# *INVESTIGATING LINKS BETWEEN THE IMMUNE RESPONSE AND HELICOBACTER PYLORI-MEDIATED DISEASE OUTCOMES*

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## COVID-19 Impact

I started my PhD on February 1st, 2020, but my laboratory work was interrupted on March 23rd due to lockdown measures. During the lockdown period, I utilized my time to write my literature review. However, working from home proved challenging due to childcare and other responsibilities. I was able to resume lab work in late July, but the implementation of a shift system resulted in reduced access to the lab and difficulties in receiving training on equipment usage. Due to social distancing measures and concerns about transmission, the majority of non-COVID clinical research has been put on hold, resulting in a decrease in the number of participants recruited for research studies and a delay in arranging the delivery of some key consumables.

## Dedication

I would like to dedicate this thesis to my family members, particularly my wonderful husband and children, for their unwavering support, sacrifices, and encouragement throughout this journey.

## Acknowledgments

I would like to express my gratitude to my thesis advisor, Dr. Karen Robinson, for her support, encouragement, attention to detail, and excellent guidance. I would also like to thank Dr. Tanya Monaghan and Dr. Kazuyo Kaneko, for their valuable advice on my research directions. My special thanks to all the members of Dr. Robinson's laboratory: Darren Letley and Joanne Rhead for providing me with a nice research environment in the laboratory. My special appreciation to the School of Medicine, and the Department of Gastroenterology. I must also acknowledge my family for their continuous encouragement and dedicated support they gave me throughout my PhD. I sincerely thank Taif University and Saudi Arabia's Ministry of Higher Education for providing me with a scholarship to support my postgraduate studies.

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- 35th Workshop of the European *Helicobacter* and Microbiota Study Group. September 8 - 10, 2022. Glasgow, United Kingdom.
- The Inaugural Translational Medical Sciences PGR showcase symposium. 3rd October 2022. The De Vere East Midlands Conference Centre, Nottingham.
- The Annual Biodiscovery Institute Research Day - 14th September 2022.
- Sue Watson PGR Oral Presentation Event on 24th March 2023.University of Nottingham, School of Medicine. (Oral presentation).
- Nottingham Digestive Diseases Center (NDDC) scientific research meeting, Biodiscovery Institute 2, Translated Medical Sciences, School of Medicine. 5th May 2023. (Oral presentation).
- GI Liver Theme/NDDC Showcase event, 13th September 2023. (Poster presentation).
- BDI Annual Research Symposium, 22nd September 2023. (Poster presentation).

## Abbreviations

| | | | |
|---|---|---|---|
| **AG** | Atrophic gastritis | **ELISA** | Enzyme-Linked Immuno-Sorbent Assay |
| **AIF** | Apoptotic-inducing factor | **ERK** | Extracellular signal-regulated kinase |
| **AMI** | Acute myocardial infarction | **ESRK** | extracellular signal-regulated kinase |
| **APC** | Antigen-presenting cell | **FBS** | Fetal bovine serum |
| **ARDS** | Acute respiratory distress syndrome | **FISH** | Fluorescent in situ hybridization |
| **Bab A** | Blood group antigen-binding adhesin | **FOXP3** | Forkhead box P3 |
| **BFA** | Brefeldin A | **FXR** | Farnesoid X receptor |
| **BMI** | Body mass index | **GAPDH** | Glyceraldehyde-3-phosphate dehydrogenase |
| **BLF** | Bronchoalveolar lavage fluid | **GERD** | Gastroesophageal reflux disease |
| **BO** | Barrett's oesophagus | **GC** | Gastric cancer |
| **CAG** | Chronic atrophic gastritis | **GF-1R** | Growth factor-1 receptor |
| ***CagA*** | Cytotoxin-associated gene | **GI** | Gastrointestinal |
| ***Cag* PAI** | *Cag* Pathogenicity Island | **GM-CSF** | Granulocytes monocytes colony stimulation factors |
| **cAMP** | Cyclic adenosine monophosphate | **GU** | Gastric ulcers |
| **CAR** | Constitutive androstane receptor | **GGT** | Gamma-glutamyl transpeptidase |
| **CCL** | Chemokine ligand | **GPBAR1** | G-protein-coupled bile acid receptor 1 |
| **CHRM** | cholinergic receptors muscarinic | **GPBAR** | G-protein bile acid receptor |
| **CK-20** | Cytokeratin 20 | **HCC** | Human hepatocellular carcinoma |
| **CRC** | Colorectal cancer | **HIV** | Human immunodeficiency virus |
| **CXCL1** | C-X3-C motif chemokine ligand 1 | **HFD** | High-fat diet |
| **CXCL10** | chemokine (C-X-C motif) ligand 10 | **HOP H** | *Helicobacter* outer membrane protein H |
| **DCA** | Deoxycholic acid | **HpGGT** | *H. pylori* GGT |
| **DC** | Dendritic cell | **HP-NAP** | *H. pylori* neutrophil-activating protein |
| **DMSO** | Dimethyl sulfoxide | ***H. pylori*** | *Helicobacter pylori* |

| | | | |
|---|---|---|---|
| **DU** | Duodenal ulcers | **HSIL** | high-grade squamous intraepithelial lesions |
| **DSBs** | Double-strand breaks | **IAV** | Influenza A virus |
| **DTH** | Delayed-type hypersensitivity | **IBD** | Inflammatory bowel disease |
| **EDTA** | Ethylene diamine tetra acetic acid | **ICIs** | Immune checkpoint inhibitors |
| **KO** | knockout | **iDCs** | immature dendritic cells |
| **LCA** | Lithocholic acid | **Ig** | Immunoglobulin |
| **LCF** | Lymphocyte chemoattractant factor | **IL** | Interleukin |
| **Le$^b$** | Lewis$^b$ | **IL-1β** | Interleukin 1 beta |
| **LFA-1** | Lymphocyte function-associated antigen 1 | **ILCs** | Innate lymphoid cells |
| **LP** | Lamina propria | **IM** | Intestinal metaplasia |
| **LOS** | *H. pylori* lipo oligosaccharide | **IMDM** | Iscove's Modified Dulbecco's Medium |
| **LPMC** | Lamina propria mononuclear cells | **IMSS** | Instituto Mexicano del Seguro Social |
| **LPS** | Lipopolysaccharide | **iTreg** | Inducible Tregs |
| **LXR** | Liver X receptor | **PAMPs** | Pathogen-associated molecular patterns |
| **MALT** | Mucosa-Associated Lymphoid Tissue Lymphoma | **PBMCs** | Peripheral blood mononuclear cells |
| **MBL** | Monoclonal B-cell lymphocytosis | **PGN** | Peptidoglycan |
| | | **PHA** | Phytohemagglutinin |
| **MCP-1** | monocyte chemoattractant protein 1 | **PMNs** | polymorphonuclear leukocytes |
| **MDM2** | Murine double minute 2 | **PKC-ζ** | protein kinase C-zeta |
| **MIP-1α** | Macrophage inflammatory protein 1 alpha | **PPIs** | proton pump inhibitor drugs |
| **MIF** | Macrophage migration inhibitory factor | **PRRs** | Pattern recognition receptors |
| **miR-155** | MicroRNA-155 | **PUD** | Peptic ulcer disease |
| **MP** | Membrane protein | **PXR** | Pregnane X receptor |
| | | | |
| **MR** | Mendelian randomization | **RA** | Rheumatoid arthritis |
| **MMP** | Metalloproteinases | **RA-FLS** | Rheumatoid arthritis- fibroblast-like synoviocytes |
| | | **RSA** | Resveratrol |
| **MRGPRX4** | MAS-related G-protein coupled receptor family member X4 | **RV-A16** | Rhinoviruses A16 |
| **moDCs** | Monocyte-derived dendritic cells | **TAMs** | tumor-associated macrophages |
| **MOI** | Multiplicity of infection | **TCR** | T cell receptor |
| **MS** | Multiple sclerosis | **Tfh** | T follicular helper |
| **MSD** | Meso Scale Discovery | **TGF-α** | tumor growth factor |

| | | | |
|---|---|---|---|
| **NAG** | Non-atrophic gastritis | **TGF-β** | Transformation growth factor beta |
| **NCGC** | Non-cardia gastric cancer | **TGR5** | Takeda G protein-coupled receptor5 |
| **NFAT** | Nuclear factor of activated T cells | **Th17** | T helper 17 |
| **NGS** | Next-generation sequencing | **TLCA3s** | Taurolithocholic acid 3-sulfate |
| **NK** | Natural Killer | **TLR** | Toll-like receptor |
| **NLD** | nuclear localization sequence | **TME** | Tumor microenvironment |
| **NOD 1** | nucleotide-binding oligomerization domain 1 | **TNF-α** | Tumor necrosis factor |
| **NSAIDs** | Nonsteroidal anti-inflammatory drugs | **T4SS** | Type IV secretion system |
| **OA-FLS** | Osteoarthritis- fibroblast-like synoviocytes | **RORγ** | retinoid-related orphan receptor gamma |
| **OD** | Odd ratio | **TME** | Tumor microenvironment |
| **OECD** | Organization for economic cooperation and development | **Timp-1** | Tissue inhibitor of metalloproteinase 1 |
| **OIP A** | Outer inflammatory protein A | **Tregs** | Regulatory T cells |
| **OMV** | Outer membrane vesicles | **TTP** | Time to disease progression |
| **OS** | Overall survival | **UC** | Ulcerative colitis |
| **Sab A** | Sialic acid-binding adhesin | **UBT** | Urease breath test |
| **SCCs** | Stem cell cancers | **VcA** | Vacuolating Cytotoxin A |
| **SNPs** | Single nucleotide polymorphisms | **VDR** | Vitamin D receptor |
| **S1PR2** | Sphingosine-1-phosphate receptor 2 | **VEGF** | Vascular endothelial growth factor |

## Abstract

**Background:** *Helicobacter pylori* is the primary cause of chronic gastritis and peptic ulcer disease and a significant risk factor for gastric adenocarcinoma. Numerous studies have reported the role of cytokines in the pathogenesis of gastric inflammation during *H. pylori* infection and disease outcomes are more likely with virulent strains that carry the Cytotoxin Associated Gene Pathogenicity Island (*cag* PAI). Interleukin-16 (IL-16) is a multifunctional cytokine implicated in various chronic inflammatory conditions, such as inflammatory bowel disease, asthma, and rheumatoid arthritis. Elevated IL-16 levels have previously been observed in the serum of gastric cancer patients compared to healthy controls. However, the connection between IL-16 production and *H. pylori* infection remained unclear. A comprehensive study profiling plasma cytokine levels across different stages of gastric cancer prognosis, including non-atrophic gastritis, atrophic gastritis, intestinal metaplasia, and gastric cancer could provide insights into the correlation between cytokines and disease progression and their potential as biomarkers for gastric cancer. Previous research demonstrated *H. pylori's* ability to induce IL-16 production in gastric epithelial cell lines. Since monocytes and dendritic cells are among the first cells to encounter *H. pylori* after it breaches the gastric epithelial barrier, the effect of *H. pylori* and its virulence factors on IL-16 production in these cells has not been explored.

**Aims;** A key objective of this thesis was to measure IL-16 concentrations in plasma samples from *H. pylori*-positive and negative patients and to investigate potential links between IL-16 and *H. pylori*-mediated gastro-duodenal disease. The study explored relationships between serum IL-16 and colonization by more virulent *cagA*-positive *H. pylori* strains, associations with gender and smoking status, the presence and severity of histopathological changes in the gastric mucosa, oesophageal diseases, and the impact of *H.*

*pylori* eradication therapy and other co-expressed inflammatory cytokines. Additionally, the thesis aimed to explore the effects of *H. pylori* infection and its virulence factors, and bile metabolites, on cytokine production by monocytes and dendritic cells. Another significant aim was to understand differential cytokine expression concerning gastric cancer progression and their potential as biomarkers for the disease.

**Materials and Methods**; Gastric biopsy and plasma samples were collected from *H. pylori*-infected and non-infected patients, including those who had received successful eradication therapy for the infection. *IL16* mRNA and plasma IL-16 levels were measured using RT-qPCR and a commercial ELISA kit respectively.

*cag* PAI-negative *H. pylori*, *cag* PAI-positive *H. pylori*, and isogenic virulence factor mutants co-cultured with human peripheral blood monocytes, monocyte-derived dendritic cells, and THP-1 and KG-1 cell lines for 24 hours. IL-6, IL-10 and IL-16 cytokines were measured by ELISA and flow cytometry.

320 plasma samples were collected from *H. pylori*-infected and uninfected patients with various gastric conditions, including non-atrophic gastritis, atrophic gastritis, intestinal metaplasia, and gastric cancer. Concentrations of cytokines and chemokines (including IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-16, IL-17, IL-18, IL-23, CCL2, CCL3, CXCL10, IFN-γ, and TNF-α) were measured using multiplex assays from Mesoscale Discovery (MSD).

