaction (PCR)-Free Library Preparation Kit (20000902, Illumina, USA); Qubit Instrument (Novogene, Tianjing, China); and NovaSeq 6000 sequencing system (Novogene, Tianjing, China).

2.2. Mouse model and H_2 intervention

Male C57BL/6 mice (aged 6 weeks) were housed in an animal facility under standard specific pathogen-free conditions at 22°C on a 12-h light/dark schedule. All animal studies were approved by the Institutional Animal Care and Use Committee of the Tongji University Affiliated East Hospital (T3LAC-015-038). After 1 week of acclimatization to a normal diet, the mice were divided into three groups: the normal control group, the HFD group, and the HFD + H_2 group, with five mice in each group. The mice of the control group were fed with ordinary diet. The model was induced by feeding the animal with HFD diet, which contained 20% protein, 20% carbohydrates, and 60% fat (Figure 1A). The experiment of the mouse model lasted for 11 weeks.

Mice in the HFD + H_2 group were subjected to H_2 inhalation for 11 weeks starting at the 12th week (Figure 1B). H_2 was provided by our self-developed device (AMS-H-01 H_2/oxygen nebulizer), which consists of a gas source device, a gas mixing device, a carbon dioxide absorption device, and a H_2 treatment chamber. The gas source unit produces a H_2-oxygen gas mixture by electrolyzing water. The flow rate of the gas mixture was adjusted through the gas mixing unit. The carbon dioxide absorption unit is mainly composed of soda lime and can absorb carbon dioxide produced by the animals in the chamber. For the H_2 absorption treatment, the mouse cage box was placed in the H_2 treatment chamber and the H_2-oxygen gas mixture (containing 33.3% H_2) was inhaled with a total flow rate of 3 L/min for 8 h, once daily.

2.3. Blood glucose and body weight measurements

Blood glucose measurement was performed as follows: the tail of each mouse was pierced by a needle, and a drop of venous blood was collected to measure blood glucose levels with a glucose meter once every 2 weeks. The weights of mice were also measured once every 2 weeks.

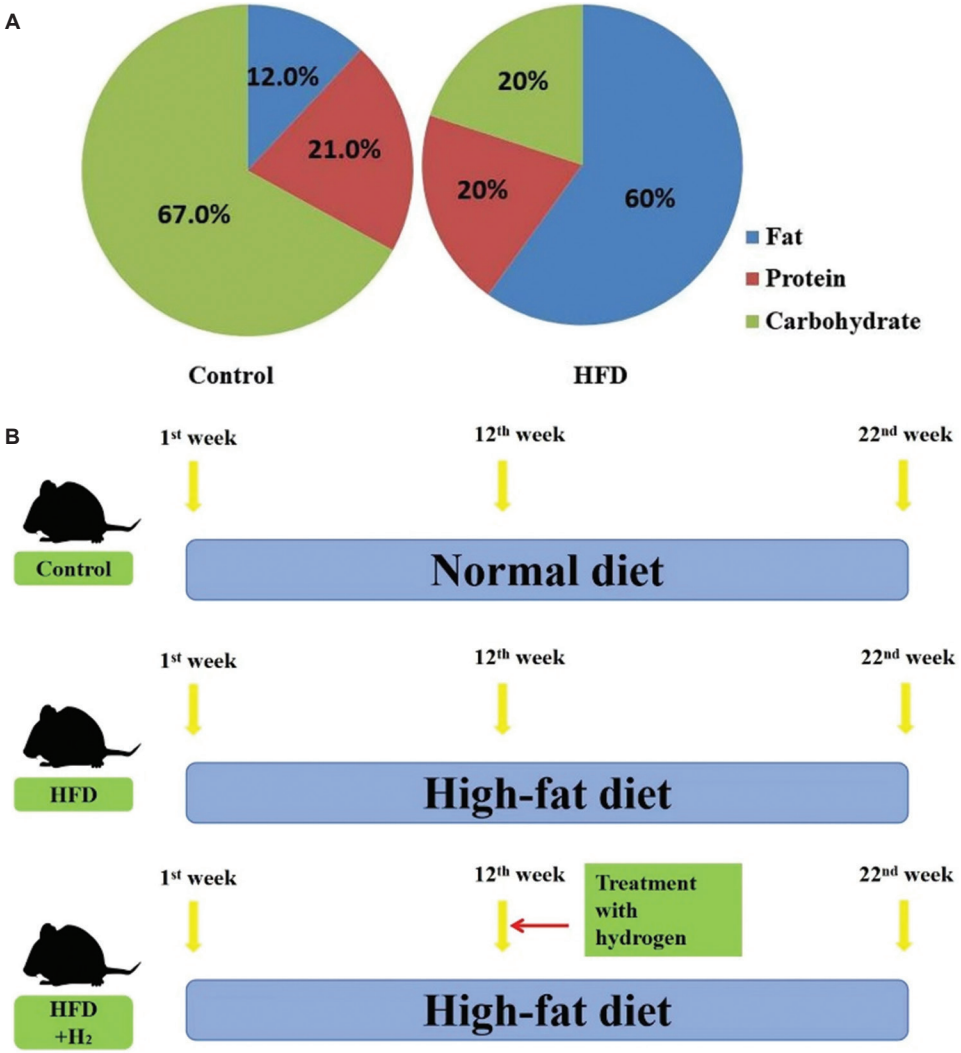

Figure 1. Mouse modeling and hydrogen (H_2) intervention. (A) The nutrient ratios of the normal feed and high-fat feed used in the experiment. (B) The experiment involves the modeling process and the H_2 intervention.

2.4. Oral glucose tolerance test (OGTT)

At 11 weeks after H_2 treatment, an OGTT was performed by glucose (2 g of glucose per kg body weight) gavage after overnight fasting for 12 h. Blood samples were collected from the mice at 0 h, 0.5 h, 1 h, and 2 h after glucose gavage, after which the blood glucose level was measured through a glucometer.

2.5. Histological morphology of the liver in mice

The mice were sacrificed by cervical vertebra dislocation after being anesthetized with isoflurane gas (3 – 4%). Liver tissues were fixed with paraformaldehyde, and 4-μm-thick paraffin-embedded sections were cut and stained with H&E solution. The other liver tissues were quickly frozen, and the sections were stained with Oil Red O solution. The morphology of the tissue was observed under a microscope.

2.6. Blood biochemistry and inflammatory factor measurement

The serum concentrations of total cholesterol (TC), triglyceride (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), LPS and TNF-α in mice were determined by ELISAs according to the manufacturer's instructions (Cusabio, Wuhan). The optical density was detected at 450 nm by a SpectraMax M5 Reader.

2.7. 16S rRNA sequencing

To investigate the effect of H_2 intervention on intestinal flora in NAFLD mice, 16S rRNA sequencing was performed.

Fresh mouse feces were collected, and DNA was extracted from the samples using a genomic DNA extraction kit with magnetic beads for soil and fecal samples. The PCR amplification procedure was as follows: PCR reaction system (30 μL): Phusion Master Mix (×2) 15 μL, primer (2 μM) 3 μL (6 μM), fecal gDNA (1 ng/μL) 10 μL (5 – 10 ng), and water 2 μL. The PCR procedure encompasses the following stages: Pre-denaturation at 98°C for 1 min; followed by 30 cycles of 98°C for 10 min, 50°C for 30 min, and 72°C for 30 min; and 72°C for 5 min. PCR products were detected by electrophoresis using 2% agarose gel. Then, the PCR products were mixed in equal concentrations according to the PCR product concentration. After mixing, the PCR products were purified by electrophoresis using 1 × TAE agarose gel at 2% concentration, and the target bands were recovered by the GeneJET Gel Recovery Kit. The TruSeq DNA PCR-free Library Preparation Kit was subsequently used for library construction. After the constructed library was quantified and analyzed with a Qubit instrument, the NovaSeq 6000 sequencing system was used for sequencing.

2.8. Statistical analysis

The values in each group were normally distributed, and the overall variance was homogeneous in the three groups. All values are presented as mean ± standard deviation for each group. One-way analysis of variance and LSD test were used to compare the statistical differences among multiple groups. $P < 0.05$ was considered significantly different. GraphPad Prism (GraphPad Software, USA) 7was used for statistical analysis.

3. Results

3.1. Effect of H_2 on the body weight of NAFLD mice

We successfully established a NAFLD mouse model that was characterized by typical obesity through an HFD feeding protocol. Compared to those in the control group, the mice in the HFD and HFD + H_2 groups stored more fat, with knotted and glossy fur, as well as a large accumulation of fat in the abdominal cavity, as was observed after dissection (Figure 2A). The body weights of the mice in both the HFD group and the HFD + H_2 group were significantly greater than those of the mice in the control group ($P < 0.0001$), but there was no significant difference between the HFD group and the HFD + H_2 group ($P > 0.05$) (Figure 2B and C).

3.2. H_2 administration improves blood glucose levels and lipid metabolism in mice with NAFLD

Given that NAFLD is strongly associated with impaired levels of TGs and glucose metabolism, we monitored blood glucose levels every 2 weeks. There was no significant difference in random blood glucose levels among the control mice at weeks 1, 11, and 22 ($P > 0.05$). The random blood glucose levels of the HFD group gradually increased compared to those of the control group at the 22nd week ($P < 0.01$) (Figure 2G and E). However, the blood glucose levels in the HFD + H_2 group were lower than those in the HFD group ($P < 0.05$), while there was no difference in the blood glucose levels between the HFD+H_2 group and the control group at the 22nd week ($P > 0.05$) (Figure 2F). These data suggest that H_2 inhalation ameliorated hyperglycemia in NAFLD mice. After the experiment finished, the mice were fasted overnight, and the OGTT was performed. The fasting blood glucose level of the HFD group was significantly higher than that of the control group ($P < 0.0001$). The fasting blood glucose level of the HFD + H_2 group was significantly lower than that of the HFD group ($P < 0.01$) but still higher than that of the control group ($P < 0.01$) (Figure 2I). The blood glucose level in the HFD + H_2 group decreased to the same level as that in the control group 2 h after glucose administration, but the blood glucose in the HFD group was significantly greater than that in the control group 2 h after glucose gavage (Figure 2H). These results suggest that an HFD induces abnormalities in glucose tolerance in mice and that H_2 inhalation can reverse HFD-induced abnormalities in glucose tolerance.

Compared with those in the control group, the levels of TC ($P < 0.05$) and TG ($P < 0.0001$) in the HFD group were significantly higher. However, the levels of TC ($P < 0.001$) and TG ($P < 0.05$) in the HFD + H_2 group were significantly lower than those in the HFD group. There was no significant difference in TG level between the HFD + H_2 group and the control group ($P > 0.05$), although the TC levels in the HFD + H_2 group were still higher than those in the control group ($P < 0.0001$) (Figure 3B and C).

3.3. H_2 administration alleviates liver damage and improves liver function in NAFLD mice

Compared with those in the control group, the livers of the mice in the HFD group were significantly larger, with a tense and smooth envelope, blunted edges, a yellow color, scattered yellow fat spots, a soft texture, and a greasy feeling. The livers of mice in the HFD + H_2 group were also enlarged in size, with a reddish color but fewer scattered yellow fat spots than those in the HFD group. H&E staining revealed that the liver structure of HFD group mice was abnormal, with incomplete hepatic lobules and dense voids. Compared with those in the HFD group, the liver morphology and structure in the HFD+H_2 group were greatly restored, and the number and area of cavities were reduced, suggesting that H_2 alleviated the structural abnormalities of the liver in NAFLD mice. Compared with those in the control group, liver lipid droplets in the HFD group were densely distributed, and the lipid droplets were larger according to Oil Red O staining. In the HFD+H_2 group, the distribution