**Results:** IL-16 was detected in all plasma samples, from both *H. pylori*-infected and uninfected patients, with wide variation in both groups, and there were no significant differences. IL-16 levels were not significantly associated with gender, age, or smoking status, nor did they differ based on whether the colonizing strain was *cagA*-positive or negative. No link was found between IL-16 concentration and histopathological changes in the gastric mucosa, or

oesophageal diseases, and there were no significant differences in plasma IL-16 levels before and after *H. pylori* eradication. The results showed that *H. pylori* virulence factors did not alter cytokine production by monocytes and dendritic cells. Interestingly, GC patients exhibited a significant reduction in IL-16 plasma levels compared to individuals with gastritis, peptic ulcer disease, and healthy controls. This outcome contradicts a previous study that reported elevated IL-16 serum concentrations in GC patients relative to healthy controls, likely due to different patient recruitment criteria. This finding's reliability was further substantiated by similar results from another ethnic group, a Mexican patient population, which showcased a reduction in both IL-16 and IL-18 plasma levels in GC patients compared to those with non-atrophic gastritis, atrophic gastritis, and intestinal metaplasia.

**Conclusion:** This comprehensive study analyzed various sample types, including plasma, gastric tissue, cell lines, and peripheral blood monocytes, utilizing techniques such as ELISA, MSD, RT-qPCR, and Flow Cytometry. Participants were categorized as healthy controls, *H. pylori*-infected and uninfected patients, individuals with different gastric conditions, and those from diverse ethnic backgrounds. The study considered multiple parameters potentially influencing plasma IL-16 levels, including age, gender, smoking status, *cagA s*tatus, and oesophageal disorders. The study concluded that *H. pylori* infection does not affect IL-16 cytokine levels.

A suggested correlation between IL-16 and IL-18 cytokines in the context of gastric cancer presents a novel research avenue. Both interleukins could be components of a larger immune regulatory network. Disruption in this network might lead to inadequate tumor control. Further research should be conducted to explore how IL-16 and IL-18 interact and influence each other in the context of cancer immunology. Investigating the combined roles of IL-16 and IL-18 can provide deeper insights into their contributions to immune response modulation.

# Chapter 1.A General Introduction

**A brief review of *Helicobacter pylori* and the cytokine interleukin-16.**

## 1.1 History

By the late nineteenth and early twentieth century, researchers had observed the occurrence of spiral bacteria in animal stomachs (Bizzozero and der Eidechsen, 1893). Similar bacteria were soon discovered in humans (Krienitz, 1906, Simpson, 2005), some of whom had peptic ulcer disease or gastric cancer. At the time, the role of these bacteria in the development of peptic ulcer disease and gastric cancer was considered, and patients were given high doses of the antibiotic chemical bismuth. This notion was later dismissed as irrelevant, most likely due to the enormous presence of these microorganisms in the stomachs of people who had no clinical symptoms. Until the early 1980s, bacteria seen in human stomachs were thought to be bacterial overgrowth or food contaminants (Marshall et al., 1987, Marshall and Warren, 1984, Warren and Marshall, 1983) .

In 1982, two Australian scientists, Barry Marshall, and Robin Warren, discovered the effective isolation and culture of a spiral bacterial species from the human stomach that would eventually become known as *H. pylori*. These bacteria can colonize the human stomach and cause inflammation of the gastric mucosa, as evidenced by self-ingestion tests by Marshall and Morris (Morris and Nicholson, 1987) and in later experiments with healthy volunteers (Morris et al., 1991). Due to their findings, Robin Warren and Barry Marshall were awarded the 2005 Nobel Prize in Physiology or Medicine for their "discovery of the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer disease."

## 1.2 Introduction

*Helicobacter pylori (H. pylori)* is a Gram-negative, microaerophilic, helical-shaped bacterial species, which is highly motile due to its unipolar bundle of sheathed flagella. This bacterium colonizes the stomachs of almost 50% of the world's population making it one of the most common bacterial pathogens. However, its prevalence varies based on geographic regions and sanitation standards. It is more commonly found in adults than in children, and the prevalence is higher in rural developing regions than in urban developed areas (Suerbaum and Michetti, 2002). The increased incidence in elderly people compared to children is explained by the fact that the majority (90%) of *H. pylori* infections are acquired in childhood and remain throughout life if left untreated rather than by a higher risk of infection at older age. Although the incidence of *H. pylori* infection in children has been decreasing due to changes in socioeconomic factors and sanitary conditions, including more frequent antibiotic use, the global prevalence in children has remained as high as 43.1% between 2011 and 2022 (Li et al., 2023). Poor socioeconomic status, overcrowded housing, having large numbers of young siblings, and poor sanitization, and hygiene conditions are risk factors for the acquisition of *H. pylori* infection (Ozbey and Hanafiah, 2017).

*H. pylori* bacteria are usually transmitted directly from one individual to another and require close contact. Person-to-person transmission includes the fecal-oral route (via aerosolized diarrhea), gastric-oral route (via aerosolized vomit or refluxed gastric juice) (Kayali *et al*., 2018), and direct oral-oral contact (Brown, 2000). However, the exact circumstances of *H. pylori* transmission are still largely unknown.

Around 80% of *H. pylori*-infected patients remain asymptomatic, but virtually all individuals with the infection develop gastritis. *H. pylori* is responsible for 90–95% of duodenal ulcers (DU) and 70–85% of gastric ulcers (GU) cases (Bakhti et al., 2019). It is thought to cause around 89% of non-cardia

gastric cancer (GC) cases (Morgan et al., 2022), the fifth most common cancer and the fourth leading cause of cancer mortality (Sung et al., 2021). Because of the association between *H. pylori* infection and the development of gastric malignancy, the World Health Organization classified *H. pylori* as a class I carcinogen (Noach et al., 1994).

The acidic pH of the gastric lumen is not a favorable environment for bacterial colonization (Schreiber et al., 2005). Additionally, the mucus layer that overlies that gastric mucosa acts as a protective barrier against invading bacteria to prevent bacterial infection (Hansson, 2012). However, *H. pylori* possesses various features that assist in successful gastric colonization and help the bacterium to avoid the gastric acidic environment and penetrate the thick, viscous mucosal layer (Scott et al., 2007, Carpenter et al., 2015). *H. pylori* produces substantial amounts of surface-associated and cytosolic urease enzyme, which hydrolyses the gastric urea to produce large amounts of ammonia and bicarbonate to buffer the pH around *H. pylori.* After entering the gastric lumen, *H. pylori* must quickly penetrate the gastric mucus layer to avoid being damaged and to establish the infection. Interestingly, the viscosity of gastric mucus is associated with gastric acidity. In general, in a highly acidic pH, the viscosity of mucus is higher than at low acidic pH. Thus, the enzymatic activity of *H. pylori* also assists the bacterial infiltration into the mucus layer (Krishna et al., 2016). Additionally, the helical shape of the bacterium facilitates its rapid movement and helps the bacterium to escape the low pH (Lee et al., 1993) (**Figure 1**).

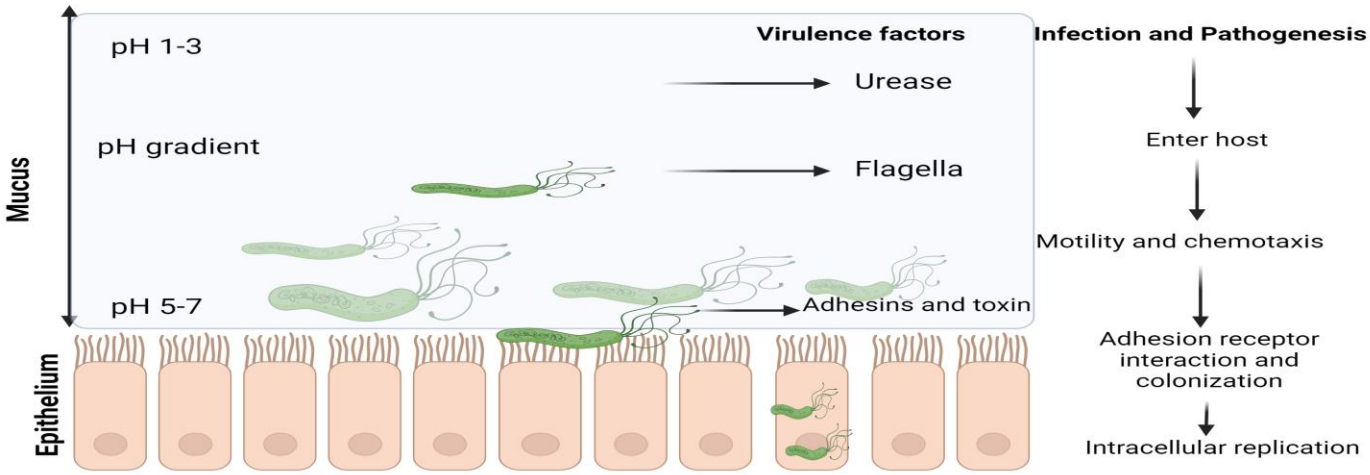



**Figure 1. Schematic diagram of *H. pylori* infection and pathogenesis**.

*H. pylori* uses its urease activity and flagella-mediated motility to survive and move towards the lower mucus gel above the epithelium. It then uses various adhesins, such as blood-antigen binding protein A (Bab A), sialic acid-binding adhesin (Sab A), and other outer membrane proteins, to interact with receptors on the host epithelial cells. Once it successfully colonizes, toxins like cytotoxin-associated gene A (Cag A) and vacuolating cytotoxin A (VacA) are responsible for damaging the host tissue and facilitating intracellular replication. Adopted from Kao CY, et al. (Kao et al., 2016).

## 1.3 *H. pylori* infection-related diseases

### 1.3.1 Peptic ulcer disease (PUD)

PUD is a commonly occurring disorder in the digestive system, typically affecting the stomach or duodenum. It is caused by damage from peptic acid, which creates a gap in the mucosa and reaches the subcutaneous layer. These ulcers are primarily found in the stomach and duodenum but can also occur in the oesophagus. In some cases, ulcers may develop in uncommon areas such as the ileum due to Meckel's diverticulum, the proximal oesophagus due to an inlet patch, or the jejunum due to excessive acid secretion in Zollinger-Ellison syndrome (Lanas and Chan, 2017). Complications of PUD can lead to serious outcomes including bleeding, perforation, penetration into nearby organs, and obstructions, which can result in death. According to a systematic review, the average mortality rate is 8.6% after bleeding and 23.5% 30 days after perforation (Lau et al., 2011).

Among the various risk factors for PUD, the most significant are infection with *H. pylori* and long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs). A meta-analysis has shown that the simultaneous presence of *H. pylori* infection and NSAID use greatly increases the risk of developing peptic ulcers, and bleeding peptic ulcers. Individuals with *H. pylori* infection who also use NSAIDs are 60 times more likely to develop peptic ulcers compared to those without infection or NSAID use, and the combination further increases the risk of ulcer bleeding by more than sixfold (McConaghy et al., 2023). Between 2009 and 2018, a cross-sectional study involving over 1 million patients who underwent an upper gastrointestinal (GI) tract endoscopy revealed that *H. pylori* infection was linked to one-fourth of duodenal ulcers and one-sixth of gastric ulcers (GU) (Sonnenberg et al., 2020). In 2019, Approximately 8.09 million cases of PUD were reported globally (Xie et al., 2022). In 2021, it is estimated that over 300,000 people in 36 Organisation for Economic Co-

operation and Development (OECD) member countries will experience hospitalizations due to PUD, making it a significant global public health concern. Encouragingly, there has been a global decline in the incidence of hospitalization and mortality rates for PUD since the beginning of the 21st century. The decrease in the incidence of PUD can be attributed to a combination of factors, including the shift towards ambulatory care, the widespread use of proton pump inhibitor drugs (PPIs), and the reduction in risk factors such as smoking, *H. pylori* prevalence, urbanization, sanitation, and access to clean water globally (Azhari et al., 2022).

In the presence of *H. pylori*, the development of an ulcer is prompted by different host and bacterial factors. Ulcers usually arise at sites where mucosal inflammation is more severe. In patients with low acid production, severe inflammation is often found in the gastric transitional zone between the corpus and antrum, increasing the risk of developing gastric ulcer disease. If the acid output increases, severe inflammation is found in the distal stomach and proximal duodenum, leading to juxta-pyloric and duodenal ulcer disease. Patients infected with more virulent *cagA*-positive *H. pylori* strains had an 18.4-fold and 2.9-fold greater lifetime risk of developing duodenal and gastric ulcers respectively (Schottker et al., 2012).

### 1.3.2 Mucosa-associated lymphoid tissue lymphoma (MALT)

*H. pylori* is associated with extra-nodal marginal zone gastric mucosa-associated lymphoid tissue (da Motta et al.) lymphoma, a type of non-Hodgkin's lymphoma that affects small, diverse B lymphocytes. This condition is responsible for 40 to 50% of primary gastric lymphomas and 1 to 6% of all gastric cancers (Isaacson and Du, 2005). While the stomach typically lacks organized lymphoid tissue, chronic inflammation caused by *H. pylori* infection can lead to the formation of this tissue. Experiments conducted *in vitro have d*emonstrated that heat-killed *H. pylori* can stimulate gastric MALT lymphoma

cells and activate *H. pylori*-specific T cells through CD40 and CD40L interactions (D'Elios et al., 1999, Hussell et al., 1996). Researchers have also observed that T cell clones from MALT lymphoma have reduced perforin-mediated cytotoxicity and Fas-mediated apoptosis (D'Elios et al., 1999). Most cases of gastric MALT lymphoma involve the presence of *H. pylori* in the gastric mucosa, and eradicating *H. pylori* through treatment typically results in complete remission of the lymphoma (Reyes, 2023, Ruskone-Fourmestraux et al., 2011, Zullo et al., 2010).

### 1.3.3 Gastric cancer (GC)

According to statistics, gastric cancer makes up 1.4% of newly diagnosed cancers in the US. It is estimated that around 26,500 individuals in the US will be diagnosed with gastric cancer in 2023, and 11,130 individuals will die from the disease (Siegel et al., 2023). *H. pylori* infection is the most important risk factor for the development of non-cardia gastric cancer (NCGC), and it is responsible for around 90% of stomach cancer cases (de Martel et al., 2020), the fifth most common cancer (Sung et al., 2021, 2023) and the fourth leading cause of cancer-related deaths (Sung et al., 2021). In 2018, 812,000 gastric cancers were recorded, including non-Hodgkin lymphoma of the gastrointestinal region, 37% of all cancers were caused by a chronic infection (de Martel et al., 2020). In 2020 GC was diagnosed in 1,089,103 people worldwide, and it claimed 768,793 lives (Ferlay et al., 2021), making *H. pylori* the most common carcinogenic pathogen. Individuals with *H. pylori* infection have a 1-5% lifetime risk of developing GC, depending on ethnicity and environmental variables. Certain populations are at a higher risk of GC after *H. pylori* infection, most likely due to genetic factors, housing conditions, and dietary habits, such as increased consumption of salted or pickled foods in East Asian populations. Additionally, indigenous populations globally and ethnic groups in the United States, including Asian Americans, have considerably greater rates of having GC. Dietary habits, socioeconomic, and lifestyle factors,

such as smoking and high salt intake, all contribute to the development of GC, but they are all dependent on the existence of *H. pylori* infection (Malfertheiner et al., 2023).

### 1.3.4 Extra-gastric diseases

*H. pylori* is a bacterium that is well known for causing gastric diseases, but it has also been associated with a range of non-gastrointestinal (GI) conditions, including type 2 diabetes (Li et al., 2017), insulin resistance (Upala et al., 2017), myocardial infarction (Liu et al., 2015), iron deficiency anaemia (Xu et al., 2017), primary immune thrombocytopenia (Satoh et al., 2009), and Parkinson's disease (Shen et al., 2017). However, the mechanisms behind these associations are still unclear. On the other hand, some studies have noted an inverse relationship between *H. pylori* and certain disorders, such as gastroesophageal reflux disease, esophageal cancer (Islami and Kamangar, 2008), childhood asthma (Chen and Blaser, 2008, Amedei et al., 2010), inflammatory bowel disease (IBD), and coeliac disease (Reyes, 2023). The inverse association between *H. pylori* and some of these conditions may be due to the bacterium's ability to induce a polarized type 1 T helper (Th1) response (Bamford et al., 1998, Karttunen et al., 1990). The cytokines produced by these Th1 cells may suppress the type 2 T helper (Th2) response, which reduces inflammation in the airways and prevents allergic disease. *H. pylori* infection may also promote the induction of regulatory T cells (Tregs) (Lundgren et al., 2003, Beswick et al., 2011), which may influence the prevention of allergic disease.

The bacterium has also been found to exert a protective effect against IBD. Initially, there was speculation that *H. pylori* infection could be a potential risk factor for IBD due to similarities in their immune response. However, several studies have suggested a contradictory relationship, showing a lower prevalence of IBD in individuals with *H. pylori* infection. There are important epidemiological differences between *H. pylori* infection and IBD. *H. pylori*

infection is more common in developing countries, whereas IBD is more prevalent in developed countries. Moreover, while the rates of IBD are increasing in developed countries, the rates of *H. pylori* infection are decreasing. In fact, individuals who are *H. pylori* seropositive tend to have a lower frequency of IBD compared to those who are seronegative (Sayar et al., 2019). A meta-analysis of 80,789 subjects (6130 patients with IBD and 74,659 non-IBD controls) further confirmed this negative correlation between IBD and *H. pylori* infection (Castano-Rodriguez et al., 2017). However, the prevalence of *H. pylori* infection is decreasing in developed countries, and rates of some of these conditions are increasing, suggesting that the relationship between *H. pylori* and non-GI disorders is complex and warrants further investigation (Reyes, 2023).

## 1.4 Factors influencing the risk of *H. pylori*-associated diseases.

The outcome of the infection is significantly influenced by the stimulation of the innate immune response in gastric epithelial cells (GECs) and immune cells by *H. pylori* effectors. However, only 1% to 3% of infected patients develop gastric adenocarcinoma, highlighting the fact that additional mechanisms, such as the genetic variables, environmental factors, and stomach microbiota induce and control mucosal innate immune response during *H. pylori* infection (Gobert and Wilson, 2022).

### 1.4.1 Genetic polymorphisms

Interactions of *H. pylori* with GECs results in the upregulation of pro-inflammatory chemokines and cytokines expression in the infected gastric mucosa, such as interleukin-8 (IL-8) (Crabtree et al., 1994), interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), interleukin-1 alpha (IL-1α), granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein-1 (MCP-1)(Jung et al., 1997), Macrophage migration inhibitory factor (MIF) (Beswick et al., 2006b) and transformation growth factor-

β (TGF-β) (Beswick et al., 2006a), IL-6, IL-12, chemokine ligand 2-5 (CCL2-5), CCL20, and chemokine (C-X-C motif) ligand 1-3 (CXCL1-3), (Peek et al., 1995, Cook et al., 2014), CXCL2 (Liu et al., 2020), CCL3 and C-X3-C motif chemokine ligand 1 (CX3CL1) (Sun et al., 2022). While this may not be an exhaustive list, it demonstrates the extensive range of cytokines produced by infected gastric epithelial cells as a well-established response to the infection (Alzahrani et al., 2014). Elevated inflammatory cytokines and chemokines levels in the infected gastric mucosa result in enhanced gastric mucosal inflammation, by binding these cytokines to their specific receptors on target T-cells. Polymorphisms in these cytokine genes and their promoter regions are fairly common, which have an impact on the expression levels of these factors, and in turn the host susceptibility to *H. pylori-related* diseases (Sugimoto et al., 2010). (***Figure 2)***

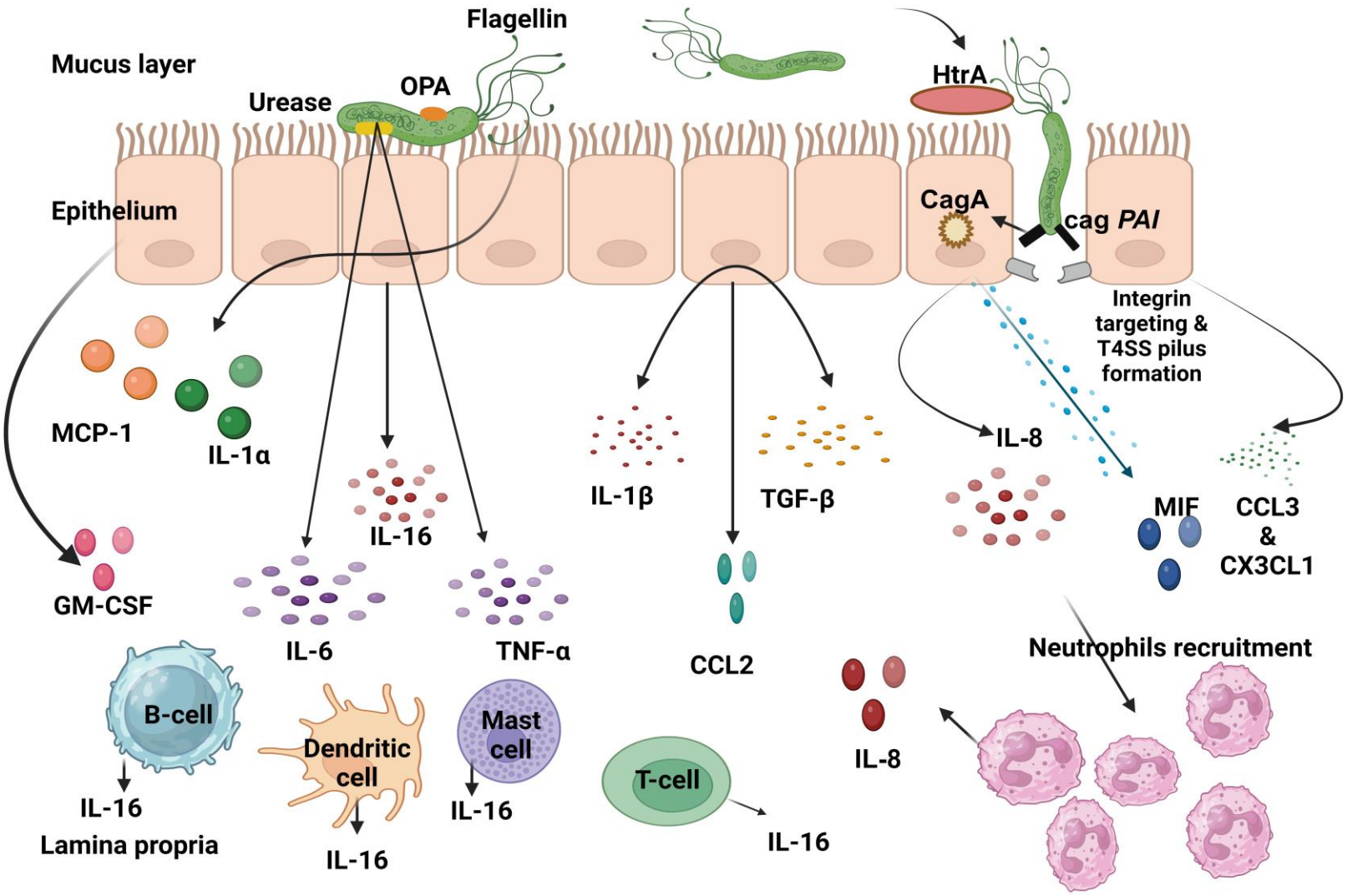

Flagellin
Mucus layer
Urease
OPA
HtrA
Epithelium
CagA
cag PAI
Integrin targeting & T4SS pilus formation
MCP-1
IL-1α
IL-16
IL-1β
TGF-β
IL-8
MIF
CCL3 & CX3CL1
GM-CSF
IL-6
TNF-α
CCL2
Neutrophils recruitment
B-cell
Dendritic cell
Mast cell
T-cell
IL-8
IL-16
IL-16
IL-16
IL-16
Lamina propria

**Figure 2** ***Cytokine production by gastric epithelial cells during H. pylori infection.***

*H. pylori* infection stimulates gastric epithelial cells (GECs) to produce proinflammatory cytokines. *H. pylori* secretes HtrA, a serine protease that breaks down epithelial junctional proteins, leading to the opening of cell junctions. This process is essential for *H. pylori's* paracellular transmigration. The T4SS pilus is activated at basolateral membranes and introduces the protein CagA. When CagA interacts with GECs, it activates nuclear factor (NF)-κB, causing changes in gene transcription and the secretion of interleukine-8 (IL-8) by GECs. This, in turn, leads to the recruitment of neutrophils. Additionally, *H. pylori* urease induces the production of IL-6 and tumor necrosis factor-alpha (TNF-α) by GECs. Other cytokines produced by GECs during *H. pylori* infection includes tumor necrosis factor-alpha (TNF-α), interleukine-1β (IL-1β), interleukin-1α (IL-1α), granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein-1 (MCP-1), migration inhibitory factor (MIF), tumor growth factor (TGF)-α, chemokine ligand 2 (CCL2), chemokine ligand 3 (CCL3), and C-X3-C motif chemokine ligand 1 (CX3CL1). OPA, outer protein attachment. This figure was Created with BioRender.com.