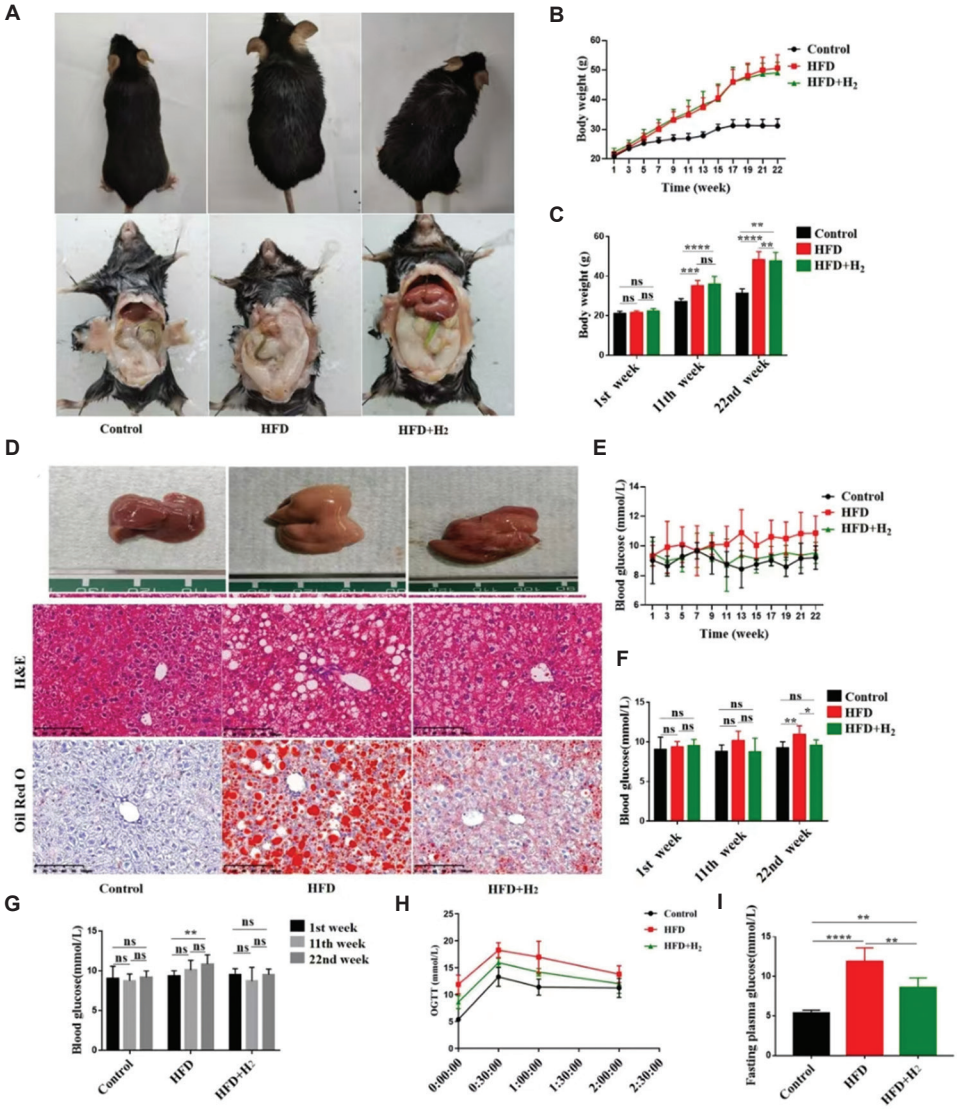

Figure 2. Hydrogen (H_2) intervention improves lipid metabolism and reduces blood glucose levels in non-alcoholic fatty liver disease mice. (A) Mouse appearance and abdominal anatomy. (B) The curve of the body weight of mice during the study. (C) The body weight of each group of mice at 3 time points during the study was compared. (D) Appearance of the mouse liver and hematoxylin-eosin and Oil Red O staining results. Scale bar: 100 μm (magnification: ×200). (E) The variation in random blood glucose levels in the three groups during the study. (F) The random blood glucose levels of each group at 3 time points in the study were compared. (G) Blood glucose levels at 3 time points during the study. (H) Blood glucose level changes of mice during the oral glucose tolerance test. (I) Fasting blood glucose levels of mice. Notes: *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; ns: P>0.05.

of lipid droplets in the liver was also dense, but the area of lipid droplets was significantly smaller than that in the HFD group, indicating that H_2 inhalation could reduce the content of lipid droplets in the liver and alleviate the burden on the liver in NAFLD mice (Figure 2D).

By performing liver function analyses, we found that the serum levels of ALT (P < 0.0001) and AST (P < 0.01) in the HFD group were significantly higher than those in the control group. Compared with those in the HFD group, the serum ALT (P < 0.0001) and AST (P < 0.01) levels in the HFD + H_2 group were significantly lower, but the differences were not significant compared with those in the control group (P > 0.05). Taken together, these results suggest that H_2 can alleviate liver metabolism abnormalities and liver damage caused by HFD (Figure 3D and E).

3.4. H2 administration decreases systemic inflammatory factors and increases the relative abundance of *A. muciniphila* in the intestinal tract of NAFLD mice

We measured the levels of systemic inflammatory factors, including LPS and TNF-α. Compared with those in the

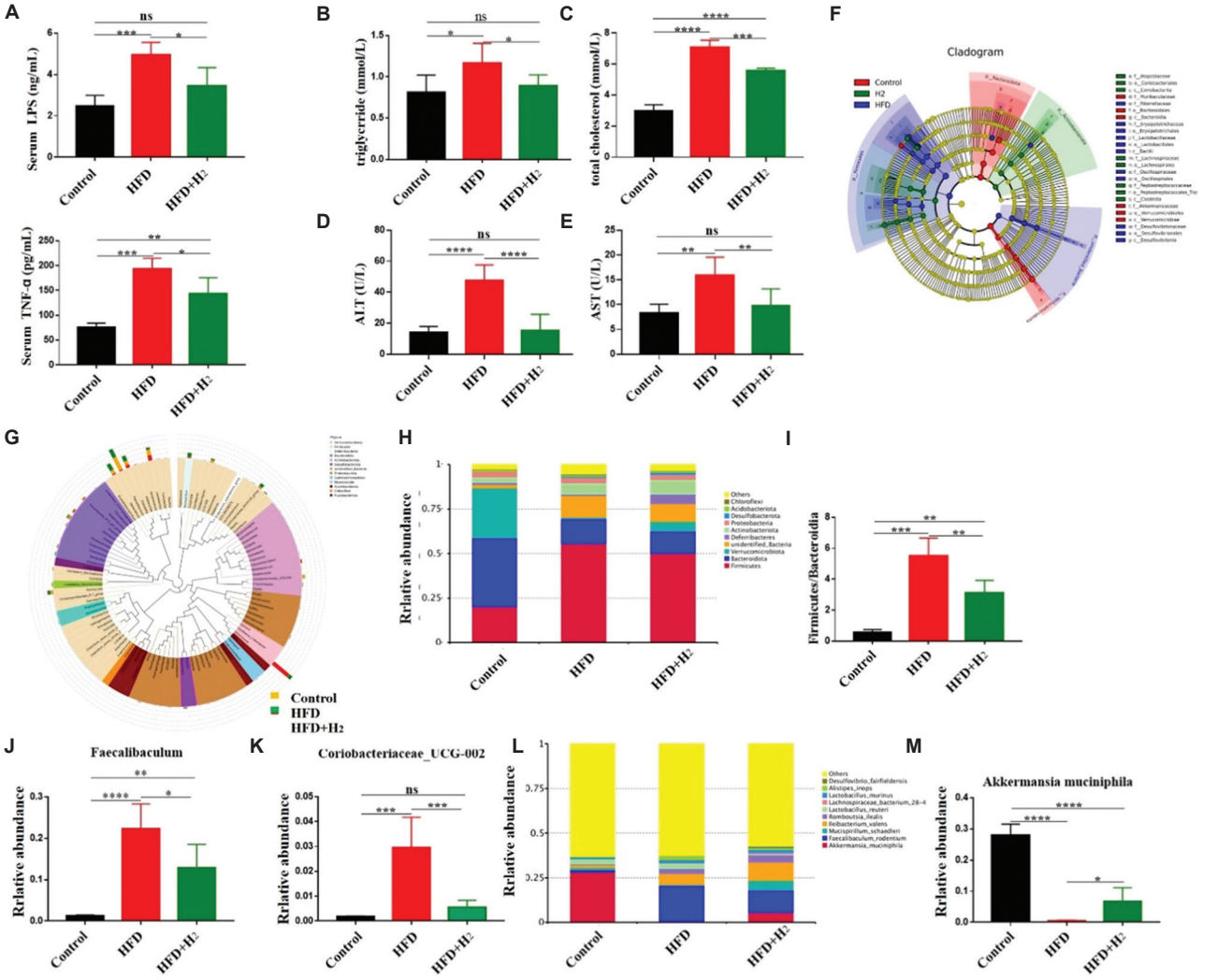

Figure 3. Hydrogen (H_2) intervention modulates liver metabolism and intestinal flora and alleviates inflammation in non-alcoholic fatty liver disease mice. (A) Serum levels of lipopolysaccharide and tumor necrosis factor-alpha (TNF-α) of the mice. (B) Serum triglyceride levels of the mice. (C) Serum total cholesterol levels of the mice. (D) Serum alanine aminotransferase levels of the mice. (E) Serum aspartate aminotransferase levels of the mice. (F) Evolutionary clade tree of the intestinal microorganisms of the three groups of mice. (G) Genus-level species evolutionary tree. (H) Relative abundance of the top 10 species at the phylum level. (I) The FBR of the three groups of mice. (J) The relative abundance of *Faecalibaculum* of the three groups of mice. (K) The relative abundance of *Coriobacteriaceae*_UCG-002 of the three groups of mice. (L) The relative abundance of the top 10 species at the species level. (M) The relative abundance of *Akkermansia muciniphila* of the three groups of mice. Notes: **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001; ns: *P*>0.05.

control group, the serum levels of LPS and TNF-α in the HFD group were significantly higher ($P < 0.001$), whereas the serum levels of LPS and TNF-α in the HFD + H_2 group were lower than those in the HFD group ($P < 0.05$). However, compared with that in the control group, the LPS level in the HFD + H_2 group was not significantly different ($P > 0.05$), but the TNF-α level was significantly higher ($P < 0.01$) (Figure 3A).

Because of the known importance of the gut microbiota in liver metabolism and host immune responses, we reasoned that the hepatoprotective function of H_2 might be attributed to the changes in the specific composition of the gut microbiota. According to 16S rRNA sequencing, the genus-level species evolutionary tree showed significant differences in the genus-level abundance of intestinal flora among the three groups (Figure 3G). *Faecalibaculum* and *Coriobacteriaceae*_UCG-002 were positively correlated with blood lipid levels.[17] Compared with the control group, the abundances of these two bacteria significantly increased in the intestinal tract of HFD group mice ($P < 0.05$). However, the level of *Faecalibaculum* and *Coriobacteriaceae*_UCG-002 in the intestinal tract of HFD + H_2 group mice were significantly lower than those in HFD group mice ($P < 0.05$) (Figure 3J and K). There

were significant differences in the abundance of important microorganisms in the mouse intestine among the three groups according to the analysis of the evolutionary clade tree (Figure 3F). The *Firmicutes*-to-*Bacteroidia* ratio (FBR) of the HFD group was significantly greater than that of the control group ($P < 0.001$) (Figure 3H and I). The FBR of the HFD + H_2 group was lower than that of the HFD group ($P < 0.01$) but higher than that of the control group ($P < 0.01$) (Figure 3I). Compared to that in the control group, the population of *A. muciniphila* in the intestinal flora of the HFD group was almost decimated ($P < 0.0001$). Surprisingly, the relative abundance of *A. muciniphila* in the HFD + H_2 group increased significantly compared with that in the HFD group ($P < 0.05$), although it still did not restore to the same level as in the control group ($P < 0.0001$) (Figure 3L and M).

NAFLD is accompanied by an impaired host immune response, and thus, an increased abundance of gut *A. muciniphila* can strengthen the integrity of the gut barrier and minimize the leakage of harmful bacterial products from the gut to portal vein circulation. Taken together, these results indicate that the beneficial effects of H_2 on NAFLD appear to be associated with increases in the relative abundance of gut *A. muciniphila* and downregulation of the expression of systemic inflammatory factors.

4. Discussion

The liver is the most important metabolic organ for maintaining normal glucose and lipid homeostasis, and this status can be deranged by chronic inflammation in obesity-associated NAFLD. NAFLD initially manifested as liver steatosis and steatohepatitis, and may gradually develop into more serious diseases such as cirrhosis and liver cancer.[18] While many previous studies have shown that NAFLD patients usually gain significantly more weight than normal individuals, it has since been found that normal-weight and underweight individuals may also have NAFLD.[19] Low-grade metabolic inflammation is a common feature of NAFLD.[20] NAFLD begins with the excessive accumulation of fat in liver tissue, which is caused by increased fat intake, deregulation of lipoprotein synthesis, and insulin resistance.[21] Subsequently, oxidative stress induced hepatocyte injury, production of pro-inflammatory factors, mitochondrial damage, and intestinal flora disorder.[22]

The gut and liver can interact through a pathway called the gut-liver axis. More than 50% of the liver's blood comes from the intestine, and the portal vein is the "bridge" between the two tissues. Therefore, the liver is one of the most vulnerable organs to enterogenic endotoxin and the "front line" to resist bacterial metabolites. It has been proposed that intestinal flora can maintain physiological dynamic balance to some extent and play an important role in the progression of obesity, metabolic syndrome, and NAFLD. Dysbiosis of the gut flora is known to lead to impaired intestinal barrier function and associated immune responses.[23] Our results showed that compared with the control group, the abundances of *Faecalibaculum* and *Coriobacteriaceae*_UCG-002 significantly increased in the intestinal tract of HFD group mice, and the FBR of the HFD group also increased significantly. Moreover, the relative abundance of *A. muciniphila* in the intestinal flora of the HFD group almost disappeared compared to that in the control group ($P < 0.0001$), suggesting that HFD induced structural changes of intestinal flora.

A. muciniphila, a gram-negative anaerobic bacterium, was successfully isolated in 2004.[24] It is also the only member of the phylum *Verrucomicrobia*.[25] As a normal bacterium in the intestines of humans and animals, *A. muciniphila* acts on mucin and uses it as its sole carbon and nitrogen source.[26] *A. muciniphila* is normally present in the gut of the human host shortly after birth, peaks shortly after birth, and decreases with age.[27] *A. muciniphila* can protect the intestinal epithelial cells and maintain the integrity of the mucus layer so that the intestinal permeability is not damaged.[28] The intestinal barrier ensures nutrient absorption and prevents the escape of microorganisms and their products. Upon damage to the intestinal barrier, the harmful products enter the bloodstream from the lumen and then activate an immune response leading to inflammation.[29] Oral administration of *A. muciniphila* increased the expression of intestinal tight junction proteins in NAFLD mice. Therefore, *A. muciniphila* was proposed to reduce endotoxemia levels and improve local inflammation by improving intestinal permeability and strengthening the intestinal barrier. Nutritionally unbalanced diets, such as HFD, can affect the intestinal microenvironment and reduce the abundance of *A. muciniphila* in the intestine.[30] However, weight loss and increased insulin sensitivity were found in obese human volunteers who were given oral 10^{10} *A. muciniphila* daily.[31] The mice treated with *A. muciniphila* also demonstrated significantly reduced serum TGs and fasting blood glucose, and increased insulin sensitivity. Due to its beneficial role in many diseases, *A. muciniphila* is expected to be the next generation of probiotics.[32]

As a colorless, odorless, non-polar, and minimal molecule in nature, inhaled H_2 can rapidly diffuse through alveoli into the blood and penetrate the cell membrane system to exert systemic effect. As early as 1975, H_2 was reported to be effective in treating skin cancer in mice.[33] In addition, the safety of breathing H_2 in humans has been demonstrated in studies related to diving-related treatments.[34] Recently, H_2 inhalation was reported to

reduce the concentration of inflammatory factors in the peripheral blood of patients with chronic obstructive pulmonary disease.[35] A study by Yao *et al.*[36] showed that H_2-rich saline treatment ameliorated inflammation and apoptosis in myocardial ischemia/reperfusion through PINK1/parkin-mediated mitochondrial autophagy. In recent years, our understanding of the association between the gut microbiota and NAFLD has improved considerably, prompting the emergence of novel treatment approaches, including H_2 administration. In this study, we confirmed the therapeutic effects of H_2 on NAFLD, such as reversing the impairment of glucose tolerance; reducing the serum concentrations of TG, TC, ALT, AST, LPS, and TNF-α; and alleviating hepatic steatosis. We clearly demonstrated that H_2 therapy led to an increase in the relative abundance of *A. muciniphila* in NAFLD model mice. Moreover, the abundances of *Faecalibacterium* and *Coriobacteriaceae_UCG-002* decreased significantly, and the FBR level decreased following H_2 treatment (Figure 4).

Dysbiosis of the gut flora is known to impair intestinal barrier function and associated immune responses.[23] Ley *et al.*[37] reported that FBR affects human susceptibility to obesity and other diseases associated with metabolic abnormalities. Many studies have shown that *A. muciniphila* regulates intestinal tight junction protein expression, and reduced *A. muciniphila* abundance could increase intestinal permeability, resulting in the leakage of harmful metabolites from the gut into the blood and further to the liver along the gut-liver axis; this leakage can damage the local microenvironment and cause liver disease.[38] Mice treated with *A. muciniphila* also exhibited significantly reduced serum TG and fasting blood glucose levels and increased insulin sensitivity. As the abundance of *A. muciniphila* in the intestinal tract of HFD-fed mice decreases and the serum levels of LPS and TNF-α consequently increase.[30] Liver exposure to high concentrations of unwanted bacterial products, particularly LPS, also promotes the activation of inflammatory pathways and the release of circulating pro-inflammatory factors (such as TNF-α and IL-6), further contributing to liver tissue damage.

Despite the interesting findings in the above, several limitations of this study should be acknowledged. Despite the encouraging hepatoprotective effect of H_2 in lowering the systematic pro-inflammatory factor levels,

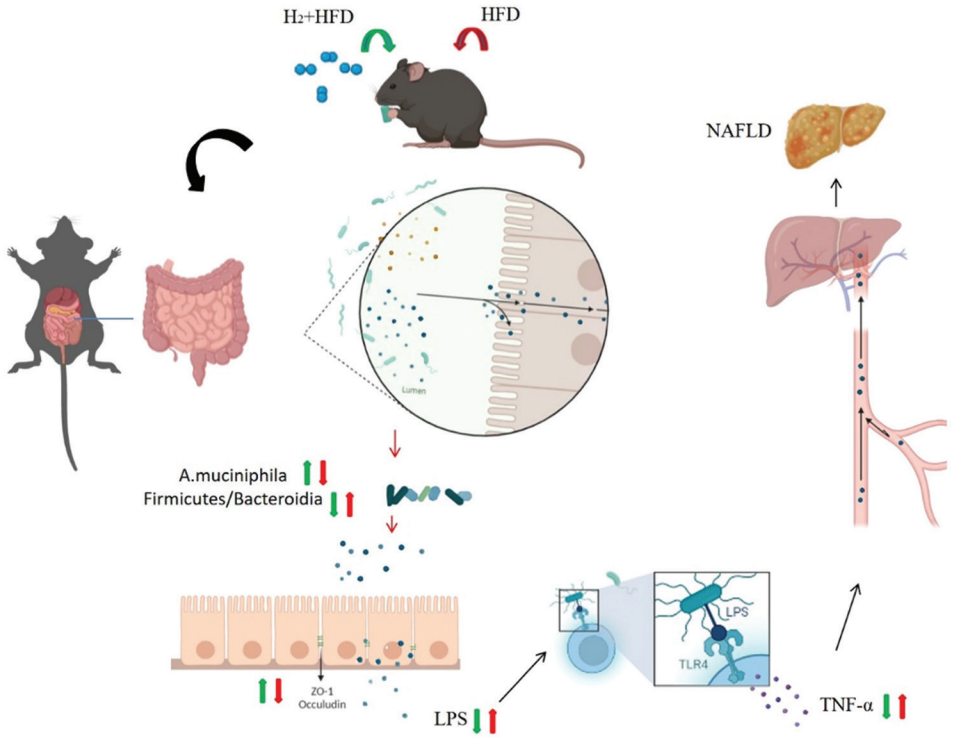

Figure 4. Effects of hydrogen (H_2) intervention on non-alcoholic fatty liver disease (NAFLD) mice. This study confirmed the efficacy of H_2 in the treatment of NAFLD. H_2 alleviated the impairment of glucose tolerance; decreased the serum levels of triglyceride, total cholesterol, alanine aminotransferase, aspartate aminotransferase, lipopolysaccharide (LPS), and tumor necrosis factor-alpha (TNF-α); and alleviated hepatic steatosis. These results suggested that H_2 might improve intestinal permeability by increasing the abundance of *Akkermansia muciniphila* in the intestinal tract and subsequently reducing the amount of LPS produced by enteric pathogens, which produce TNF-α, in the liver through the portal vein. Thus, H_2 has promising therapeutic potential for NAFLD based on the increasing abundance of *A. muciniphila*.

the transcriptional basis in this regard remains unknown. Thus, characterizing the complete gene-expression profile in hepatocytes and stromal cells in the liver with and without H_2 treatment will be critical to a better understanding of the exact mechanisms. Furthermore, although *A. muciniphila* seems to be the critical middle factor in mediating H_2-induced NAFLD reversal in terms of symptoms and biochemistry, other bacteria may also play a role. Further work will be needed to determine to what extent *A. muciniphila*, on its own, could influence the NAFLD pathogenic process. Finally, the animal experiments substantiated the beneficial effects of H_2 treatment on FBR and *A. muciniphila* abundance, but the exact mechanism regarding how H_2 affects the gut microbiota remains to be unveiled.

5. Conclusion

In summary, these results provide a better understanding of the effects and possible mechanisms of H_2 treatment in mice with NAFLD. In fact, H_2 is naturally present in the colon due to anaerobic bacterial fermentation, and its abundance is important for maintaining a healthy anaerobic ecosystem. H_2 delivery by direct inhalation or the transplantation of engineered H_2-producing *A. muciniphila* is two attractive approaches to pursue in translational medicine. We demonstrated that the gut microbiota can be safely and positively altered by H_2 administration. Thus, H_2 has promising therapeutic potential for NAFLD by increasing the abundance of *A. muciniphila*, although H_2 therapy is fraught with several clinical limitations, including the involvement of adequate and expensive medical facilities.

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Conflict of interest

The authors declare they have no competing interests.

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All animal studies were approved by the Institutional Animal Care and Use Committee of the Tongji University Affiliated East Hospital (T3LAC-015-038).

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Not applicable.

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

Investigation of hydrogenase enzymes and the presence of orthologs in the human proteome

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Abstract

Hydrogenase enzymes catalyze the reversible oxidation/reduction of hydrogen (H_2) and play a crucial role in microbial energy metabolism, with significant implications for human immunity. H_2, produced by gut microbes during fermentation or administered exogenously, is vital in modulating oxidative stress and inflammation. In the gastrointestinal tract, microbial H_2 production can reach up to 13 L/day, with approximately 71% of commensal bacteria capable of metabolizing H_2. By interacting with complex I, particularly the NDUFS7 subunit, H_2 may reduce mitochondrial electron leakage and limit the generation of reactive oxygen species (ROS). Excessive ROS can trigger pro-inflammatory signaling and impair immune responses. This study investigated the presence of hydrogenase orthologs in the human proteome, particularly within mitochondrial complex I, and their potential role in immune function. This novel research highlights a possible evolutionary link between microbial hydrogenases and human immunity, suggesting that microbial-derived H_2 may support immune homeostasis by mitigating oxidative stress and inflammation. Although human homologs of nickel/iron hydrogenases, such as NDUFS2 and NDUFS7, likely lack classical hydrogenase activity, sequence similarities between NDUFS7 and hydrogenase subunits in Asgard archaea and δ-proteobacteria indicate the conservation of potential redox-active sites. Redox activity, occurring at the N2 iron-sulfur cluster in NDUFS7, may influence mitochondrial oxidative stress responses, which are integral to immune regulation. These findings open new avenues for exploring the therapeutic potential of H_2 in immune regulation.

Keywords: Complex I; Evolution; Hydrogen; Hydrogenases; Redox activity; NDUFS7; Oxidative stress

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1. Introduction

The intestinal microbiome plays a fundamental role in regulating immune function and mitigating oxidative stress, serving as a critical interface between host systems and the external environment. Comprising a vast array of microorganisms, the gut microbiome not only modulates innate and adaptive immune responses but also defends against pathogens while maintaining immune homeostasis.[1] A key mechanism through which the microbiome influences immune health is the production of short-chain fatty acids

(SCFAs), such as butyrate, acetate, and propionate, during the fermentation of indigestible dietary fibers. SCFAs strengthen the gut epithelial barrier, thereby reducing the translocation of bacterial endotoxins that can trigger systemic inflammation and immune dysregulation.[2] Furthermore, SCFAs exhibit potent anti-inflammatory properties and are known to regulate regulatory T cells, which play a crucial role in maintaining immune tolerance and preventing excessive immune responses.[3]

In addition to the favorable effects of microbial metabolites on adaptive immunity, the intestinal microbiome is pivotal in reducing oxidative stress, a major contributor to inflammation and cellular damage. The immune system relies on a delicate balance of oxidative and antioxidative processes to function effectively. Oxidative stress, a phenomenon that can lead to erratic immune signaling, occurs when the production of reactive oxygen species (ROS) exceeds the body's ability to detoxify them. ROS, including superoxide and hydroxyl radicals, are highly reactive molecules that participate in a network of signaling pathways. Furthermore, ROS are known to influence cellular stress responses, including the expression of proinflammatory chemokines and cytokines, as well as apoptosis.[4] While ROS signaling is essential for immune responses, such as the destruction of pathogens, excessive levels can damage healthy cells and tissues, leading to inflammation and impaired immune responses.[1,2] Disruption of this balance may result in either an overactive immune response, contributing to autoimmune diseases, or a weakened somatic response, increasing susceptibility to infections. Many microbial metabolites, including hydrogen (H_2) and glutathione, act as antioxidants, either by directly neutralizing ROS and preventing oxidative damage or by activating signaling pathways, such as nuclear factor erythroid 2-related factor 2, which promotes the expression of antioxidant enzymes and enhances cellular defenses against oxidative stress.[5]

The gastrointestinal tract hosts more than 10^{12} microorganisms, collectively referred to as the gut microbiota. This diverse array of archaea, bacteria, bacteriophage, fungi, and viruses contributes to various physiological processes, including immune function.[1,6] During the fermentation of undigested carbohydrates by commensal bacteria, H_2 gas is produced as a metabolic byproduct. This H_2 can be absorbed into the bloodstream and expelled via the lungs, or it can be utilized by methanogens and sulfate-reducing bacteria through a process known as interspecies H_2 transfer.[7-9] It is estimated that the intestinal microbiome can produce approximately 13 L of H_2 each day, with around 71% of commensal bacteria capable of metabolizing H_2,[7] indicating the potential significance of H_2 in regulating intestinal immune function.

Emerging research suggests that H_2 acts as an effective antioxidant and anti-inflammatory agent, with numerous studies showing that H_2, whether produced endogenously by microbes or administered exogenously, can reduce oxidative stress, inflammation, and modulate immune responses,[10-14] For instance, H_2 has been shown to stimulate the production of butyrate, an essential SCFA known for its anti-inflammatory properties,[8] The ability of the gut microbiota to modulate H_2 production and utilization is crucial for maintaining gut health. *Methanobrevibacter smithii*, for example, uses H_2 to reduce carbon dioxide into methane (CH_4), a less reactive byproduct, thus preventing excess accumulation of H_2.[9] Interspecies H_2 transfer plays a key role in sustaining gut microbial diversity and contributes to overall gut health. In addition, recent studies indicate that H_2 positively affects mitochondrial structure and function, enhancing adenosine triphosphate production, mitigating oxidative stress, and stabilizing membrane potential.[15-18] These findings suggest that H_2 supports favorable energy dynamics in somatic cells.

Over the past decade, H_2 has gained interest as a modulator of oxidative stress and inflammation,[10-12,16] with several studies showing that H_2 can attenuate inflammation in various models of intestinal diseases, including colitis and inflammatory bowel disease. For example, a study conducted by Song *et al.*[19] demonstrated that H_2-rich water, which mimics the effects of microbial-produced H_2, significantly reduced colonic inflammation in a mouse model of ulcerative colitis by decreasing levels of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α). Significant increases in glutathione concentration and inhibition of pathogenic bacteria, including *Enterococcus faecalis*, *Clostridium perfringens*, and *Bacteroides fragilis*,[19] were also noted. As a non-polar, electrochemically neutral, and lightweight (molecular weight: 2.016 g/mol) diatomic molecule, H_2 can traverse biological membranes and target intracellular compartments, playing a crucial role in the interaction between intestinal microbes and the immune system.