#### 1.4.1.1 Interleukin-1 beta (IL-1β)

IL-1β is encoded by the *IL1β* gene and is mainly produced by activated macrophages. It acts as an inflammatory mediator and involves cell proliferation, differentiation, and apoptosis. Besides its role as a potent inflammatory cytokine (Sugimoto and Yamaoka, 2009), it is also an effective inhibitor of stomach acid output. This cytokine is crucial for both the beginning and the intensification of the inflammatory response to *H. pylori* infection (Atherton, 1997, El-Omar et al., 2000).

*IL1β* T-31C and *IL1β* C3954T are two single nucleotide polymorphisms (SNPs) detected in the *IL1β* gene known to result in decreased IL-1β expression levels. Consequently, a decrease in gastric acid production provides a favorable environment for *H. pylori* to initiate infection in the stomach. Increased IL-1β production is linked to the polymorphic allele (*IL1β-511*T*), which has thymine (T) instead of cytosine (C) at position -511 in the regulatory region of the *IL1β* gene (Segal et al., 2001).

The *IL1RN* gene encodes the IL-1 receptor antagonist (IL-1ra), an anti-inflammatory cytokine that binds to IL-1 receptors competitively, moderating the potentially harmful effects of IL-1. The *IL1NR* gene contains a variable number of tandem repeat genes, which results in either a short (S) or long (L) allele. Increased IL-1β production is linked to the *IL1*R N * S allele. *H. pylori* infection in people having this allele may result in chronic inflammation, mucosal damage, and GC (Machado et al., 2001).

*IL1β 511* TT or TC and *IL1RN* SS are the most common genotypes among GC patients. In Portugal, Figueiredo *et al* reported that the combination of *IL1β511* TT or TC/*I L1RN* LL with IL*1RN* SS increases the host's odds ratio (OR) of intestinal gastric carcinoma by 3.8 and 6.5, respectively (Figueiredo et al., 2001). The combination of infection with an *H. pylori* strain expressing the *cagA* virulence factor and an *IL1β* 511 TT or TC allele in the human host is associated with an elevated risk of GC development. However, the highest risk

correlated with the development of GC is the combination of *H. pylori* expressing the VacA m1/s1 virulence factor with *IL1β* 511 TT or TC allele polymorphisms (Figueiredo et al., 2002). Previous studies from Poland and China have reported that patients in these populations are at very high risk of developing GC due to the combination of these polymorphisms and the most virulent *H. pylori* strains (*vacA* s1/m1 and *cagA* positive)(El-Omar et al., 2000). A study conducted in Pakistan by Raza et al. found that *IL1β* gene polymorphism at two locations (-511 and IRN) significantly increases the risk of gastric cancer in patients with *H. pylori* infection (Raza et al., 2017). Another recent study from Pakistan showed that *IL1β*-31 polymorphism is only expressed at higher levels in *H. pylori*-infected patients with gastric disease compared to *IL-1B*-511 polymorphism. Additionally, the *IL1B*-511 and *IL1B*-31 CT polymorphism, which were also observed in healthy individuals, may increase the risk of *H. pylori* infection (Kalsoom et al., 2020).

#### 1.4.1.2 Interleukin-8 (IL-8)

IL-8, a member of the CXC chemokine family, was originally known as a potent chemoattractant for neutrophils and lymphocytes. IL-8 is generated by gastric epithelial cells during *H. pylori* infection **(Figure 2**), specifically in the *cag*-pathogenicity-island-positive strain of *H. pylori*, one of the major virulence factors. Furthermore, IL-8 protein levels in gastric cancer are 10-fold higher than in normal gastric tissue, and they are strongly related to tumor vascularity. Increased IL-8 levels may intensify the inflammatory response to *H. pylori* by attracting neutrophils and monocytes, resulting in progressive gastritis and, eventually, predisposition to gastric cancer (Yamaoka et al., 2001)

The *CXCL8* gene encodes IL-8 and may have CXCL8-251T>A or CXCL8-845T>C polymorphisms in its promoter region. The A/A mutation in position -251, which results in enhanced CXCL8 promoter activity, is linked to higher stomach neutrophil infiltration, atrophy, intestinal metaplasia, and GC in *H.*

*pylori*-infected Japanese, Chinese, and Korean patients than the A/T or T/T genotypes (Ohyauchi et al., 2005). In European Caucasian patients, the identical A/A genotype is markedly elevated in patients with gastritis but is unrelated to GC (Szoke et al., 2008). Another South Korean study found that the T/A and A/A genotypes were associated with higher levels of IL-8 in the gastric mucosa of *H. pylori*-infected patients than the T/T genotype, and the A allele significantly increased the risk of severe atrophic gastritis and GC. The A/T genotype is associated with duodenal ulceration in *H. pylori*-infected patients from Eastern Europe and with GC in Mexican patients(Garza-Gonzalez et al., 2007). Interestingly, in a population of Chinese Veterans infected with *H. pylori*, the CXCL8-251T/T genotype was associated with higher CXCL8 transcriptional activity and a higher risk of GC(Lee et al., 2005). Together, these findings show that mutations in the *CXCL8* gene promoter region are linked to higher levels of IL-8 production, inflammation, and the probability of developing neoplastic transformation.

#### 1.4.1.3 Interleukin-10 (IL-10)

IL-10 is an anti-inflammatory cytokine that suppresses cell-mediated immune responses and cytotoxic inflammatory responses. IL-10 is upregulated during *H. pylori* infection to inhibit the immune response and allow the infection to persist (Assis et al., 2014). Low-level IL-10 expression is associated with increased severity of gastric inflammation and an increased risk of gastric cancer in *H. pylori*-positive patients (Akdogan et al., 2014, Wroblewski et al., 2010b)

The *IL10* gene has been found to include three single nucleotide polymorphisms that decrease the production of IL-10 at positions 1082 (G>A), 819 (C>T), and 592 (C>A) in the promoter region(Gobert and Wilson, 2022). El-Omar et al. discovered that patients who are Caucasian and have the low *IL10*-producer -1082A/ 819T/592A haplotype had a considerably higher risk of

developing GC (El-Omar et al., 2003). Different outcomes have been seen for other populations: In Taiwanese (Wu et al., 2003) and Japanese patients (Sugimoto et al., 2007), the high *IL10*-producer haplotype 1082G/ 819C/592C is more prevalent in GC than the low *IL10* haplotype (ATA). Comparably, in China, individuals with *H. pylori* infection and IL10-1082G carriers exhibit an enhanced risk of GC (Lu et al., 2005b). The *IL10*-592C/C and *IL10*-1082G/G genotypes raise the risk of IM, dysplasia, and GC in Mexico and Venezuela, respectively (Sicinschi et al., 2006, Kato et al., 2006). The discovery that Caucasians and East Asians had significantly different allele frequencies for the *IL10*-1082 polymorphism can be used to explain the differences between American and Asian patients (Won et al., 2010). However, *IL10* polymorphisms affect the degree of illness and inflammation triggered by *H. pylori*.

In general, patients with high producer alleles of pro-inflammatory cytokines, and low producer alleles of anti-inflammatory cytokines have more severe gastric mucosal inflammation caused by *H. pylori* infection, which increases the risk of developing gastric cancer and gastric ulcer. In contrast, modest stomach mucosal inflammation is seen in low-producer allele carriers of pro-inflammatory cytokines and high-producer allele carriers of anti-inflammatory cytokines (**Figure *3***) (Sugimoto et al., 2010) .

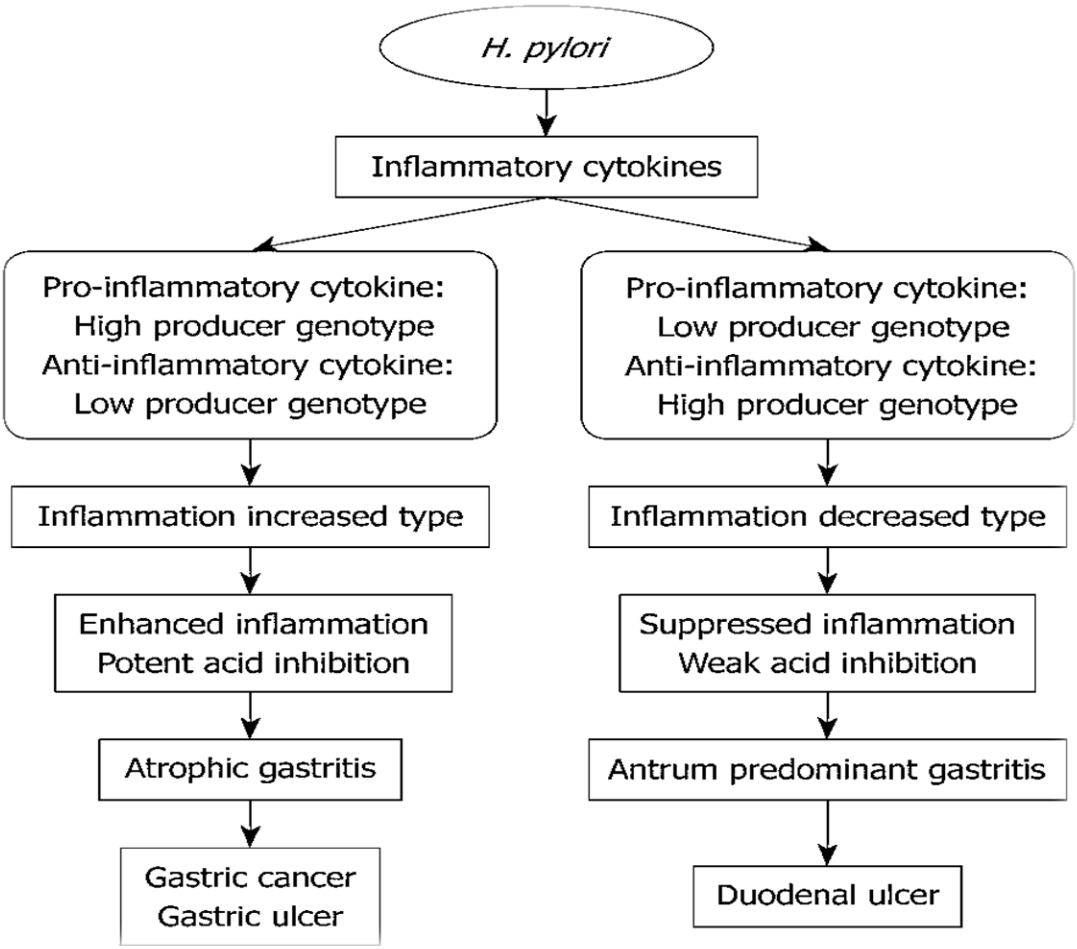



**Figure 3. Scheme of the association of inflammatory cytokine polymorphisms and gastroduodenal disease development.**

(Sugimoto et al., 2010).