Many reports describe the anti-inflammatory effects of H_2 treatments, supported by scientific evidence indicating that H_2 suppresses biological markers of oxidative stress and pro-inflammatory peptides (for example, TNF-α), interleukins (IL; for instance, IL-6 and IL-1ß), and nuclear factor kappa B (NFκB).[10-12,16] These protective mechanisms involve not only interactions with multiple cellular processes, as described above but also the regulation of p38 mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase signaling cascades responsible for initiating the biosynthesis of proinflammatory cytokines.[20,21]

To understand the origins of these complex regulatory pathways, the ancient environmental conditions that may have contributed to the development of early biological membranes should be examined. Given the volatile chemistry of the Hadean Earth, circa 4 billion years ago, it is plausible that the first membranes were formed not from organic materials but from minerals such as mackinawite.[22-27] These semi-permeable, inorganic protomembranes could form in the vicinity of submarine alkaline hydrothermal vents, where reductive, electron-rich volcanic detritus is exposed to the acidic, proton-rich oceanic waters, potentially providing the chemical disequilibrium and minerals necessary for organic life.[22,23] As the Earth's early atmosphere was likely composed of reducing gases such as carbon monoxide, H_2, and CH_4, it has been suggested that the ability to utilize iron (Fe)- induced catalysis of H_2 as a means of supplying electrons and protons for energy production, evolved billions of years ago.[24] Preiner *et al.*[27] propose that minerals such as mackinawite and magnetite could serve as prebiotic hydrogenases, facilitating organic reactions. Therefore, from an evolutionary standpoint, it is possible that H_2 was one of the first reducing agents exploited in early energy metabolism, a phenomenon that now predominantly occurs in the mitochondria of animals.

Hydrogenase enzymes catalyze the reversible oxidation/reduction of H_2 ($H_2 \leftrightarrow 2H^+ + e^-$) and are found in all single-celled organisms and many multicellular organisms;[28-30] however, they are not known to exist in animals and humans. Recent research by Lu.[31] indicates that complex I may have retained hydrogenase-type activity. Hydrogenases can be categorized into specific phylogenic groups, namely (i) iron only, (ii) iron-iron, and (iii) nickel-iron ([Fe], [FeFe], and [NiFe], respectively).[28,29] Among these, the [NiFe] hydrogenases are the most commonly occurring, present in a wide range of microbial species.

[NiFe] hydrogenases can be further divided into four classes: (i) membrane-bound hydrogenases (Mbh), (ii) nitrogen-fixing cytoplasmic hydrogenases, (iii) cytoplasmic hydrogenases that utilize 8-hydroxy-5 deazaflavin (coenzyme F420) as a low-potential redox co-factor, and (iv) oxygen (O_2)-sensitive, membrane-bound, energy-converting hydrogenases.[28,29] Of these subgroups, membrane-bound group iv hydrogenases exhibit activity resembling that of mitochondrial complex I,[28,31] making this group particularly relevant to the present discussion.

The interaction between H_2, commensal microbes, and the immune system forms a complex network with significant implications for health and immune function. A deeper understanding of this relationship could lead to novel therapeutic strategies for gastrointestinal and immune-related diseases. Within the context of innate immunity, the evolution of complex I is central to immune regulation. It is conceivable that H_2 influenced the development of immune defense mechanisms by modulating the metabolic pathways responsible for ROS generation.

The syntrophy theory of evolution[32,33] emphasizes H_2 as a crucial intermediary in metabolic exchanges, particularly involving an H_2-consuming, sulfate-reducing δ-proteobacterial host, an H_2-releasing Asgard archaeon, and a sulfide-oxidizing α-proteobacterium. Therefore, it is reasonable to propose that H_2 metabolism, facilitated by hydrogenase enzymes, played a key role in the early bioenergetic processes essential for the evolution of complex life.

The primary objective of this research was to investigate the potential evolutionary connection between hydrogenases and the functional subunits of complex I across various microorganisms that may have contributed to the evolution of eukaryotic cells. This study sought to elucidate how hydrogenases might have influenced the development of bioenergetic systems critical to the evolution of complex cellular and somatic functions, including the modulation of cellular redox potential and immune responses.

2. Methods

To explore the potential evolutionary link between hydrogenases and the functional subunits of complex I, this study examined three microorganisms implicated in the syntrophic hypothesis of evolution: a δ-proteobacterium (*Desulfovibrio carbinolicus*), an Asgard archaeon (*Candidatus Heimdallarchaeota*), and an α-proteobacterium (*Rhodobacter sphaeroides*).

Mbh investigated in this study include: (i) *Ca. Heimdallarchaeota* MbhJ (Uniprot# A0A1Q9PFW3) and MbhL (Uniprot# A0A1Q9PFM5), (ii) *D. carbinolicus* with subunits Hyd494 (Uniprot# A0A4P6HTH3) and Hyd258 (Uniprot# A0A4P61469), and (iii) *R. sphaeroides* HupL (Uniprot# Q3J0L7), HupS (Uniprot# O86467), HupU (Uniprot# O86466), and HupV (Uniprot# Q3J0M0).

2.1. Basic local alignment search tool (BLASTp) analysis

To assess whether the hydrogenase proteins of interest retained homology within the human proteome, the BLASTp was employed to analyze the [NiFe] hydrogenase enzyme sequences from all three species.[34]

2.2. Dot plot analysis

Using the [NiFe] sequences, a bioinformatic matrix analysis was conducted using the EMBOSS program to

identify regions of similarity between complex I subunits and [NiFe] hydrogenases.[35]

2.3. Clustal Ω

Prokaryotic sequences displaying similarities with the human proteins NDUFS2 and NDUFS7 were then aligned and analyzed using the Clustal Ω program.[36]

2.4. ScanProsite

Using selected motifs of interest, an analysis was performed with ScanProsite to identify predicted sites of post-translational modifications, including N-glycosylation, N-myristoylation, and phosphorylation, known to support pro-oxidative and proinflammatory signaling pathways. This investigation focused on pinpointing redox-sensitive sites that could potentially facilitate H_2-driven antioxidant and anti-inflammatory signaling activity.[37]

3. Results

3.1. BLASTp

The BLASTp analysis identified the NDUFS2 subunit as most comparable to the large membrane-bound subunits of [NiFe] hydrogenases, which are responsible for H_2 catalytic activity, and the NDUFS7 module as most similar to the non-catalytic small subunit. With 27% sequence identity, the homology between the human protein NDUFS2 and the large MbhL subunit of *Ca. Heimdallarchaeota* hydrogenase is higher than that between NDUFS2 and Hyd494 of *D. carbinolicus* (23%) and *R. sphaeroides* (<20%).

Exceeding the 25% significance threshold,[38] the BLASTp analysis of *Ca. Heimdallarchaeota* showed a 35% similarity with NDUFS7. Notably, the BLASTp search indicated 35% homology between the [NiFe] hydrogenases of *D. carbinolicus* and the NDUFS7 subunit of mitochondrial complex I. Conversely, the BLASTp search indicated <20% homology between the [NiFe] hydrogenases of *R. sphaeroides* and the NDUFS7 subunit.

3.2. Dot plot analysis

Consistent with previous findings,[29] the BLASTp analysis confirmed that NDUFS2 is most similar to the large membrane-bound subunits of [NiFe] hydrogenases responsible for hydrogenase activity. To further explore whether other regions of [NiFe] hydrogenases may be represented in NDUFS2, a similarity matrix – referred to as a dot plot analysis – was created to visualize sequence homology (Figure 1).

In accordance with previous research, this analysis also confirmed that NDUFS7 is most similar to the small membrane-bound subunits of [NiFe] hydrogenases. To further explore whether other regions of [NiFe] hydrogenases may be represented in NDUFS7, a similarity matrix was created (Figure 2).

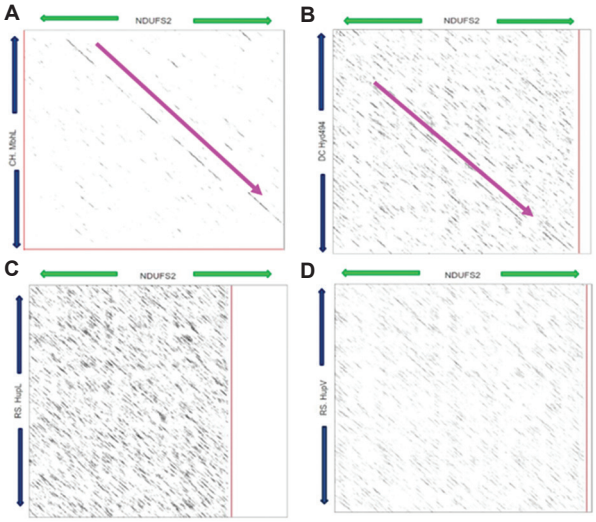

Figure 1. Similarity matrix analyses of NDUFS2 and hydrogenases from (A) *Candidatus Heimdallarchaeota* MbhL B (418aa), (B) *Desulfovibrio carbinolicus* Hyd494 (494aa), (C) *Rhodobacter sphaeroides* HupL (596aa), and (D) *Rhodobacter sphaeroides* HupV (475aa)
Notes: Dot plot analysis of the NDUFS2 protein (463aa) sequence which is on the X-axis (green arrows indicate sequence direction). The hydrogenase of interest is listed on the Y-axis (blue arrows indicate sequence direction). Clear alignments denote areas of homology. The horizontal red line indicates the amino acid sequence length of NDUFS2. The vertical red line represents the amino acid sequence length of hydrogenases. Regions of interest are marked with pink arrows. (A) identifies a close similarity between NDUFS2 and the MbhL protein of *Candidatus Heimdallarchaeota* (CH), as evidenced by a clear diagonal pattern. (B) displays some similarities between NDUFS2 and the large [NiFe] hydrogenase subunit of *Desulfovibrio carbinolicus* (DC; Hyd494). In contrast, (C and D), derived from the sequences of *Rhodobacter sphaeroides* (RS) HupL and HupV, respectively, show minimal homology with NDUFS2.

3.3. Clustal Ω analysis

To determine whether the catalytic site is well-conserved, a Clustal alignment was performed between NDUFS2 and the hydrogenases of interest (Figure 3).

To examine whether the small uptake subunit of hydrogenases is well-conserved, a further Clustal alignment was performed between NDUFS7 and the hydrogenases of interest (Figure 4).

4. Discussion

The anti-inflammatory effects of H_2 gas produced by the gut microbiota have gained significant attention in recent years.[39-42] This microbial-derived H_2 is a byproduct of the fermentation processes carried out by specific gut bacteria, particularly from the phylum *Bacteriodetes* and *Firmicutes*.[43] These bacteria generate H_2 during the

anaerobic metabolism of dietary fibers that are indigestible by the human body. H_2 produced by commensal bacteria can be utilized by other microbial populations, particularly methanogenic archaea, sulfate-reducing bacteria, and acetogenic bacteria, which employ H_2 as an electron donor in their respective metabolic pathways.[8] This symbiotic relationship between H_2 producers and consumers helps maintain a balanced intestinal environment. Imbalances, such as an overproduction of H_2, can lead to gastrointestinal disturbances like small intestine bacterial overgrowth.[44] In contrast, insufficient H_2 utilization, which is a more common occurrence, may impair microbial metabolic efficiency, leading to an increase in ROS and subsequent inflammation.

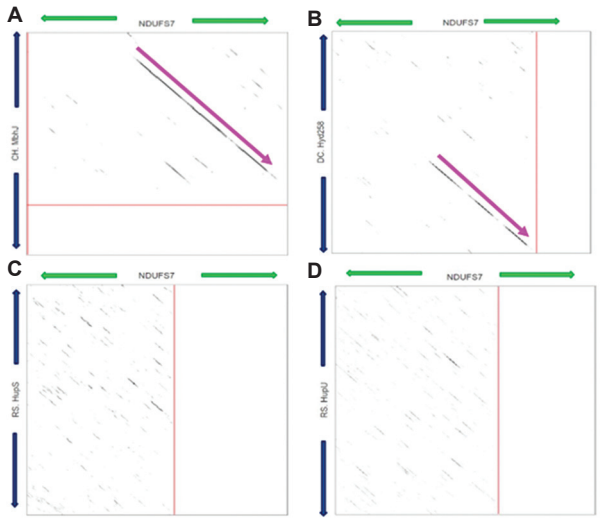

Figure 2. Similarity matrix analyses of NDUFS7 and hydrogenases from (A) *Candidatus Heimdallarchaeota* MbhJ (155aa), (B) *Desulfovibrio carbinolicus* (DC) Hyd258 (258aa), (C) *Rhodobacter sphaeroides* (RS) HupS (369aa), and (D) *Rhodobacter sphaeroides* (RS) HupU (330aa)
Notes: Dot plot analysis of the NDUFS7 protein (213aa) sequence. X-axis (green arrows) indicates NDUFS7 sequence direction. Y-axis (blue arrows) indicates the sequence direction of the hydrogenase of interest. Pink arrows denote regions of clear homology. The horizontal red line marks the truncation of the amino acid sequence of the hydrogenase. The vertical red lines indicate the truncation of the NDUFS7 amino acid sequence.

The gut microbiome is well established as a modulator of systemic inflammation through various mechanisms, including the regulation of immune cell activity,[45] cytokine production,[46] and the maintenance of intestinal epithelial integrity.[47] A healthy microbiome, enriched in H_2-utilizing microbes, is critical for preventing leaky gut syndrome, which can lead to systemic inflammation by allowing bacterial endotoxins to enter the bloodstream.[48] Furthermore, microbial-produced H_2 has been shown to promote the growth of beneficial bacteria that outcompete pathogenic species linked to inflammation.[43] For instance, butyrate-producing bacteria benefit from the H_2 economy in the intestinal environment, as their metabolic pathways help regulate inflammation and enhance intestinal barrier function.[8] Thus, the role of H_2 in gut health and immune function highlights an underexplored yet critical intersection between the intestinal microbiome, metabolic byproducts, and inflammatory regulation.

```
NDUFS2      -------------NDVDPPKDTIVKNITLNFGPQHPAAHGVLRLVMELSGEMVRKCDPHI    113
MBHL        -------------TLTDGSDSPPGADHHIFIGPQHPWAEEPAHFIIHLKGERVVEADIRI    57
494         GCPARPEEPRPAPGVTDHFRLRGEEAHEVAVGPVHAGIIEPGHFRFQCSGEDVYHLEISL    170
HupV        ------------------------MTRLVVGPFN-RVEGDLEVHLEVAEGA-VTAARVN    33
HupL        -----------MVATPNGFNL-DNSGRRIVVDPVT-RIEGHMRCEVNVDDQGIIRNAVST    47
                                     : ..*        .  ..

NDUFS2      GLLHRGTEKLIEYKTYLQALPYFDRLDYVSMMCNEQAYSLAVEKLLNIRP--PPRAQWIR    171
CH.MbhL     GFNLRGIEKAMENRTWRQNTMLVPRACGICSAVHQNVYVRVVEKLAGVEDQISERARLIR    117
DC.Hyd494   GFQHRGIEARLIGGPDKRTIHFMETLAGDTTIGHSLAHAALVEALT--ETAVPARGRAIA    228
RS.HupL     GTMWRGLEVILKGRDPRDAWAFTERICGVCTGTHALTSVRAVEDALGISI--PDNANSIR    105
RS.HupV     APLYRGFERMLEGRDPRDALTITPRICGICSISQSVAAARALGAAMGLAP--APAGERVA    91
            .   ** *  :                        :  .    :           .. :

NDUFS2      RMHAAYIRPGGVHQDLPLGLMDDI-----------------YQFSKNFSLRLDELEELL    262
MBHL        RVNPALMLPGGVKRDIPKDKADKA-----------------RPMLNKLIKQVEYHKKVF    208
494         RFGRGLVRPGGVAFDLDKPTIREL-----------------LSRLELTRRAAFGAAELL    319
HupV        WPHTLAVQPGGVTRA--------PG--AAERMRILSSLRSFRRHLERTLFGGPLEAFAAL    211
HupL        NPHPNWLV-GGVPCPINIDGVGAVGAINMERLNLVSSIIDQCIQFTNNVYLP-----DVV    279
                  :  ***                                 .           .
```

Figure 3. Short sections of the Clustal alignment between NDUFS2 and the large hydrogenase subunits
Notes: Histidine 88 is highlighted in bold and underlined. Blue highlights denote the motifs and conserved residues of interest. Grey highlights indicate the -HXXAHXVLR- motif and its conserved residues. Italics show the region surrounding the active site of hydrogenases. Pink highlights tyrosine 151 (human). Asterisks "*" identify identical residues across all sequences. Colons ":" represent strongly similar residues. Full stops "." denotes weakly similar residues.
Abbreviations: *CH: Candidatus Heimdallarchaeota; DC: Desulfovibrio carbinolicus; RS: Rhodobacter sphaeroides.*

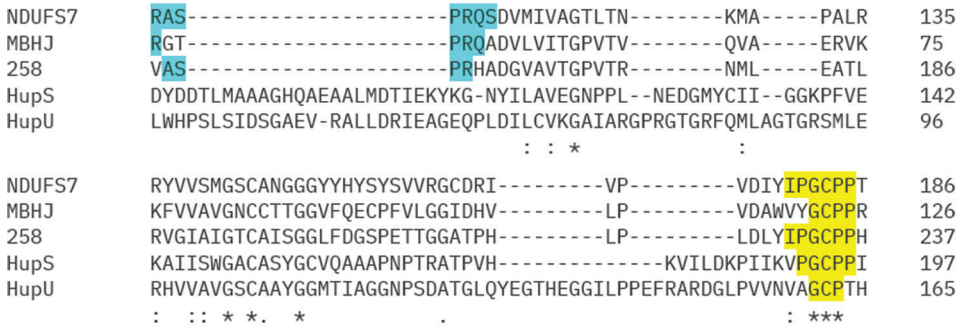

Figure 4. Short sections of the Clustal alignment between NDUFS7 and the small hydrogenase subunits
Notes: Blue highlights indicate conserved residues within the protein kinase C phosphorylation site -RASPRQS-. Yellow highlights mark the -IPGCPP- N-myristoylation site. Asterisks "*" identify identical residues across all sequences. Colons ":" show similar residues. Full stops "." denotes weakly similar residues.

As previously noted, the inflammatory response is significantly influenced by the redox status of cells and their compartments, with ROS playing essential roles in both normal cellular functions and pathological conditions. In healthy cells, ROS participate in signaling pathways essential for homeostasis and immune responses. Nevertheless, during inflammation, ROS production is often elevated. Overproduction of ROS or deficiencies in antioxidant defense mechanisms can lead to oxidative stress, damaging cellular components such as lipids, proteins, and DNA, thus exacerbating inflammatory responses.[49,50] ROS acts as a signaling molecule that activates transcription factors, including NFκB and activating protein-1, which are responsible for upregulating proinflammatory cytokines such as TNF-α and various ILs (for instance, IL-6 and IL-1β), thereby amplifying the inflammatory cascade.[51,52] While ROS is necessary for cell signaling and pathogen defense, their prolonged presence can lead to chronic inflammation and tissue damage, contributing to aging and various health conditions. Therefore, maintaining a balance between ROS production and antioxidant defenses is vital for regulating inflammation and preventing disease progression.

Due to its nearly constant redox activity and abundant electron supply, complex I serves as a prominent source of superoxide within the mitochondria. There are potentially two sites within complex I where O_2 can accept electrons from the nicotinamide adenine dinucleotide co-factor: (i) the flavin mononucleotide (FMN) module and (ii) the ubiquinone binding site.[53] Duong *et al.*[54] identified through *in silico* modeling that the FMN module is likely the putative site for ROS production, concluding that ROS generation is further stimulated by the absence of ubiquinone at the ubiquinone/complex I interface. In the absence of ubiquinone, the intraprotein channel becomes accessible to O_2, exposing it to a region where electrons are concentrated, thus enhancing the potential for ROS formation. In addition, incomplete reduction of ubiquinone at the Q module can lead to the production of semiquinone, a negatively charged intermediate capable of contributing to oxidative stress and proinflammatory signaling.[55] The redox midpoint potential of the ubiquinone/semiquinone couple (−0.163 V)[56] is similar to that of the oxygen/superoxide couple (−0.16 V),[57] suggesting that semiquinones may also significantly contribute to ROS formation in this context. Notably, reports indicate that the subunits of complex I that form the ubiquinone docking channel may have originated from hydrogenase enzymes.[28,29,31]

Numerous empirical and pre-clinical studies have identified H_2 as an effective redox mediator and regulator of the immune response.[58-64] In single-celled organisms, H_2 is metabolized by hydrogenase enzymes that catalyze the reversible oxidation/reduction of H_2. Among the various hydrogenase groups, [NiFe] hydrogenases are the most prevalent, found in diverse microbiota, fungi, and plants.[65-67] All characterized [NiFe] hydrogenases comprise a large subunit containing the active H_2 deprotonation site and a smaller subunit housing up to nine iron-sulfur (FeS) clusters. Similarly, complex I of the mitochondrial electron transport chain also relies on a series of FeS clusters to transfer electrons to the terminal N2 cluster, where they reduce ubiquinone to ubiquinol.

In the present study, initial BLASTp analysis identified NDUFS2 as the most similar to the catalytic units of the [NiFe] hydrogenases investigated. Supporting these findings, matrix analysis (Figure 1) identified a relatively strong correlation between NDUFS2 and NDUFS7 with the [NiFe] hydrogenases from *Ca. Heimdallarchaeota* (27% and 35%, respectively) and δ-proteobacterium *D. carbinolicus* (23% and 35%, respectively). In contrast, the α-proteobacterium *R. sphaeroides* showed less than 20% homology, indicating greater evolutionary distance.

Given the potential homology between hydrogenases and complex I subunit proteins, the active site is particularly interesting, as similar sequence alignments could indicate retention of form and function. Notably, the -RGXE- motif in the catalytic site (Figure 3) is conserved across all sequenced proteins, suggesting a distant evolutionary relationship between NDUFS2 and the large subunits of [NiFe] hydrogenases. The conserved glutamic acid residue (glutamic acid 119 in NDUFS2) may be pertinent to proton transfer.[65]

While this study identified limited homology between the aligned regions of NDUFS2 and microbial hydrogenase active sites, the redox-sensitive tyrosine 151 residue may be involved in the electron transfer chain, conserved only in *Ca. Heimdallarchaeota*. This is significant, as a crystal structure analysis of NDUFS2, conducted by Kampjut and Sazanov,[68,69] demonstrated that the ubiquinone molecule docks within 4.5 Å of tyrosine 108 in the ovine NDUFS2 module, corresponding to the human equivalent, tyrosine 151. In NDUFS2, the redox-sensitive tyrosine 151 residue may be involved in redox-regulated reactions central to the complex's function. Given that tyrosine 95 in MbhL is also conserved (Figure 2), this could indicate a similar role in electron transport within *Ca. Heimdallarchaeota*. Furthermore, tyrosine residues often contribute to protein stability through hydrophobic interactions and H_2 bonding.[70,71] Therefore, preventing the oxidation of these conserved tyrosines may be critical for H_2 in maintaining the protein's structural integrity, especially in regions vital for its function.

The structural analysis of NDUFS2 further identifies histidine 59 and asparagine 160 (in ovine), corresponding to histidine 112 and asparagine 182 (in humans), as potential candidates for proton translocation.[69] It has been proposed that a proton shuttling mechanism among this triad of residues (asparagine, histidine, and tyrosine) could create a negative charge, enhancing ubiquinone binding potential and lowering the redox potential of the N2 FeS cluster, thereby facilitating electron transfer.[69] Therefore, although the catalytic ability may have been lost through evolution, H_2 could still influence complex 1 dynamics through interactions with redox-sensitive residues.

Wirth *et al.*[72] further suggest that redox functionality likely exists within the β1–β2 helices of NDUFS2 at positions 88 – 96 (human), showing the -HPXAHXVLR- arrangement (Figure 3). Histidine 88 and histidine 92 of this sequence are situated in close proximity to both ubiquinol and the terminal 4Fe-4S cluster (N2) of NDUFS7, which may provide the redox sensitivity required for electron transfer to ubiquinone. However, this region is not well conserved across the species studied. Nonetheless, the alignment does reveal conserved homology immediately preceding the -HPXAHXVLR- motif. The preceding -GPQHP- sequence, positions 85 – 91 (human), contains histidine 88 and two redox-active proline residues (positions 86 and 89), likely close to ubiquinol, which could be key in the partial-to-full reduction of ubiquinone and downstream immune signaling responses.

Further along the sequence, at positions 228 – 232, there is another conserved motif, -RPGGV-. Although it has not yet been identified as a site for protein modification, its conservation throughout the examined hydrogenases suggests that it may play a critical role in protein structure and function. Although speculative, the idea that NDUFS2 serves as a key oxygen-sensing module and a regulator of complex I activity[73] suggests that these residues may be relevant as sites of H_2 activity. H_2 could protect these sites from autoxidation, thereby facilitating optimal protein function.

The structural relationship between the proton-transferring NDUFS2 and the FeS (N2)-containing NDUFS7 subunit indicates that this specific region of the ubiquinone binding module is likely responsible for electron transfer. By reducing electron leakage and subsequent ROS formation through structural maintenance, H_2 could have significant downstream cellular effects. For example, the protein kinase C (PKC) phosphorylation site (-RASPRQS-) in the smaller subunit shares the most sequence identity among species (Figure 4). If H_2 modulates phosphorylation in this region, it could influence the cellular signaling cascades that regulate the expression of proinflammatory factors such as NFκB and TNF-α in mammalian physiology. Interestingly, H_2 is noted to influence other protein kinase pathways, including MAPK.[20,21] Therefore, this raises the question of whether H_2 could influence the activity of this relatively well-conserved region, which is likely crucial for understanding H_2 bioactivity in humans.

Recent investigations utilizing a combination of mass spectroscopy and *in silico* modeling of the NDUFS7 unit identified that bovine arginines 108 and 112 (arginine 111 and 115 in humans) within the highly conserved C-terminal -RASPRQ- motif were integral for retaining ubiquinone in the hydrophobic cavity.[74] Nevertheless, it remains unclear whether direct electron transfer activity occurs at either of these moieties. If H_2 stabilizes this region or prevents oxidative damage, it is likely that a steady supply of electrons would be available for the complete reduction of ubiquinone to ubiquinol, thereby enhancing the immediate antioxidant potential of the mitochondria. This could have significant downstream effects, including an increased membrane potential and reduced cellular stress response.[75,76]

Further along the sequences (at positions 180 – 185 in humans; Figure 4), there is a well-conserved - IPGCPP- motif rich in redox-active proline residues, along with a cysteine thiol residue at position 183. The abundance of conserved residues associated with redox chemistry suggests an important conserved function in this region. Notably, the -IPGCPP- motif in *D. carbinolicus* is fully conserved, raising further questions about whether this region is integral to the function of hydrogenase remaining and potentially linking it to the electron transfer function of NDUFS7. In addition, the -IPGCPP- site is significant due to N-myristoylation, a post-translational modification crucial for regulating innate immune responses, including toll-like receptor-dependent inflammatory reactions.[77] Consequently, H_2 might modulate N-myristoylation and thereby influence the innate immune response, although further research is needed to validate this hypothesis.