### 1.4.2 *H. pylori* and the gastric microbiota

Before the discovery of *H. pylori,* the stomach was thought to be free of microbes because of its harsh and acidic environment (Yang et al., 2013). Then, based on culture-dependent techniques, *H. pylori* was long assumed to be the only microorganism capable of replicating in the harsh gastric environment (Stockbruegger, 1985). However, according to culture-independent approaches such as next-generation sequencing (NGS) techniques, fluorescent in situ hybridization (FISH), and dot-blot hybridization

analysis of the gastric microbiome, the bacteria in the human stomach are denser and more diverse than previously thought (Li et al., 2009). The gastric microbiota consists of several microorganisms, which have been found in the human body since birth. *Proteobacteria* and *Firmicutes* are the most abundant phyla in the gastric microbiota, followed by *Bacteroides*, *Actinobacteria*, and *Fusobacteria* (Cao and Yu, 2015, Rinninella et al., 2019). These five major phyla are found in both *H. pylori*-infected and uninfected individuals. However, the composition of the gastric microbiota differs between those with and without *H. pylori* infection and people with *H. pylori* infection had a larger prevalence of several genera (Liu et al., 2018). Thus, the role of *H. pylori* in gastric disorders may be mediated by the local gastric microbiota. In *H. pylori*-positive patients with antral gastritis, the abundance of the phylum *Proteobacteria* was decreased in the gastric mucosa and *Firmicutes* was increased in comparison to *H. pylori*-negative patients. In atrophic gastritis patients, the abundance of *Prevotella spp* decreased whereas *Streptococcus spp.* increased compared to healthy controls. Additionally, patients with chronic gastritis displayed a higher rate of bacterial growth than people without gastritis **(*Figure 4*).**

*H. pylori* infection, and host genetic and environmental variables are all risk factors for gastric cancer. Although *H. pylori* infection is a well-studied risk factor for gastric adenocarcinoma, cancer risks vary substantially between populations with similar *H. pylori* frequency. One of the possible reasons is the interplay of different *H. pylori* strains and the composition of the microbiota in the stomach. A study found that two populations in the same nation with differing risks of GC had varied compositions of the stomach microbiome. They discovered that individuals with high stomach cancer risks (Túquerres town) had greatly abundant operational taxonomic units (OTUs) assigned to *Leptotrichia wadei* and the species *Veillonella*. OTUs assigned to *Staphylococcus* and *Neisseria flavescens* were abundant in those with a lower chance of developing gastric cancer (Tumaco town)(Yang et al., 2016). **Table 1**

**Table 1. Summarizes key findings on the gastric microbiota, its interaction with *H. pylori*, and its potential role in gastric cancer risk.**

| | |
|---|---|
| Presence in *H. pylori*+/- individuals | The five major phyla are present in both *H. pylori*-infected and uninfected individuals, but their composition differs. |
| Gastric cancer and microbiome | In high-risk populations for GC (e.g., Túquerres town), the microbiome showed an increased abundance of *Leptotrichia wadei* and *Veillonella.* Low-risk populations (e.g., Tumaco town) showed higher *Staphylococcus* and *Neisseria flavescens levels.* |

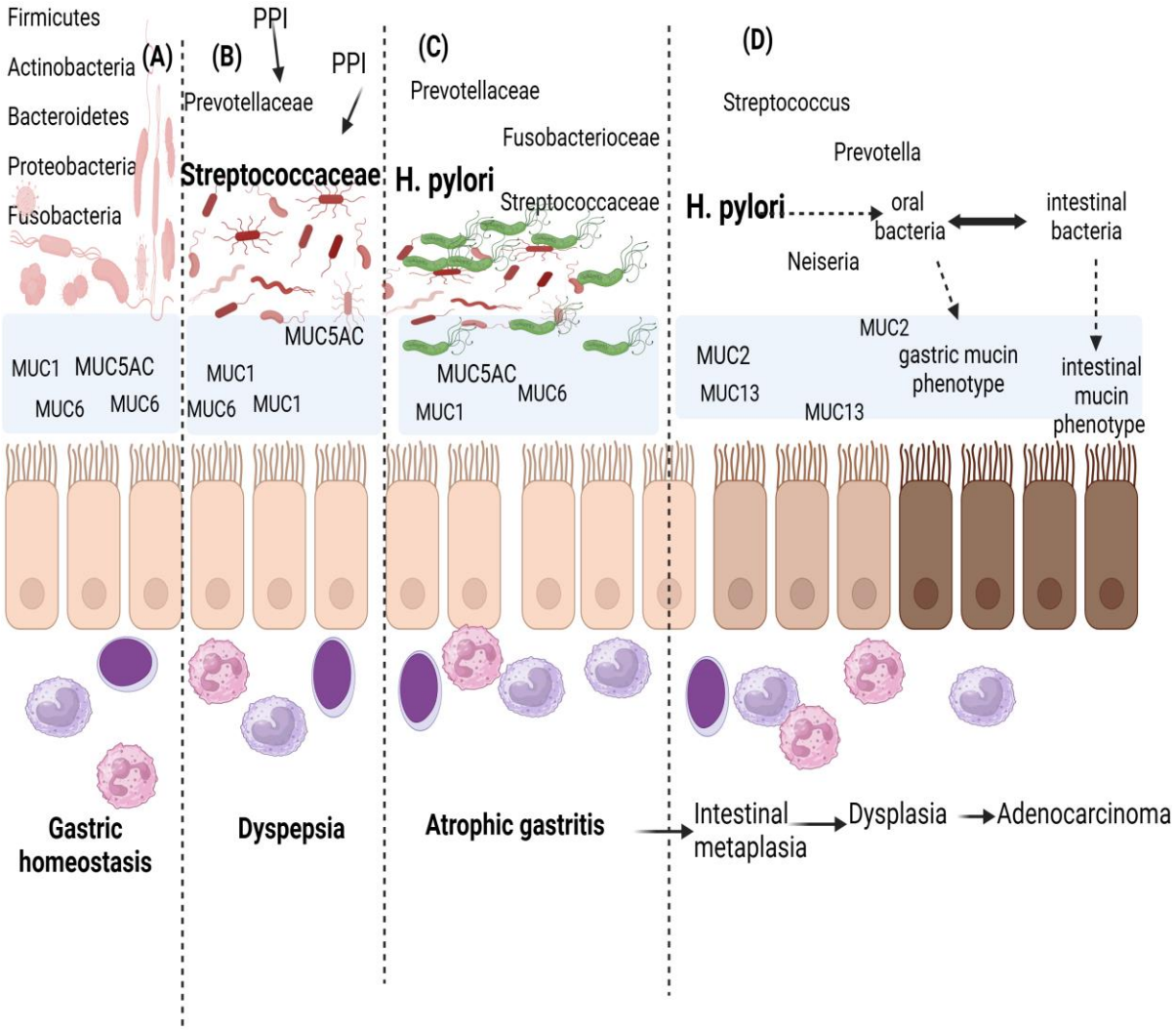



**Figure 4. Schematic model representation of the gastric microbial and mucosal composition in healthy and diseased stomachs.**

In a healthy stomach, the gastric mucosa consists of a thick mucus layer, a single layer of epithelial cells, and the inner lamina propria containing innate and adaptive immune cells. MUC1, MUC5AC, and MUC6 mucins serve as a protective barrier and specialized niche for the gastric microbiota, *with Helicobacter pylori* as a core species in low abundance. In dyspeptic patients treated with PPIs, bacterial profiles are similar to those in healthy untreated controls, but with differences in ranks regarding abundant bacterial families. In *H. pylori*-positive patients with chronic (atrophic) gastritis, the *Helicobacter* genus dominates the gastric mucosa, resulting in decreased bacterial

diversity. Other families, such as *Streptococcaceae, Fusobacteriaceae*, and *Prevotellaceae,* are also present but to a lesser extent. As the pathway of carcinogenesis advances towards intestinal metaplasia, the microbiota in this pre-neoplastic lesion can be seen as an intermediate group towards gastric cancer, with a decrease or depletion of *H. pylori*, except in cases of incomplete (type II/III) metaplasia where *H pylori* can still be found in cells expressing gastric mucins. In gastric cancer, oral or intestinal-type bacteria can be found. This difference in abundancy might be assigned to the mucin phenotype (gastric or intestinal) present in the tumor and thus to niche-specific interactions of the gastric cancer microbiome. Adapted from Rajilic *et al*. (Rajilic-Stojanovic et al., 2020)

### 1.4.3 *H. pylori* virulence factors:

#### 1.4.3.1 The *cag* pathogenicity island (*cag* PAI)

Besides the role of host factors and environmental conditions of the stomach, *H. pylori* virulence factors also contribute to the development of more severe clinical outcomes. Several bacterial virulence factors are involved in the inflammatory response towards *H. pylori.* Of those, the *cag* pathogenicity island (*cag* PAI), *cagA*, and *vacA* are the best studied. The *cag PAI* is a 40 kb genomic island, composed of approximately 32 genes (depending on the strain) that encode a bacterial type IV secretion system (T4SS). The *cag PAI* is found in approximately 60–70% of Western *H. pylori* strains and 100% of East-Asian *H. pylori* strains (Tomb et al., 1997, Akopyants et al., 1998, Censini et al., 1996). Even though all *H. pylori* strains induce gastritis, strains that have the *cag* PAI (assigned as *cag*+) enhance the risk for severe gastritis, dysplasia, and gastric cancer in comparison to strains that lack the *cag PAI* (*cag* −)(Blaser et al., 1995). *H. pylori cag*− strains are more likely to be free-swimming in the gastric mucus layer, while *cag*+ strains are found near to or attached to gastric epithelial cells, indicating that *cag* genotype has an impact on the area of colonization within the stomach (Camorlinga-Ponce et al., 2004). The *cag*-T4SS translocates substrates, such as peptidoglycan peptides, and effector molecules, and the cytotoxin CagA, from the bacteria into the host epithelial

cell. Translocated CagA then interacts with different host proteins, leading to the activation of host signaling pathways including activation of nuclear factor kB (NF-kB), expression of IL-8 chemokine, and recruitment of inflammatory cells to the site of the infection (Shibata et al., 2005).

The degree of *cagA* expression, the amount of CagA translocation into host cells, and the biological activity of CagA can be used to further stratify the risk of gastric cancer in *H. pylori* infectors that are *cagA*-positive. *cagA* expression level is greater with the presence of the genetic AATAAGATA motif upstream of the translation-starting site, which was linked to a higher risk of advanced gastric precancerous lesion. The amount of CagA translocation is greater in strains containing an amino acid sequence polymorphism (Y58E59) in the CagL of T4SS, which improves its binding affinity with integrin receptor $\alpha5\beta1$ on the gastric epithelial cell. As a result, gastric cancer risk was enhanced by 4.6-fold in patients infected by the *cagL*-Y58E59 strain compared with those infected by the non-Y58E59 strain (Chang et al., 2018).

#### 1.4.3.2 Peptidoglycan (PGN)

In addition to CagA, *H. pylori* peptidoglycan (PGN) peptides are translocated into gastric epithelial cells through the *cag*-T4SS secretion system and outer membrane vesicles (OMV)(Kaparakis et al., 2010). Inside the host cell, the PGNs are sensed by the nucleotide-binding oligomerization domain 1 (NOD1), an intracellular pathogen pattern-recognition molecule(Boughan et al., 2006, Viala et al., 2004), which causes the activation of different signaling molecules, such as NF-kB, p38, and Erk, and the production of proinflammatory chemokines, antimicrobial peptides, and cytokines, e.g. MIP-2, β-defensin, and IL-8 (Allison et al., 2009).

#### 1.4.3.3 Vacuolating Cytotoxin A (VacA)

Another major protein secreted by *H. pylori* is the pore-forming cytotoxin, VacA (McClain et al., 2003). *vacA* is a 140 kDa pro-toxin that has an N-terminal signal peptide, a central region that forms the toxin, and a C-

terminal domain for transportation function. After processing, the central region (~88 kDa), also known as the mature virulent form of the toxin, is released via the type V autotransporter secretion pathway (Cover and Blanke, 2005) and further processed into two different subunits of 33 (A subunit) and 55 kDa (B subunit), respectively. It has been thought that the p33 form is responsible for pore formation, while the p55 form acts as the cell binding component (Reyrat et al., 1999, McClain et al., 2003). However, recently both subunits have been suggested to be involved in binding and vacuole formation (Torres et al., 2005, Gonzalez-Rivera et al., 2010). The exact mechanism of cell entry is still ambiguous since several receptors have been proposed, but binding to sphingomyelin seems to be aid in the process(Gupta et al., 2008). VacA binds to the target host T-cells and is internalized leading to the formation of large vesicle 'vacuolation'. In addition to inducing cell vacuole formation, VacA causes a variety of cell functional alterations such as permeabilization of the plasma membrane, autophagy, cell death, inhibition of T cell activation and proliferation, and disruption of lysosomal and endosomal trafficking(Sharma et al., 1998, Cover and Blanke, 2005, Cover et al., 1992). This toxin has also multiple effects on different immune cells such as dendritic cells, B cells, platelets, and macrophages **(Figure 5).** Although all identified *H. pylori* strains possess the *vacA* gene, there is a remarkable sequence diversity in *vacA* genes across *H. pylori* isolate strains (Atherton et al., 1995, Gerhard et al., 1999, Rhead et al., 2007), which is identified in three regions: the signal sequence region (*s*-region), mid-region (*m*-region) and intermediate-region (*I*-region). *s1* or *s2* and *m1* or *m2*, are the two types of allelic variations in the *s*-region and *m*-region respectively (Atherton et al., 1995). The *s2* type encodes a VacA protein with an additional N-terminal amino acid segment, while the *s1* type encodes a VacA protein only. The presence of this additional segment suppresses the *s2-type* toxin from vacuole formation (McClain et al., 2001). Numerous studies have reported that *H. pylori* with *s1/m1* and *s1/m2 vacA* cause more severe chronic inflammation when

compared to the other genotypes. Infection with *H. pylori* strains containing the *s1/m1* allele combination is also correlated with increased risk of gastric malignancy (Atherton et al., 1995, Gerhard et al., 1999, Miehlke et al., 2000, Miehlke et al., 2001). The *i1* allele is strongly associated with the production of CagA and the presence of the *s1* type allele. Thus, it has been suggested that the intermediate region is involved in the development of the severe outcomes of chronic *H. pylori* infection (Chung et al., 2010)