Figure 4 also illustrates that the PKC phosphorylation site -RASPRQS-, as identified by Yoga *et al.*,[74] is relatively well conserved. Notably, the phosphorylation target, serine residue 113 in humans, is represented by threonine in *Ca. Heimdallarchaeota*, suggesting a retained function, as threonine can also be phosphorylated.[78] There is no correlation between the phosphorylation target -RASPRQS- in *R. sphaeroides*, indicating divergence in form and function within this region. The correlation between the smaller subunit of the [NiFe] hydrogenase (Hyd258) and NDUFS7 also reveals the retention of the serine residue -RASPRQS- motif. This suggests that in δ-proteobacteria, this segment may be significant for understanding the influence of H_2 on protein phosphorylation and cell signaling events.[79] Understanding whether H_2 has any influence on the molecular activity of this conserved region could be crucial for advancing our knowledge of its biological effects.

5. Future perspectives

The interplay between hydrogenase enzymes, microbial H_2 metabolism, and immune function represents a promising area of research, particularly regarding the role of H_2 in modulating oxidative stress and inflammation. Future studies should explore several key areas to enhance our understanding of the evolutionary links between microbial hydrogenases and human immune regulation.

One significant avenue for future research is to further investigate the potential redox activity of human complex I subunits, particularly NDUFS2 and NDUFS7, and their interactions with microbial-derived H_2. In accordance with Lu,[31] who posits that complex I may exhibit hydrogenase-like activity, our findings suggest that while classical [NiFe] hydrogenase activity has been lost, NDUFS7 may retain significant redox functionality, with the potential to influence mitochondrial oxidative stress responses. Therefore, one of the initial steps should involve determining the crystal structures of the identified human hydrogenases NDUFS2 and NDUFS7 using *in silico* modeling and X-ray crystallography, followed by probing their functional analyses with techniques such as cryo-electron microscopy[80] and redox-sensitive fluorescent probes.[81] These investigations could clarify whether the NDUFS2 and NDUFS7 subunits directly interact with H_2 and how this interaction affects mitochondrial electron flow and ROS production in human cells.

As evidence increasingly highlights the gut microbiota's role in shaping systemic immune responses,[82] it is essential to investigate the broader immunological effects of microbial H_2 production within the gastrointestinal system. Given the significant H_2 output from commensal bacteria and its potential impact on mitochondrial redox states, research should determine whether microbial-derived H_2 directly influences immune cells or operates through the gut-liver or gut-brain axes. Longitudinal studies using germ-free and hydrogen-supplemented animal models, such as those demonstrated by Yang *et al.*,[83] alongside human clinical trials, could elucidate the immunomodulatory effects of H_2 in both health and disease contexts.

Another key avenue for research lies in exploring the therapeutic potential of H_2 in managing immune-related conditions. While studies have demonstrated its anti-inflammatory and antioxidant effects,[10-17] the precise mechanisms remain unclear. For instance, investigating how H_2 modulates the phosphorylation and N-myristoylation of mitochondrial proteins, such as NDUFS7, could provide valuable insights into its role in reducing electron leakage and mitigating ROS production. Further clinical studies in autoimmune diseases, inflammatory disorders, and age-related immunosenescence could offer practical applications for harnessing these benefits.

Finally, extending research into microbial hydrogenases beyond the intestinal microbiome – specifically in the skin, oral, and respiratory microbiomes – may uncover additional pathways through which H_2 influences immune function. Understanding the interplay between hydrogenase activity, microbial H_2 metabolism, and immune regulation offers a promising frontier for therapeutic exploration. Such investigations could unlock novel strategies to leverage microbial interactions for maintaining immune homeostasis and mitigating inflammation.

6. Conclusion

This report provides evidence that the closest human homologs of [NiFe] hydrogenases are unlikely to possess

classical hydrogenase activity due to the absence of a functional Ni-Fe di-metal core. A detailed analysis of protein sequences reveals stronger similarities between the human proteins NDUFS2 and NDUFS7 – key components of the ubiquinone binding channel in complex I – and their homologous proteins in the *Ca. Heimdallarchaeota*, with hydrogenase similarity percentages of 27% and 35%, respectively. In contrast, similarities with the α-proteobacterium *R. sphaeroides* are less than 20%, and those with the δ-proteobacterium *D. carbinolicus* are 23% and 35%. These findings suggest a potential evolutionary link[38] between archaeal hydrogenases and complex I subunits, although further research is necessary to confirm this hypothesis.

Of particular relevance to microbial and immunological research, this study identifies two motifs within the NDUFS7 subunit – the PKC phosphorylation site (-RASPRQS-) and the N-myristoylation site (-IPGCPP-) – as potential sites for H_2 activity. If H_2 is found to support the structure and function of these motifs, it could mitigate electron leakage, reduce the formation of ROS, and prevent oxidative damage. Such outcomes would have significant implications for maintaining redox balance and limiting pro-inflammatory signaling, thereby highlighting a promising therapeutic avenue for regulating immune responses and microbial interactions in human cells.

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Conflict of interest

This work was part-funded by Water Fuel Engineering, a manufacturer of oxy-hydrogen inhalation devices. Grace Russell is the Guest Editor of this special issue but was not involved in the editorial or peer-review processes for this paper, either directly or indirectly.

Author contributions

This is a single-authored manuscript.

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Not applicable.

Consent for publication

Not applicable.

Availability of data

The data supporting the findings of this study are available from the corresponding author on request.

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ACCSCIENCE PUBLISHING

Microbes & Immunity

CASE SERIES

Rapid diagnosis of culture-negative *Klebsiella pneumoniae* liver abscesses by next-generation sequencing: A case series

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Abstract

Diagnosis of *Klebsiella pneumoniae* (Kp) pyogenic liver abscesses is usually achieved by imaging and isolation of the bacterium. However, when blood and other cultures were negative, laboratory diagnosis of Kp liver abscess may be challenging. Herein we report two patients with culture-negative Kp liver abscess with atypical presentations diagnosed by metagenomic next-generation sequencing (mNGS). For the first non-diabetic patient, computed tomography examination of the abdomen showed multiple round low-density foci with untidy margin in the liver, which mimicked hydatid cysts; while the second patient presented with acute cholecystitis/cholangitis. mNGS analysis of the blood sample from the first patient revealed 144 sequence reads of Kp; and that of the second revealed 153 sequence reads of Kp as well as other latent or non-pathogenic microorganisms. Both patients responded promptly to antibiotics treatment. mNGS is a useful tool for laboratory diagnosis of culture-negative Kp liver abscess.

Keywords: *Klebsiella pneumoniae;* Pyogenic liver abscess; Next-generation sequencing; Rapid diagnosis

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1. Introduction

Traditionally, bacterial pathogens commonly associated with pyogenic liver abscesses include flora of the gastrointestinal tract, such as *Escherichia coli* and other members of the enterobacterales, *Enterococcus* species, and anaerobes. In the past few decades, multiple studies have shown that *Klebsiella pneumoniae* (Kp) has emerged as the most important cause of pyogenic liver abscesses.[1] In Western countries, Kp accounted for about a quarter of all cases of pyogenic liver abscesses.[2] In East Asians, such as

the Chinese, Taiwanese, Koreans and Japanese, there was a disproportionately high incidence of pyogenic liver abscesses and other pus-forming lesions, such as endophthalmitis and pyomyositis, associated with Kp.[3-5] Furthermore, these Kp pyogenic liver abscesses were also strongly associated with diabetes mellitus.[6] In addition to this distinct entity of Kp pyogenic liver abscesses in East Asians, this bacterium is also associated with liver abscesses due to recurrent pyogenic cholangitis, another unique infection in our population due to *Clonorchis sinensis* infections, pigment stone formation and recurrent cholangitis of the biliary tree.[7-9]

Diagnosis of Kp liver abscess is usually achieved by radiological examination and isolation of the bacterium from clinical samples such as blood or pus obtained by drainage. However, in rare situations, when blood culture and culture of other clinical specimens were negative, laboratory diagnosis of Kp liver abscess may be challenging. Yet, it is extremely important to distinguish Kp liver abscess from other culture-negative liver abscess-like lesions, such as amebic liver abscess, hydatid cyst, or even tumors, as treatment of these diseases is radically different. In the past few years, next-generation sequencing (NGS) has emerged as a technology for laboratory diagnosis of many culture-negative infections.[10-13] In this study, we describe the use of NGS for rapid diagnosis of Kp culture-negative liver abscesses.

2. Case presentation

2.1. Case 1

A 60-year-old Chinese woman was admitted to The University of Hong Kong–Shenzhen Hospital because of a fever for 6 days. Six days before admission, she had acute onset of fever and dysuria and was investigated in a clinic. Urinalysis and microscopic examination of the urine revealed proteinuria, hematuria and white blood cells in the urine. C-reactive protein (CRP) was 66.9 mg/L. She was treated with oral cefuroxime but the fever persisted. On admission, her body temperature was 38°C. There was no localizing sign. Total white cell count was 11.13 × 10^9/L, with neutrophils 8.6 × 10^9/L. Her platelet count was 375 × 10^9/L and hemoglobin was 112 g/L. Liver enzymes were mildly elevated. Serum urea and creatinine levels were normal. Random blood glucose was 8.3 mmoL/L. The oral cefuroxime seemed useless, which could not cover the pathogens, that CRP was elevated. CRP was 183.5 mg/L. Blood and urine culture were performed and empirical intravenous amoxicillin-clavulanate was commenced. Plain computed tomography (CT) scan of the abdomen revealed multiple round low-density foci of varying sizes (the largest one measuring 32 mm × 16 mm in segment VI) with untidy margin in both lobes of the liver (Figure 1A and B).

On day 7 after admission, fever persisted. Blood and urine culture showed negative results. The patient's blood was sent for mNGS analysis. Contract-enhanced CT scan of abdomen showed multiple round low-density foci with double rim enhancement and untidy margin in the liver and spleen, which were suspected to be hydatid cysts or abscesses (Figure 1C-F). Hepatitis A virus immunoglobulin M (IgM), hepatitis B virus surface antigen, hepatitis C virus antibody and hepatitis E virus IgM were negative. On day 9, mNGS analysis of the blood sample revealed 144 sequence reads of Kp. Amoxicillin-clavulanate was continued, resulting in gradual resolution of her fever and normalization of transaminase levels. Antibodies for *Echinococcus granulosus*, *Schistosomiasis japonicum*, *Paragonimus westermani*, *C. sinensis*, *Spirometra mansoni*, *Taenia solium,* and *Angiostrongylus cantonensis* were negative. Drainage of the lesions in the liver was not performed as the patient responded to the antibiotic treatment. Amoxicillin-clavulanate was continued for a total of 6 weeks. Interval ultrasonographic scan of the liver during and after treatment showed reduction in both the size and number of low-density foci in the liver (Figure 1G and H). The patient remained asymptomatic 6 months after discharge.

2.2. Case 2

An 82-year-old Chinese man was admitted to The University Hong Kong–Shenzhen Hospital because of fever, chills, rigor and right upper quadrant abdominal pain for 1 day. The patient had histories of hypertension, diabetes mellitus and coronary heart disease. He started to develop dizziness and vomiting 2 days before admission. On the day of admission, he developed fever, chills, rigor and severe right upper quadrant pain of the abdomen. His body temperature was 39.6°C. Upper right quadrant tenderness was detected. Total white cell count was 10.66 × 10^9/L, with neutrophils 7.8 × 10^9/L. His platelet count was 195 × 10^9/L and hemoglobin was 129 g/L. Liver and renal function test results were normal. The prothrombin time was 13.5 s and the activated partial thromboplastin time was prolonged to 50.4 s. His CRP was 215.09 mg/L and procalcitonin was 4.42 ng/mL. Contrast CT scan of the abdomen revealed enlarged gallbladder with cholecystitis, mild dilation of the intra- and extra-hepatic bile ducts and pancreatic duct, and multiple round low-density foci with surrounding abnormal perfusion in the liver, which were suspected to be infected liver cysts with surrounding small abscesses (Figure 2A and B). In addition to the multiple cystic lesions in the liver, ultrasonographic scan also revealed a stone (7.7 mm × 5.3 mm) and a large

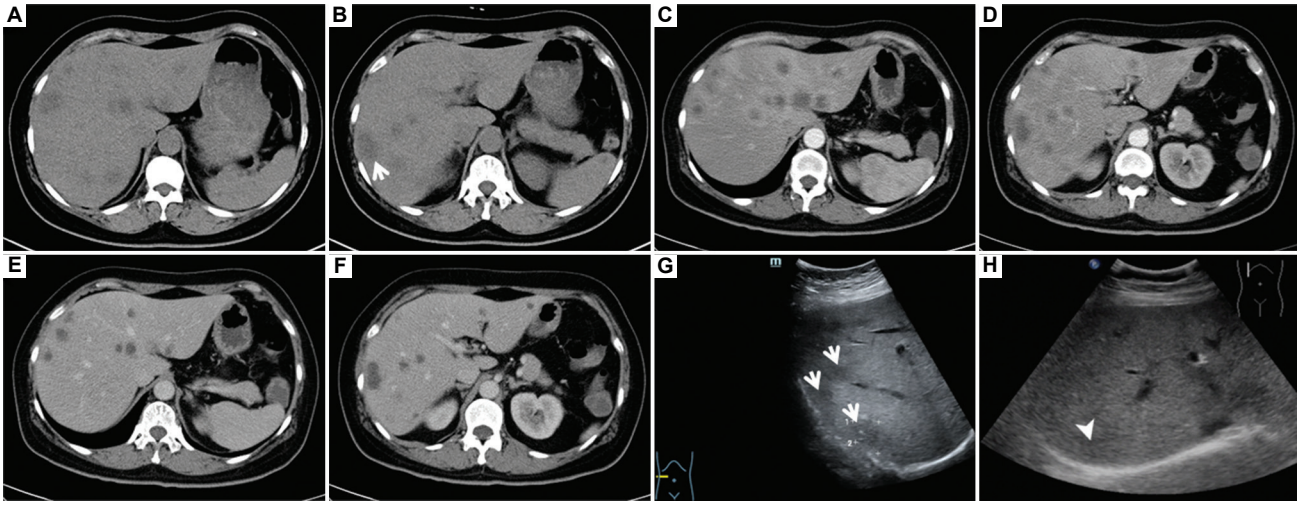

Figure 1. Computed tomography (CT) and ultrasonographic scan of Case 1. (A and B) Plain CT scan of the abdomen on day 5 after admission, showing multiple round lesions with hypoattenuation and untidy margin in both lobes of the liver and spleen, with the largest one measuring 32 mm × 16 mm (arrow) in the segment VI of the liver. (C and F) Contrast enhanced CT scan of the abdomen on day 7 after admission, showing peripheral rim enhancement around the low-density lesions in both lobes of the liver in the arterial phase (C and D) and portal venous phase (E and F). (G) Ultrasonographic scan of the liver on day 9 after admission, showing multiple hypoechoic lesions (arrow) in the right lobe of the liver, with the largest one measuring 33 mm × 25 mm. (H) Ultrasonographic scan of the liver on day 48 after admission, showing reduction in size and number of the hypoechoic lesions (arrowhead) in the right lobe of the liver.

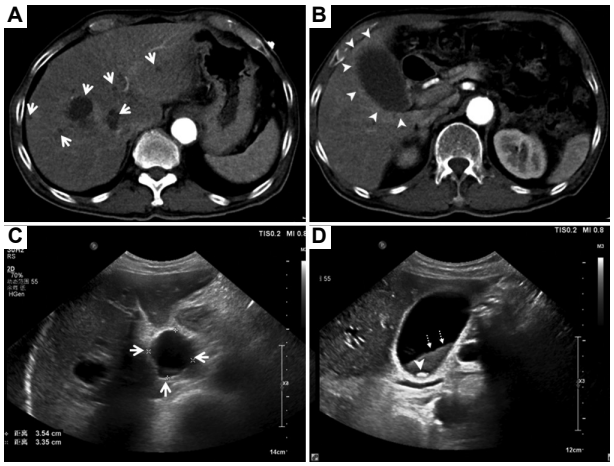

Figure 2. Computed tomography (CT) and ultrasonographic scan of Case 2 on day 2 after admission. (A) Contrast enhanced CT scan of the abdomen, showing multiple well-demarcated water-attenuation lesions (arrows) with peripheral transient abnormal perfusion and blurred margins in some of the lesions. (B) Contrast enhanced CT scan of the abdomen, showing the enlarged gall bladder with transient abnormal perfusion (arrowheads) in the adjacent lobe of the liver. (C) Ultrasonographic scan of the liver, showing multiple anechoic unilocular fluid-filled spaces with imperceptible walls and posterior acoustic enhancement, with the largest one measuring 35 mm × 33 mm (arrows) in the caudate lobe of the liver. (D) Ultrasonographic scan of the liver, showing an echogenic focus casting an acoustic shadow (arrowhead) and a large amorphous collection of sludge not casting an acoustic shadow (dotted arrows) within the gallbladder.

amorphous collection of sludge in the gallbladder (Figure 2C and D). Stool analysis showed no parasitic infections. One set of blood culture was performed, and then empirical intravenous piperacillin-tazobactam was commenced. On day 2, oral doxycycline was added. On day 4, the fever persisted and his blood was sent for mNGS.

On day 5, mNGS analysis of the blood sample revealed sequence reads of Kp (n = 153), *Klebsiella variicola* (n = 256), human herpes virus 6 (n = 3), Torque teno virus (n = 37), Epstein–Barr virus (n = 1) and adenovirus D (n = 1), but blood culture was negative. Piperacillin-tazobactam and doxycycline were continued. His fever gradually subsided, and CRP and procalcitonin returned to normal ranges. Surgical treatment was declined by the patient and his relatives. After 11 days of piperacillin-tazobactam treatment, the antibiotic regimen was switched to oral amoxicillin-clavulanate, which was sustained for another 5 days. The patient remained asymptomatic 4 months after discharge.

3. Discussion

Herein we report two extremely rare cases of Kp culture-negative liver abscess diagnosed by mNGS. Both patients were not the typical Kp liver abscess cases in which the bacterium was readily isolated from blood, liver pus, and other samples collected from the secondary lung abscess, brain abscess, pyomyositis, *etc.*[3-5] In fact, Case 1 did not even have diabetes mellitus, which was observed in most East Asians with Kp liver abscess. Interestingly, she presented with refractory upper urinary tract infection, and the liver abscess was incidentally revealed only after

Table 1. *Klebsiella pneumoniae* liver abscess diagnosed by mNGS

Case no.	Reference	Sex/Age	Diabetes mellitus	Clinical presentation	Bacterial culture		mNGS		Outcome
					Specimen	Result	Specimen	Microbe (no. of reads)	
1	Present report	F/60	No	Incidentally discovered during investigation of upper urinary tract infection	Blood	Negative	Blood	*Klebsiella pneumoniae* (144)	Survived
2	Present report	M/82	Yes	Fever, right upper quadrant pain	Blood	Negative	Blood	*Klebsiella pneumoniae* (153), *Klebsiella variicola* (256), HHV-6 (3), Torque teno virus (37), EBV (1), adenovirus D (1)	Survived
3	Zeng *et al.* (2021)[14]	F/59	Yes	Discovered during the investigation of acute meningitis	CSF	Negative	CSF	*Klebsiella pneumoniae* (13470)	Survived
					Blood	Negative	Blood	*Klebsiella pneumoniae* (5318)	
					Liver pus	Negative			
4	Xie and Zhu (2021)[15]	F/56	Yes	Fever, pain on percussion of the abdomen	Drain fluid	Positive	Drain fluid	*Klebsiella pneumoniae* (119331)	Survived
5	Luo *et al.* (2023)[16]	M/71	Yes	Incidentally discovered during investigation of subacute pneumonia syndrome	Blood	Positive	BAL	*Klebsiella pneumoniae* (not mentioned)	Died
					Liver pus	Positive			

Abbreviation: BAL: Bronchoalveolar lavage fluid; CSF: Cerebrospinal fluid; EBV: Epstein–Barr virus; F: Female; HHV: Human herpes virus; M: Male; mNGS: Metagenomic next-generation sequencing.

CT examination of the abdomen. Among the three cases of Kp liver abscesses described in the literature in which NGS played a crucial role in their diagnosis, only one patient was culture-negative (Case 3, Table 1).[14] In contrast to the two cases in the present study, that patient actually presented with central nervous system infection, fever, headache, neck stiffness, and positive Kernig's sign. Lumbar puncture and cerebrospinal fluid (CSF) analysis also revealed extremely high white cell count. The CSF was sent for mNGS analysis because CSF culture did not reveal any positive findings. Kp liver abscess syndrome was only suspected upon the revelation of the mNGS analysis results of Kp sequence reads. Subsequent CT scan of the abdomen revealed liver abscess. Blood and liver abscess pus cultures were negative. Subsequent mNGS analysis of the blood also revealed Kp sequences. Since culture-negative liver abscess could be due to a variety of causes, such as amebic liver abscess and hydatid cyst, confirming the identity of the microorganism involved in these cases would be of paramount importance because specific antimicrobial treatment could be immediately commenced, and there was no need to spend extra resources and effort on additional laboratory tests for delineating the microbiological cause. In fact, for Case 1 in the present study, the possibility of hydatid cyst has been entertained by the radiologist, but the subsequent positive mNGS results and negative serology tests for parasitic diseases have resolved the diagnosis.

In addition to identifying cases of culture-negative Kp liver abscess, mNGS was also useful for making rapid diagnosis of Kp liver abscesses, as presented in Cases 4 and 5 (Table 1), whose cultures were positive for Kp a few days after the positive mNGS results.[15,16] For Case 4, the patient presented with fever and chest tightness but no abdominal symptoms, although there was mild pain during percussion of the right upper quadrant.[15] After a CT examination of the abdomen showed a liver abscess, ultrasound-guided drainage of the liver abscess was performed. mNGS of the drained fluid was positive for Kp sequences and a culture of the bacterium confirmed the presence of Kp 3 days later. As for Case 5, the patient presented with subacute pneumonia syndrome, and liver abscess was only discovered through CT scan of the abdomen.[16] mNGS analysis of his bronchoalveolar lavage fluid showed sequence reads of Kp, as well as *Candida albicans* and *Aspergillus flavus*. Both the blood and liver abscess pus were subsequently culture-positive for Kp.

4. Conclusion

mNGS is a useful tool for making a rapid diagnosis of Kp culture-negative liver abscesses. The advanced technology provides comprehensive detection of microbial DNA in clinical samples, accurately identifying pathogens even in complex clinical scenarios where conventional methods have failed. The application of mNGS in diagnosing

culture-negative Kp liver abscess not only improves the timeliness and accuracy of diagnosis but also facilitates appropriate antibiotic therapy, thereby improving patient outcomes and reducing morbidity associated with this potentially severe infection.

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Conflict of interest

Patrick C. Y. Woo has provided scientific advisory/laboratory services for Gilead Sciences, Incorporated; International Health Management Associates, Incorporated; Merck and Corporation, Incorporated; and Micología Molecular S.L. and Pfizer, Incorporated. Patrick C. Y. Woo is also an Editorial Board Member of this journal, but was not in any way involved in the editorial and peer-review process conducted for this paper, directly or indirectly. Separately, other authors declared that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper. The funding sources had no role in study design, data collection, analysis, interpretation, or writing of the report.

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Writing – review & editing: All authors

Ethics approval and consent to participate

Ethics approval for this retrospective study was endorsed by the Institutional Review Board of The University of Hong Kong–Shenzhen Hospital ([2022]120), and written informed consent was obtained prior to patients' participation in this project.

Consent for publication

Written informed content was obtained from the patients for the publication of this case report.

Availability of data

The data that support the findings of this study are available from the corresponding author, Patrick C.Y. Woo.

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ACCSCIENCE PUBLISHING

Microbes & Immunity

CASE REPORT

Cedecea lapagei as an emerging extensively drug-resistant microorganism: A case report in a patient with pleural empyema and literature review

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Abstract

Cedecea lapagei is a Gram-negative bacterium that belongs to the Enterobacteriaceae family and is said to be pathogenic for humans. Herein, we report a case of extensively drug-resistant *C. lapagei* in a patient with pleural empyema and offer a literature review of the already documented case reports on individuals infected with *C. lapagei*. A 60-year-old female patient was brought to the emergency department at AIIMS Bathinda with a history of breathing difficulty since one day after she sustained multiple injuries from a road accident. She was diagnosed with pleural empyema. Subsequently, an intercostal drain tube was inserted into the patient, and the drain content was sent for culture, which revealed growth of *C. lapagei*. Our literature retrieval work gathered a total of 13 relevant cases. In conclusion, early identification of *C. lapagei* and administering suitable treatment is important for good patient outcome.

Keywords: *Cedecea lapagei*; Intercostal drain; Multidrug resistance

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1. Background

Cedecea are Gram-negative bacteria belonging to Enterobacteriaceae family with six known species.[1] Among them, *Cedecea davisae*, *Cedecea lapagei,* and *Cedecea neteri* are regarded as human pathogens.[1,2] *Cedecea* are catalase-positive, oxidase-negative, motile, non-lactose fermenting, non-sporing, and non-encapsulated bacteria that are capable of reducing nitrates to nitrites. First discovered in 1977, it was not recognized as a potential pathogen to humans until 2006. This bacterium was first described in a 55-year-old man with hypertension and a recent history of liver transplant on ambulatory peritoneal dialysis. He developed peritonitis, and his peritoneal fluid specimen was sent for culture, which revealed growth of *C. lapagei.*[1] *C. lapagei* was further isolated in patients with pneumonia, bacteremia, soft-tissue infection, peritonitis, sepsis, hemoptysis, and urosepsis.[2,3]

In this paper, we report a case of isolation of *C. lapagei*, which was extensively drug-resistant, from a patient with pleural empyema after sustaining traumatic chest injuries

due to a road accident, and offer a literature review of the *Cedecea* cases documented.

2. Case presentation

A 60 year old female patient was referred to the emergency department with a history of breathing difficulty along with pain on her right side of chest since one day. She met with a road traffic accident one week before. She had been to the private hospital for management of her conditions. A computed tomography (CT) of the chest revealed comminuted fracture of the right scapula, fracture third to eighth rib, and moderate right hemothorax that caused right-side lung collapse. Head CT showed temporal bone fracture extending into the right parietal lobe. Laboratory results showed hemoglobin 6.9 g/dL, white blood cells $10 \times 10^3/\mu L$, and platelets $377 \times 10^3/\mu L$. Liver function test showed increased serum glutamic-oxaloacetic transaminase and serum glutamic pyruvic transaminase. The patient was conscious and oriented to time, place, and person.