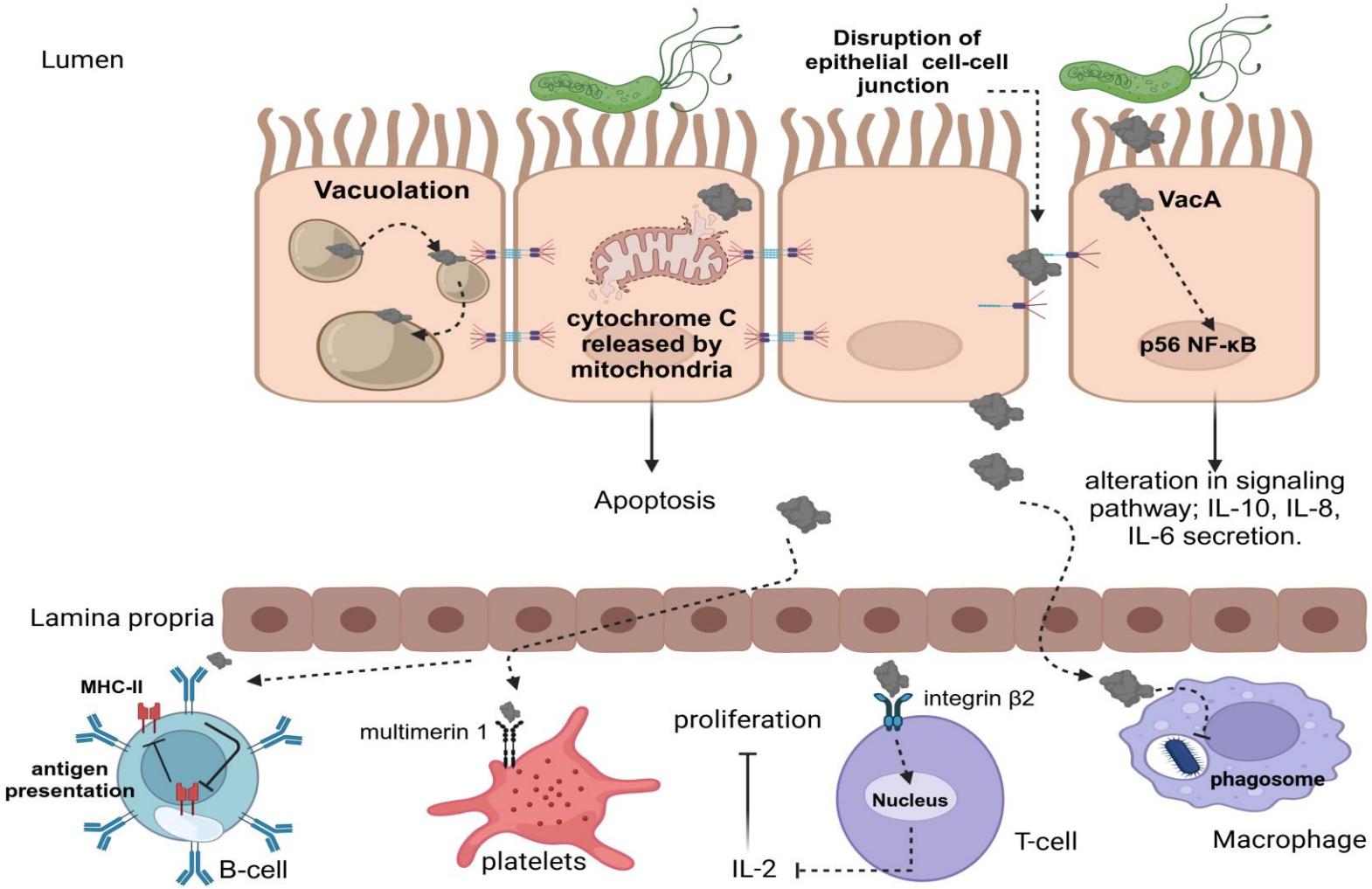

Lumen
Disruption of epithelial cell-cell junction
Vacuolation
cytochrome C released by mitochondria
VacA
p56 NF-κB
Apoptosis
alteration in signaling pathway; IL-10, IL-8, IL-6 secretion.
Lamina propria
MHC-II
antigen presentation
B-cell
multimerin 1
platelets
proliferation
IL-2
integrin β2
Nucleus
T-cell
phagosome
Macrophage

**Figure 5. VacA, a multi-functional toxin.**

The VacA toxin produced by *H. pylori* has various effects on different types of cells. It binds to receptors on the surface of epithelial cells and enters the cells. Once inside, VacA has multiple functions, including inducing cytokine secretion by gastric epithelial cells, causing the cells to form vacuoles, releasing cytochrome c from mitochondria which leads to cell apoptosis, and altering cell signaling pathways. *H. pylori* also disrupts the junctions between cells, allowing VacA to cross the epithelial cells. VacA interferes with antigen presentation in B-cells by affecting MHC class II. In T-cells, VacA binds to the surface receptor, preventing nuclear translocation of the nuclear factor of activated T-cells and downregulating *IL2* gene transcription, thus inhibiting T-cell proliferation. VacA also interacts with Multimerin 1 on human platelets, resulting in platelet activation. Additionally, VacA interacts with macrophages and inhibits their maturation when present in their phagosome. This figure was created with BioRender.com.

#### 1.4.3.4 Urease

Urease is another potent virulence factor and probably the most abundant protein expressed by bacteria. It represents 10% of the total protein by weight (Bauerfeind et al., 1997). Urease assists in gastric colonization by neutralizing gastric acid and providing ammonia for bacterial protein synthesis. This process facilitates bacterial movement through the mucus layer overlay of the epithelial cells by decreasing the viscosity of the mucus layer and increasing the pH (Celli et al., 2009). Besides its major function in gastric acid neutralization, it is also thought to be involved in the progression of gastric cancer via the induction of angiogenesis. This process is essential for tumor growth and metastatic dissemination (De Palma et al., 2017, Macedo et al., 2017). Accordingly, greater urease activity might be correlated with a higher risk of induction of histopathological changes within the gastric mucosa and further gastric carcinogenesis. It is thought that suppressing urease activity could be a useful therapeutic approach for avoiding illnesses caused by *H. pylori* (Baj et al., 2020).

### 1.4.4 Environmental factors

In addition to genetic variations and *H. pylori* virulence factors, environmental factors such as diet and smoking, are also involved in the risk of *H. pylori*-associated illnesses. According to a number of epidemiological studies, a diet high in fruits, vegetables, and dietary antioxidants is linked to a lower risk of stomach cancer (Setiawan et al., 2001, Lunet et al., 2005). On the other hand, a diet high in red meat or salty foods is directly linked to an elevated risk of stomach cancer (Joossens et al., 1996, Gonzalez and Riboli, 2006). Furthermore, compared to people with adequate blood ferritin levels, patients with low serum levels of the iron-binding protein, ferritin, have experienced more severe *H. pylori* infection outcomes (Akiba et al., 1991)

In people who are seropositive for *H. pylori*, current smoking is the most important behavioral risk factor for stomach cancer (Butt et al., 2019). Furthermore, smokers are more likely than non-smokers to develop stomach malignancies, including in the cardia and non-cardia (Ladeiras-Lopes et al., 2008). Smoking may also decrease the effectiveness of antibiotics, increasing the chance that eradication therapy may fail (Suzuki et al., 2006).

## 1.5 *Helicobacter pylori* adhesion to gastric epithelial cells

*H. pylori* found within the gastric mucus layer either swim freely (Hazell et al., 1986) or attach to gastric epithelial cells (Hessey et al., 1990). To achieve successful colonization, *H. pylori* carries several outer membrane proteins (Reyes Velez et al.) which allows tight adherence of the bacteria to gastric epithelial cells (Hessey et al., 1990). Furthermore, the attachment of *H. pylori* to the gastric epithelium serves as a shield against clearance mechanisms like liquid flow, peristaltic movement, and mucus shedding. This attachment not only provides a source of nutrients for the bacteria from damaged epithelial cells but also facilitates the delivery of bacterial toxins and other virulence factors to the host cells, which ultimately contributes to the establishment of a persistent infection (Matos et al., 2021).

### 1.5.1 Blood group antigen-binding adhesin (Bab A)

BabA is the first outer membrane protein discovered and another significant virulence factor. BabA adheres to the Lewis$^{b}$ antigens (Le$^{b}$), a blood group antigen that is expressed on the gastric epithelial cells and cell-surface mucins. This closer attachment may lead to DNA damage, via inducing DNA double-strand breaks (DSBs) in the nuclear DNA and triggering a DNA-damage response (DDR) in infected cells, translocation of CagA into the GECs through syringe-like structure (T4SS) and subsequently release pro-inflammatory cytokines by GECs (Toller et al., 2011, Ishijima et al., 2011)

(**Figure 6A**).

According to reports, the interaction between the BabA adhesin and its ligands is sensitive to pH. The acidic conditions in the stomach are important for *H. pylori* to adhere and bind strongly to ABO glycan antigens, enabling its survival in this specific environment. However, under low pH, this binding is inhibited, causing the bacteria to be released and moved to a more neutral microenvironment. This allows the bacteria to escape physiological processes like the shedding of epithelial cells and mucus turnover (Bugaytsova et al., 2017).

### 1.5.2 Sialic acid-binding adhesin (SabA)

In the early stages of infection, binding of BabA to $Le^b$/ABO is important, however, in the ongoing infection phase, expression sialyl-$Lewis^x$ antigen ($sLe^x$), a glycosphingolipid expresses on GECs and acts as *H. pylori* receptor, is also increased. *H. pylori* uses SabA, an outer membrane protein, to bind to sialyl-$Lewis^X$. Therefore, it was suggested that *H. pylori* SabA is essential for the adherence of the bacteria to the inflamed gastric mucosa due to the higher expression level of sialylated glycans on gastric mucosa (Mahdavi et al., 2002) **(Figure 6B).** The association of SabA in the development of gastroduodenal diseases has been investigated in different epidemiologic studies, and the association of SabA with atrophic gastritis, intestinal metaplasia, and gastric cancer has been reported (Yamaoka et al., 2006).

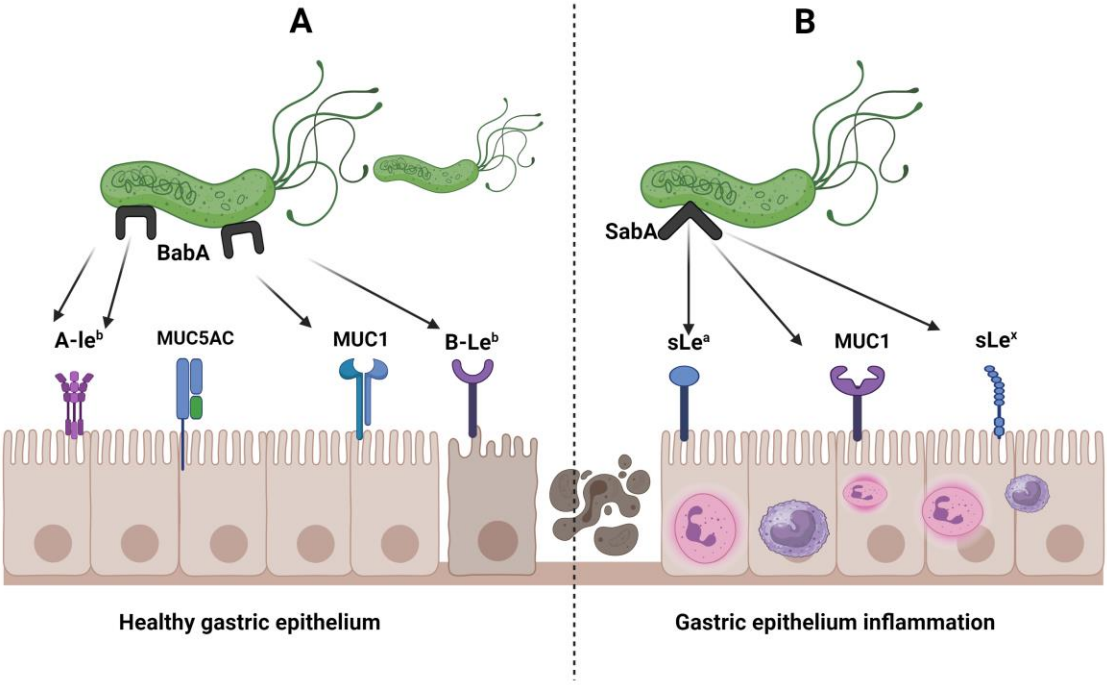



**Figure 6. Schematic of *H. pylori's* anchoring facilitated by BabA and SabA**.

*(*A) BabA can bind to different antigens, including A/B-Le$^b$, MUC5AC, and MUC1 in a healthy gastric epithelium cell. This interaction is crucial for the initial adherence process on the gastric epithelial cells. (B) In inflamed gastric epithelium cells, sLe$^x$ and sLe$^a$ antigens are upregulated. As a result, SabA may have a more significant role in anchoring to epithelium cells during an ongoing inflammation process. Adapted from Doohan, D. *et al* (Doohan et al., 2021).

### 1.5.3 Outer inflammatory protein A (OipA)

OipA, also known as *Helicobacter* outer membrane protein H (HopH) and primarily found in *cagA*+ strains, is a member of the Hop family of proteins. In both human and mouse models, functional OipA is linked to higher levels of *H. pylori* colonization, neutrophil infiltration, and mucosal IL-8 responses. Interestingly, after a 4-hour infection or with a high multiplicity of infection (1000), CagA translocation and cytokine induction in AGS cells is not affected by *oipA* deletion. This may indicate that OipA is involved, but not essential, in

inducing the innate response of GECs in the later stages of infection (Gobert and Wilson, 2022).

## 1.6 Innate immune response to *H. pylori*

The immune response against *H. pylori* starts with the recognition of the pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) located on gastric epithelial cells and innate immune cells and subsequently leads to the initiation of the adaptive immune responses. However, this bacterium applies multiple evasion strategies to escape the host immune response to survive in the stomach and sustain the infection (Mogensen, 2009). PRRs include the toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), which recognize PAMPs such as lipopolysaccharide (LPS), flagellins, unmethylated CpG DNA, and cell wall peptides (Takeda and Akira, 2004). Some *H. pylori* PAMPs are modified to evade recognition by a wide variety of PRRs that are important for detection of other Gram-negative bacteria (Peek et al., 2010). For example, the activity of the tetra-acetylated *H. pylori* LPS is 1000 times lower than the Hexa-acylated LPS of *Escherichia coli* (Stead et al., 2008). In addition, a low negative charge on LPS that results from the deletion of phosphate groups from two positions of lipid A in LPS contributes to its poor recognition by TLRs (Cullen et al., 2011).

### 1.6.1 Flagellin

Flagellin is the protein component of bacterial flagella required for motility and colonization. *H. pylori* employs five or six polar flagella made of two separate subunits, FlaA and FlaB, to facilitate movement within the gastric mucus (Gewirtz et al., 2004). Flagellin is also the ligand for TLR5 expressed by GECs. *H. pylori* can escape recognition by TLR5, by encoding different amino acids than that of other bacteria in the TLR5 recognition site and having

a compensatory mutation that maintains bacterial motility. Alteration of the N-N-terminal recognition domain of flagellin reduces the affinity of binding to TLR5 and inhibits activation of the innate immune response (Gewirtz et al., 2004). TLR9 is a receptor for unmethylated CpG motif present in bacteria and viruses. *H. pylori* DNA shows a high rate of methylation. Thus, it can avoid the detection of its DNA by TLR9(Suarez et al., 2006). A member of the human nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR) family (NOD1) is expressed by antigen-presenting cells (APCs) and GECs, and this recognizes small molecules derived from PGN, a layer outside the plasma membrane of the bacterial cell wall. Unlike TLRs which are associated with the plasma membrane or endosomal vesicles, NOD1 is expressed in the cytosol of innate cells (Strober et al., 2006). NOD1 signaling is also involved in the development of human gastric inflammation as GECs isolated from *H. pylori*-associated gastritis patients express a high level of NOD1 as compared with healthy controls or *H. pylori* non-associated gastritis (Rosenstiel et al., 2006). Soluble PGN components are delivered into the host GECs via the *cag* type IV secretion system (Viala et al., 2004). Also, they can enter the cells in a *cag*PAI-independent manner, through bacterial outer membrane vesicles (OMVs) released from *H. pylori* and activate NOD1 in GECs (Minaga et al., 2018).

### 1.6.2 Neutrophils and Macrophages

Neutrophils are the first leukocytes that migrate to the inflammatory site in early inflammation, to clear pathogens by phagocytosis, degradation, and extracellular traps. Migration of neutrophils or polymorphonuclear leukocytes (PMNs) into infected gastric mucosa is also a characteristic feature of *H. pylori* infection. *H. pylori-infected* patients have a lower peripheral blood neutrophil/lymphocyte ratio than *H. pylori*-negative patients (Guclu and Faruq Agan, 2017). *H. pylori* plays a key role in promoting the recruitment of neutrophils by the secretion of the virulence factor *H. pylori* neutrophil

activating protein (HP-NAP), which acts as a chemotactic factor (Evans et al., 1995, Wen et al., 2021). Furthermore, following *H. pylori* infection, GECs express chemokines to attract neutrophils which immediately produce IL-8, IL-1β, and TNF-α cytokines. Additionally, to enhance neutrophil infiltration, *H. pylori* induces the expression of hepatoma-derived growth factor, which is a mediator in gastric carcinogenesis (Chu et al., 2019). This extensive activation and recruitment of neutrophils to the gastric mucosa during *H. pylori* infection causes severe inflammation and mucosal damage (Toyoshima et al., 2018). However, *H. pylori* can resist phagocytosis and the subsequent oxidative burst to survive. *H. pylori* avoids opsonization in different ways. The low gastric pH and mucins prevent antibody binding to the bacterial surface (Berstad et al., 1997). Urease production by *H. pylori* inhibits the deposition of complement component C3 (Rokita et al., 1998). Furthermore, *H. pylori* infection causes upregulation of decay-accelerating factor and CD59, both of which prevent complement-mediated opsonization (Sasaki et al., 1998). *H. pylori* can also avoid phagocytosis by several mechanisms. The *cag* type IV secretion system plays a key role in inhibiting phagocytosis (Ramarao et al., 2000). Moreover, *H. pylori* engages a novel phagocytic pathway in macrophages that delays phagocytosis and is regulated by protein kinase C-zeta (PKC)-ζ, which is different from conventional phagocytosis of pathogens that is dependent on PKC-α and PKC-δ (Allen and Allgood, 2002). *H. pylori* which lacks the *cag* PAI and expresses the s2/m2 form of VacA displays enhanced survival in macrophages in comparison with virulent strain types. *H. pylori cag PAI*$^{+}$ and *vacA*$^{+}$ strains were shown to reside in compartments with early endosome properties and did not fuse with lysosomes (Zheng and Jones, 2003). This emphasizes the importance of *H. pylori* virulence factors in preventing phagocytic killing of *H. pylori* (Zheng and Jones, 2003). However, other studies proposed this to occur independent of the bacterial *cagA* and *vacA* status. (Baldari et al., 2005, Rittig et al., 2003). Finally, lipid components of the *H. pylori* outer membrane impact bacterial engulfment by phagocytes.

Glucosylation of cholesterol in the outer bacterial membrane enhances the ability of *H. pylori* to evade phagocytosis, while an excess of cholesterol leads to augmented phagocytosis (Wunder et al., 2006).

## Adaptive immune response to *H. pylori*

Adaptive immune responses against *H. pylori* start after failure of innate immune response to clear the bacteria. Both the innate and the adaptive immune responses cause damaging gastric inflammatory responses and subsequently enable the persistence of the infection (Ihan et al., 2012).

### 1.7.1 Humoral and B cell immune response

The immune system is activated in response to the invasion of *H. pylori* into the stomach, leading to an inflammatory reaction. This reaction persists until *H. pylori* is eliminated. Various cells and mediators, including IL-8, IL-1$\beta$, TNF$\alpha$, IL-6, and IL-12, are involved in mediating this inflammatory reaction. When the gastric epithelium's PRR detects *H. pylori*, these mediators are secreted. They attract immune cells such as neutrophils, macrophages, dendritic cells, natural killer cells, and lymphocytes to the infection site. The dendritic cells then activate $CD4^+$ T cells, also known as T helper cells, which in turn stimulate B-cells to differentiate into plasma cells. Plasma cells secreted three different isotypes of antibodies: immunoglobulin M (IgM), immunoglobulin G (IgG) (Rinninella et al.), and immunoglobulin A (IgA). Each of these antibodies has its correlation with *H. pylori* density in gastric mucosa. An increased level of anti-*H. pylori* IgM in the bloodstream indicates recent colonization of *H. pylori* in the stomach (Rosenstock et al., 2000), whereas an elevated level of anti-*H. pylori* IgG is observed when the density of *H. pylori* in the gastric mucosa increases (Plebani et al., 1996, Hsu et al., 1997). An elevated level of anti-*H. pylori* IgA in the bloodstream is associated with mild inflammation in the gastric mucosa (Oluwasola et al., 2012). Specific

antibodies are found in serum and gastric aspirate of *H. pylori*-infected patients. Additionally, increased titers of IgG and IgA antibodies against urease, flagellin, *H. pylori* adhesion A (HpaA), LPS, and membrane protein (MP) have been detected in infected individuals. However, asymptomatic patients and patients with duodenal ulcers show no differences in antibody levels (Ihan and Gubina, 2014). Plasma cells, which are known to play an important role in several chronic inflammatory conditions (Ansar et al., 2016) are thought to accumulate in the gastric mucosa due to the presence of a large number of *H. pylori* bacteria. This accumulation results in the production of antibodies that circulate in the bloodstream at high levels. However, a study investigating the relationship between the density of *H. pylori* bacteria and the total number of plasma cells in the infected mucosa found no statistically significant correlation. Therefore, the presence of plasma cells in the gastric mucosa is not a specific indicator of *H. pylori* infection (Hartecia et al., 2020).

*H. pylori* can manipulate the survival and cell death pathways of B cells to sustain the infection. It does this by translocating the apoptotic-inducing factor (AIF) and causing apoptosis in B cell lines, which has been linked to the persistence of *H. pylori* (Singh et al., 2006). On the other hand, when the *H. pylori* CagA protein is translocated by the bacterial T4SS, it leads to increased survival of B cells *in vitro. This* translocation of CagA stimulates extracellular signal-regulated kinase (ERK) and MAPK phosphorylation, as well as the upregulation of the anti-apoptotic proteins BCL-2 and BCL-XL. These findings suggest that while apoptosis may aid in the persistence of *H. pylori* by eliminating protective B cells, the increased survival of B cells has been associated with the development of *H. pylori*-induced lymphoma (Singh et al., 2006, Lin et al., 2010). However, further investigation is needed to determine whether one or both mechanisms occur *in vivo during H. pylori* infections (Nothelfer et al., 2015).

Typically, both innate and adaptive responses contribute to protecting against *H. pylori*, but they are not enough to eliminate the bacteria (Blosse et

al., 2018, Rosser et al., 2014, Yoshizaki et al., 2012). Regulatory B cells (Breg) cells have a suppressive function by producing anti-inflammatory cytokines such as IL-10, and IL-35, and transforming growth factor beta (TGF-β). Additionally, previous research has demonstrated that Breg cells can inhibit the proliferation of $CD4^+$ T cells, decrease the number of T helper 17 (Th17) cells, and increase the population of Treg cells (Kessel et al., 2012, Hong et al., 2019). Previous data suggests that B cells producing IL-10 expand earlier than Foxp3+ Treg cells. Additionally, previous study conducted in a mouse model has shown that after *H. pylori* infection, the number of Breg cells increases in the early stages of infection before Treg cells are induced (Wei et al., 2014).

### 1.7.2 Cellular immune response

#### 1.7.2.1 Unconventional T cells

Natural killer (NK) cells are essential cells of the innate immune system and are characterized by the expression of an adhesion molecule, CD56 (Cooper et al., 2001). NK cells kill virus-infected cells and tumor cells (Herberman, 2002). Additionally, NK cells are potent inducers of IFN-γ, which in turn stimulates many parts of the immune system such as phagocytosis and antigen presentation. When exposed to both *H. pylori* antigens and IL-12, NK cells obtained from peripheral blood exhibit a significant increase in IFN-γ production (Boehm et al., 1997, Xing et al., 2001). This suggests that the activation of NK cells within the mucosa infected with *H. pylori* may contribute to reducing the bacterial load. Thus, NK cells play a crucial role in the defense against *H. pylori* infection (Yun et al., 2005).

Natural killer T (NKT) cells possess characteristics of both NK cells and T cells. Like conventional T lymphocytes, NKT cells express a T cell receptor (TCR) that is formed through genetic rearrangement. However, unlike conventional T cells that have a wide range of TCR diversity, the majority of

NKT cells have a partially invariant TCR repertoire (Godfrey et al., 2004). Instead of peptides presented by CD1d molecules, these cells are activated through the recognition of glycolipid antigens, leading to the production of cytokines that can either activate or suppress other immune cells (Nishioka et al., 2018). NKT cells possess the capability to promptly generate many cytokines upon TCR stimulation. These cytokines include Th1 cytokines like IFN- $\gamma$ and tumor necrosis factor (TNF), as well as the typical Th2 cytokines such as IL-4, IL-10, and IL-13. Additionally, NKT cells can produce IL-2, TGF-β, and several chemokines, and a subset can even generate the Th17 cytokine IL-17 (Michel et al., 2007).