On the day of admission, oxygen therapy was initiated and the patient underwent blood transfusion, blood pressure control, and fluid resuscitation. Empirical antibiotic therapy with cefuroxime and levofloxacin was initiated, and the patient was transferred to the surgical intensive care unit. The patient had increased breathlessness, and subsequently intercostal drain (ICD) tube was inserted. On day 7, an ultrasound was performed, revealing residual right-side hemothorax with a thickness of 3 cm. On day 20, the patient developed a fever and exhibited a raised total leukocyte count ($20 \times 10^3/\mu L$). Her ICD content and blood samples were sent for microbiological culture and antibiotic susceptibility. A direct Gram stain was conducted on the ICD samples, showing numerous pus cells with Gram-negative bacteria (Figure 1). The content was then inoculated on blood agar and MacConkey agar, which were subjected to incubation at 37°C. Her blood culture was sterile but ICD fluid content showed growth of non-lactose fermenting colonies after 24 h of incubation (Figure 2). The Gram stain showed non-capsulated Gram-negative rods. The growth was analyzed in Vitek™ 2 compact system (bioMerieux, France) for bacterial identification and antibiotic susceptibility. The results revealed that the pathogen was *C. lapagei*, which showed resistance to amoxicillin-clavulanate, piperacillin-tazobactam, cefuroxime, cefepime, ceftriaxone, ertapenem, imipenem, meropenem, tetracycline, doxycycline, amikacin, gentamicin, ciprofloxacin, cotrimoxazole, and ceftazidime-avibactam but was susceptible to minocycline only. Repeat sample was taken after changing the ICD tube to exclude the contamination and to confirm the suspected bacteria. Same microorganism was identified

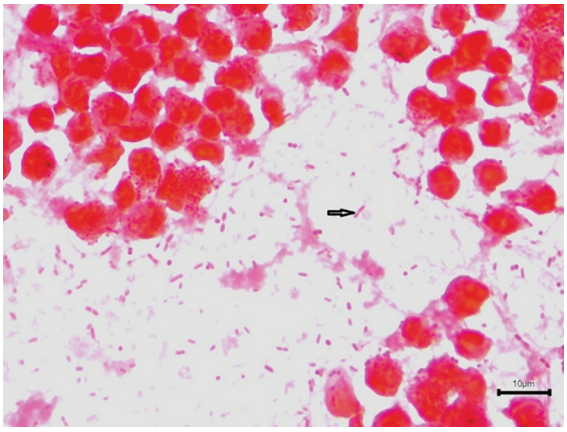

Figure 1. Microscopic examination of Gram-negative bacteria present in the intercostal drain sample of the patient. Scale bar: 10 µm. Magnification: ×100

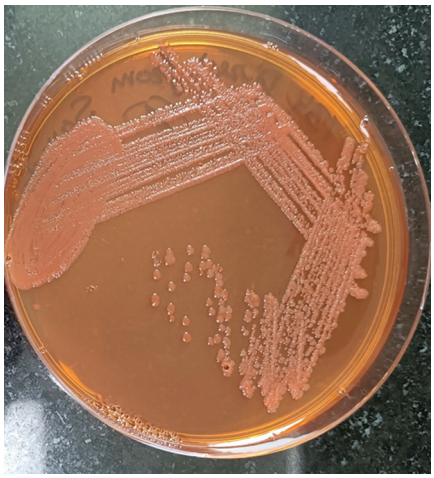

Figure 2. The growth of non-lactose fermenting colonies on MacConkey agar

from the analysis of repeated ICD content with a similar susceptibility pattern obtained. The patient was put on minocycline, and after three days, new samples were tested sterile after 48 h of incubation. The patient became afebrile and the ICD tube inserted was removed. Afterward, the patient was discharged under stable conditions.

3. Discussion and literature review

The *Cedecea* cases documented were searched on the PubMed using the terms "*Cedecea lapagei*" and "*Cedecea*." The literature search procedure also extended to seeking references cited in the collected articles. Our literature retrieval work revealed that only 13 cases of *C. lapagei* are available in the literature thus far. Based on the retrieved articles, the microorganism of interest was isolated from different specimens, such as blood (6), sputum (2), knee wound (1), pus (1), exudates (1), urine

(1), and Bronchoalveolar lavage (1) (Table 1). Out of the 13 cases, four were neonatal patients and nine were adult patients. In most cases, the *C. lapagei* infection was diagnosed with the aid of Vitek™ 2 compact system, Phoenix 100 (Becton Dickinson, USA, and API20E kit, Biomerieux, France), MALDITOF-MS (Bruker

Table 1. Literature review of *Cedecea*

Study	Year	Diagnosis	Sample	Instrument for diagnosis	Treatment	Patient outcome
Davis *et al.*[1]	2006	55-year-old male with CAPD-related peritonitis, having received liver transplant	Peritoneal fluid	NA	Initially, vancomycin and gentamicin followed by ceftazidime and gentamicin	Discharged
Yetkin *et al.*[6]	2008	38-year-old male patient with COPD	BAL	Phoenix 100	Amikacin and meropenem	Expired
Dalamaga *et al.*[5]	2008	47-year-old male with bacteremia and wound infections from cement-related chemical burns, and diabetes mellitus	Blood and left knee wound	Phoenix™	Cefotaxime and amikacin	Discharged
Lopez *et al.*[7]	2013	34-year-old patient with pneumonia and acute promyelocytic leukemia	Sputum sample	Vitek 2 Analysis (bioMerieux Inc.)	Tigecycline	Discharged
Hong *et al.*[8]	2015	76-year-old male with pneumonia and COPD	Sputum and blood	MALDITOF-MS (Bruker Daltonik GmbH, Bremen, Germany) and Vitek 2 GN system (bioMerieux, Marcy l'Etoile, France)	Cefpodoxime	Discharged
Biswal *et al.*[9]	2015	50-year-old male with superinfection and malignant oral ulcer with squamous cell carcinoma of right buccal mucosa	Pus	Vitek 2 Analysis (bioMerieux Inc.)	Ciprofloxacin	Discharged
Islam *et al.*[10]	2016	Neonate female with neonatal sepsis	Blood	VersaTREK blood and body fluid culture system	Ciprofloxacin and amikacin	Discharged
Kury *et al.*[14]	2017	Neonate male with ventilator-associated pneumonia and sepsis	Blood	MicroScan WalkAway-96 system	Meropenem	Discharged
Ahmed *et al.*[11]	2017	Neonate female with late-onset sepsis in preterm	Blood	BD Phoenix™ 100 Automated Microbiology System using panel NMIC/ID-55	Amikacin and cefotaxime	Discharged
Arishi *et al.*[15]	2017	Patient with necrotizing enterocolitis and peritonitis	NA	NA	Piperacillin- tazobactam for 14 days	Discharged
Chavez Herrera *et al.*[3]	2018	52-year-old Mexican man with liver cirrhosis and treated hypertension	Bullae fluid sample	MicroScan WalkAway 96 plus system	Imipenem and clindamycin	Expired
Ramaswamy *et al.*[13]	2019	Neonate male with nosocomial pneumonia in late preterm	Blood	NA	Piperacillin- tazobactam	Discharged
Mohamed *et al.*[12]	2021	55-year-old man with acute exacerbation of renal failure and irritative voiding symptoms and chronic renal failure, diabetes mellitus and hypertension	Urine sample	Eosin methylene blue agar	Levofloxacin	Discharged
Xu *et al.*, China[16]	2021	42-year-old with multiple injuries	Exudates	Vitek™ 2 compact system	Ampicillin/sulbactam	Discharged

Abbreviations: BAL: Bronchoalveolar Lavage; CAPD: Continuous ambulatory peritoneal dialysis; COPD: Chronic obstructive pulmonary disease; NA: Not applicable.

Daltonik GmbH, Bremen, Germany) and Vitek™ 2 GN system (bioMerieux, Marcy l'Etoile, France), MicroScan WalkAway 96 plus System (Beckman Coulter, USA), and VersaTREK blood and body fluid culture system (TREK diagnostic, Cleveland, Ohio).[4]

Cedecea was first recognized as a human pathogen in 2006 by Davis *et al.*[1] who reported the first case of the microorganism in a patient having peritonitis related to continuous ambulatory peritoneal dialysis. In this case, reported by Davis *et al.*, the patient first received a separate treatment of vancomycin and gentamicin, followed by gentamicin and ceftazidime. In 2008, Dalamaga *et al.*[5] reported another case of *C. lapagei*, with a sample obtained from an infected wound of a patient suffering from cement-related chemical burns. In this case, the patient recovered after treatment. Another case study in the same year was reported by Yetkin *et al.*[6] in a patient with chronic obstructive pulmonary disease (COPD), but the patient expired. Lopez *et al.*[7] and Hong *et al.*[8] also described *C. lapagei*-related pneumonia in 2013 and 2015, respectively. In 2015, Biswa *et al.*[9] reported a *Cedecea* superinfection in a 50-year-old Indian man with a malignant oral ulcer with squamous cell carcinoma of the right buccal mucosa. In addition, Islam *et al.*,[10] Ahmed *et al.*[11] and Ramaswamy *et al.*[13] have reported neonatal cases of *Cedecea* infection from India.

Cedecea is intrinsically resistant to colistin (polymyxin E). Data on the *Cedecea* resistance to drugs remains limited in the literature. It is important to note that, according to all of the previous studies, this microorganism showed signs of multidrug resistance (Table 2). Combined with the literature review, the current case report establishes this organism as extensively drug-resistant.

C. lapagei is an emerging multidrug-resistant pathogen in India, presenting a huge treatment challenge. The ever-increasing complexity of its resistance pattern adds further difficulty to the treatment efforts. It is worthy to note that *C. lapagei* infection is mainly acquired from the hospital, similar to our case. Hence, proper infection control practices and early management of this rare microorganism should be implemented to improve patient outcome.

Table 2. Antimicrobial resistance pattern of *Cedecea* in different studies

Antibiotics	Hong *et al.*[8]	Dalamaga *et al.*[5]	Lopez *et al.*[7]	Islam *et al.*[10]	Ahmed *et al.*[11]	Chavez Herrera *et al.*[2]	Ramaswamy *et al.*[13]	Mohamed *et al.*[12]	Xu *et al.*[16]	Current report
Ampicillin/sulbactam	-	-	I			I (16/18)			I (16)	R (≥32)
Amikacin	-	S (8)	R			S (≤4)	R	S	I (≤2)	R (≥64)
Ampicillin	-	-	R			R (>16)		R	-	
Aztreonam	-	S (≤2)	R		R	S (≤8)			S (≤1)	
Ceftriaxone	-	-	R	R	R	S (≤8)		R	S (≤1)	R (≥64)
Ceftazidime	S	S (≤1)		R		S (≤1)	R	R	-	R (≥64)
Cefazolin	-	-	R			R (≥16)		R	R (≥64)	
Cefotaxime	S	S (≤2)			R	S (≤2)			S (4)	R (≥64)
Ciprofloxacin	S	S (≤0.25)	R	S		S (≤1)	S	S	I (2)	R (≥4)
Cefepime	S	S (≤2)	R		R	S (≤2)			S (≤1)	R (≥32)
Cefuroxime	-	-		R		S(≤4)			-	R (≥32)
Cefotetan	-	-				S (≤16)			-	
Gentamicin	-	R (>8)	I	R		S (2)	R		I (8)	R (≥16)
Imipenem	-	-	R	S	R	R (≥8)		S	R (4)	R (≥16)
Levofloxacin	-	S (≤1)				S (≤2)	S	S	I (2)	R (≥8)
Meropenem	-	S (4)	R		R	S (≤4)	R		-	R (≥16)
Moxifloxacin	-	-	R			S (≤2)			-	
Piperacillin/tazobactam	-	-	R	R		S (≤8)	S		S (≤4)	R (≥128)
Tobramycin	-	R (>8)	I			-			I (8)	
Cotrimoxazole	S	-	-	R		-	S	S	R (≥320)	R (≥320)
Tigecycline	-	-	S			-			-	
Minocycline										S (≤1)

Notes: I: Intermediate; R: Resistant; S: Sensitive values in parentheses indicate the minimum inhibitory concentration (MIC) μg/mL.

4. Conclusion

Cedecea is a rare nosocomial pathogen that is commonly associated with pneumonia and bacteremia in immunocompromised patients, such as those with diabetes, chronic heart disease, and renal disease, as well as prematurely born infants and neonates with nosocomial pneumonia. This organism shows extensive clinical manifestations by virtue of the isolation from urine, wound, pus and exudates. This case report describes a case of extensively drug-resistant *C. lapagei* in an Indian patient with pleural empyema. Early identification of this bacteria and suitable treatment is crucial for ensuring good patient outcome.

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Conflict of interest

The authors declare they have no competing interests.

Author contributions

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Writing – review & editing: Bhawna Sharma, Jai Ranjan, Kamla Kant

Ethics approval and consent to participate

Written informed consent was taken from the patient.

Consent for publication

The patient gave consent to publish her data in the given study.

Availability of data

Not applicable.

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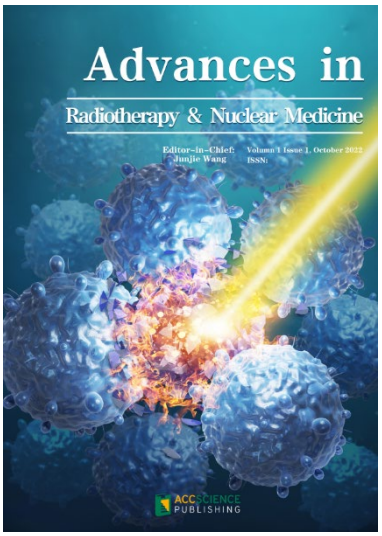

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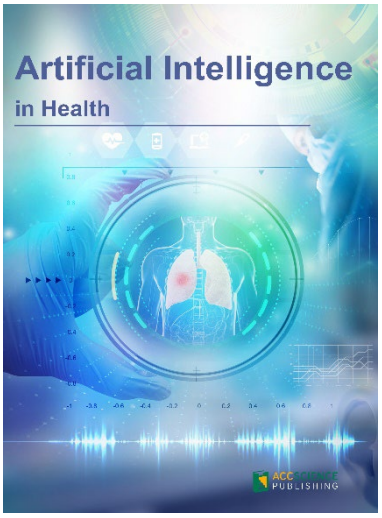

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