NKT cells make up approximately 14-35% of the T-cell population in the epithelial layer and around 16-25% of T cells in the lamina propria of the gastric mucosa. The presence of a substantial population of T cells indicates that these cells likely play a role in the immune response at the local level where studies have identified a novel subset of T cells in the adult human gastric mucosa that express NK cell markers including CD56, CD161, and CD94, as observed in antral biopsy samples. It is important to mention that there are significant variations in the populations of NKT cells in individuals infected with *H. pylori* compared to those who are not infected. In infected patients, there is a decrease in the number of NKT cells expressing CD56 and CD94 in both the epithelium and lamina propria layers of the mucosa. On the other hand, there is an increase in the number of cells expressing CD161, implying possible variations in the function of these NKT cells during *H. pylori* infection (O'Keeffe and Moran, 2008). Considering the high presence of NKT cells in the normal antral mucosa, alterations in the frequencies of different NKT cell subsets during *H. pylori* infection, and the crucial role of these T cells in fighting against tumors, any changes in the quantity or activity of NKT cells in response to *H. pylori* could potentially impact the development of localized cancer in the gastric mucosa. Previous studies have investigated the potential

interaction between NKT cells and Treg cells. NKT cells can modulate the function of Treg cells through IL-2-dependent mechanisms. When activated, NKT cells release IL-2, which promotes the proliferation of Treg cells without affecting their suppressive capabilities. Conversely, Treg cells can suppress the functional activities of NKT cells by inhibiting their proliferation, cytokine production, and cytotoxic functions. Therefore, it can be hypothesized that NKT cells and Treg cells mutually regulate each other in the gastric mucosa infected with *H. pylori*. Given that Tregs cells have an inhibitory role in anti-tumor immunity (Shevach, 2002), and NKT cells contribute to tumor surveillance (Terabe and Berzofsky, 2004), the direct effects and interactions between these T cells may significantly impact the development of *H. pylori*-associated gastric cancer (O'Keeffe and Moran, 2008).

Mucosal-associated invariant T (MAIT) (Yamaoka et al.) cells, which account for up to 10% of peripheral T cells, are a type of innate-like T cells. They are found in abundance in both human blood and tissue. These cells are classified as TRAV1-2+ (TCR V$\alpha$7.2+) T cells and are distinguished by the MHC class I-related molecule (MR1)(Huang et al., 2005). MR1 specifically recognizes non-peptide riboflavin biosynthesis intermediates that are conserved among bacteria and fungi.

MAIT cells play a complicated role in *H. pylori* infection and are involved in the chronic stages of pathogenesis. The discovery of MAIT cells in the gastric LP and their ability to mount a response against *H. pylori* infection was first demonstrated by Booth et al, which led to further investigations on the involvement of MAIT cells in chronic *H. pylori* infection and its effects on gastric pathology (Booth et al., 2015). Using *H. pylori* infection mouse models, as developed by D'Souza et al., it was observed that MR1-/- mice exhibited lower levels of lymphocytic infiltration and gastric atrophy in comparison to MAIT TCR-transgenic mice and wild-type (Ramaker et al.) mice. This suggests a strong association between MAIT cells and gastritis, as MAIT cells attract not

only other MAIT cells but also immune cells like macrophages and DCs to the infection site (D'Souza et al., 2018). In a recent study, it was found that MAIT cells producing IL-9 could potentially exacerbate inflammation in patients with *H. pylori*-induced gastritis. This finding supports the notion that MAIT cells could worsen gastric pathology during bacterial infection (Ming et al., 2021). These discoveries suggest that MAIT cells have the potential to both control *H. pylori* infection and contribute to gastric pathology due to their intrinsic antimicrobial properties. In high-risk populations, chronic *H. pylori* infection has the potential to promote the development of gastric cancer. Studies have shown that MAIT cells secrete IFN-γ, TNF, and IL-17A in response to *H. pylori*-infected cells. IL-17 has been associated with pro-tumorigenic and proinflammatory effects, while IFN-γ is known for its anti-tumor response and strong immunostimulatory properties. However, it is still unclear whether MAIT cells play a role in promoting cancer development or contribute to cancer immune surveillance following *H. pylori* infection (Michel et al., 2007).

#### 1.7.2.2 Classical T cell

The defense against *H. pylori* infection heavily relies on the cellular immune response. Upon entry into the body, antigen-presenting cells such as dendritic cells and macrophages recognize and process *H. pylori* antigens, presenting them to T cells. The effector T cells, predominantly $CD4^+$ T cells, differentiate into Th1, and Th17 cells, which generate pro-inflammatory cytokines including TNF-$\alpha$, IFN-$\gamma$, and IL-17A. These cytokines activate other immune cells, such as neutrophils and macrophages, to eliminate *H. pylori* and clear the infection. Anti-inflammatory Tregs are another $CD4^+$ T cell subset induced by *H. pylori* infection, which produces immune-modulating cytokines such as IL-10 and transforming growth factor beta (TGF-$\beta$) to suppress the immune response and maintain homeostasis. Th2 and Th22 responses have also been reported (Buzzelli et al., 2015, Robinson et al., 2008)**(*Figure 7*)**.

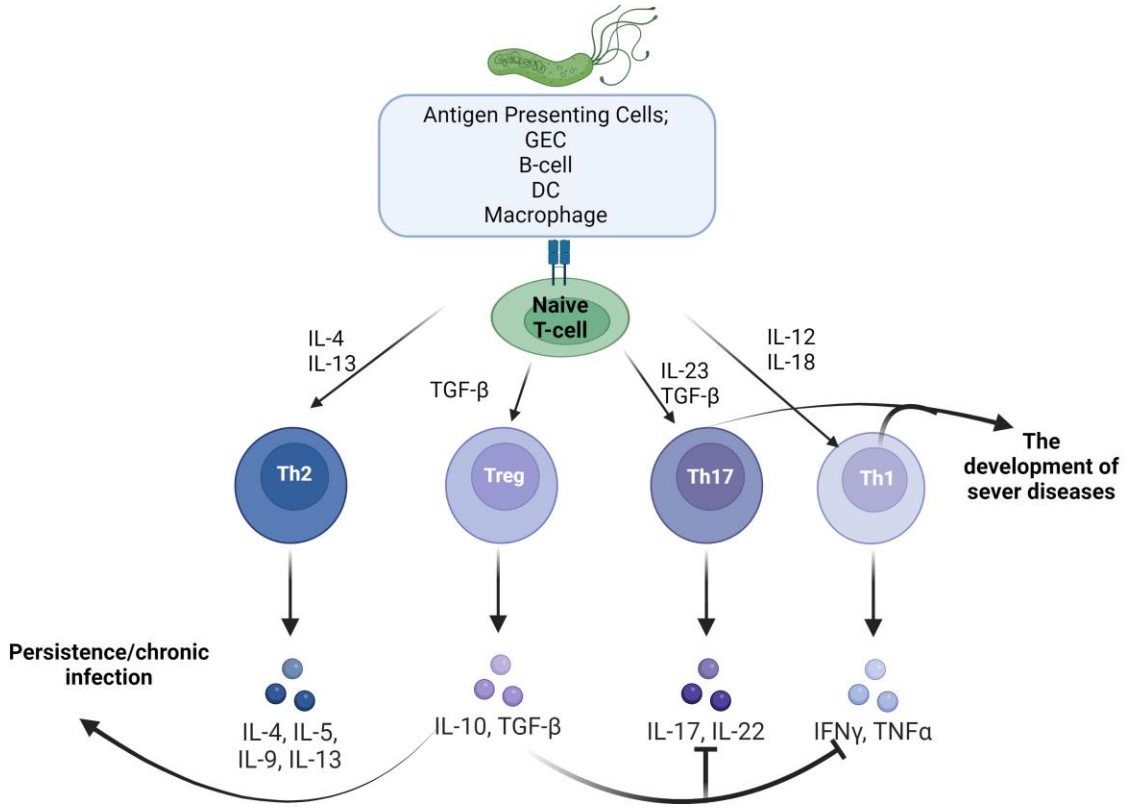



**Figure 7. Immunological determinants of *H. pylori*-induced inflammation.** The innate immune system recognizes *H. pylori*, which causes differentiation of various T-cell lineages in the adaptive immunological response. *H. pylori*-induced gastritis is distinguished by a strong Th1/Th17 response that is controlled by Treg cells. The various T-cell lineages secrete distinct cytokines that control the immunological response, which is a significant element in the development of serious diseases such as ulcers or gastric cancer. This figure was created with BioRender.com.

#### 1.7.2.2.1 T helper 1 (Th1) cells

Activated antigen-presenting cells, such as DC, macrophages, and neutrophils, are the primary producers of IL-12. This cytokine plays a crucial role in the differentiation of naive helper T cells into Th1 cells (Hsieh et al., 1993), which are responsible for generating cell-mediated immunity via the production of cytokines such as IFN-γ and IL-2. Th1 cells are particularly essential for protection against intracellular infections. Additionally, the

transcription factors STAT4 and T-bet are key factors in regulating Th1 cell differentiation (Szabo et al., 2000).

In *H. pylori* infection, CD4+ T cells are abundant in the gastric mucosa, which contributes to the development of chronic gastritis. This is evidenced by the fact that mice lacking CD4+ T cells do not develop gastritis. Furthermore, the development of gastritis is impaired in mice deficient in IFN-γ, while the absence of IL-4 increases gastritis development. Therefore, the production of IFN-γ by Th1 cells plays a significant role in gastritis development. Additionally, *H. pylori*-specific T cells stimulate the infiltration of neutrophils and macrophages into the infected gastric mucosa. In addition to the presence of T cells and activated neutrophils and macrophages, *H. pylori*-induced gastritis is characterized by an increase in the secretion of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and TNF-α in infected patients (Bagheri et al., 2018).

Thus, Th1 could potentially lead to PUD as TNF-α and IFN-γ can induce functional changes in gastric epithelial cells and increase gastric acid secretion (Tourani et al., 2018).

The severity of gastritis in *H. pylori*-infected individuals is correlated with the number of IFN-γ-secreting cells in the gastric mucosa. IFN-γ itself plays a crucial role, as its infusion into mice, even in the absence of *H. pylori* infection, induces gastric atrophy, metaplasia, and dysplasia, which are pre-cancerous conditions (Eaton et al., 2006). A study has shown that Th1 cell responses are increased in GC tissue or in peripheral blood mononuclear cell populations, and mainly in lymph node metastasis of patients with GC. This data suggests that Th1 cell infiltration may contribute to GC development and metastasis (Su et al., 2014).

#### 1.7.2.2.2 T helper 17 (Th17) cells

Th17 cells were first identified as a distinct subset of T helper cells in recent years (Harrington et al., 2005, Park et al., 2005), and their development is regulated by the retinoic acid receptor-related orphan receptor-γt (RORγt) (Ivanov et al., 2006). The differentiation of Th17 cells can be induced by IL-1β (Pachathundikandi et al., 2016), IL-23 (Harrington et al., 2005), or a combination of IL-6 and TGF-β (Mangan et al., 2006). These cells are named after their ability to produce IL-17, including IL-17A and IL-17F (Harrington et al., 2005). IL-17 is a pro-inflammatory cytokine that acts on both immune and non-immune cells, inducing the production of antibacterial peptides, pro-inflammatory cytokines, chemokines, and prostaglandins. IL-17 also promotes the recruitment of neutrophils through the induction of chemokines such as CXCL1, CXCL2, CXCL5, and IL-8 (Laan et al., 1999a, DeLyria et al., 2009), as well as CCL20, which plays a role in cell recruitment to mucosal surfaces (Acosta-Rodriguez et al., 2007). Th17 cells are associated with inflammation, autoimmune diseases (Langrish et al., 2005), and immune responses against extracellular pathogens like *H. pylori* (Khader et al., 2009). The differentiation of Th17 cells is inhibited by IL-27 (Hirahara et al., 2012), which also promotes the differentiation of Th1 cells (Yoshida et al., 2001). IL-17A has been found to have a role in inflammation caused by *H. pylori* colonization. Studies using mouse models have shown that wild-type (WT) mice infected with *H. pylori* have a significant increase in neutrophil infiltration in the stomach compared to *IL-17A*$^{-/-}$ mice (Conti et al., 2016). The longer the WT mice are infected with *H. pylori*, the higher the level of neutrophil infiltration. However, in *IL-17A*$^{-/-}$ mice or *IL-17AR*$^{-/-}$ mice infected with *H. pylori*, there is no significant increase in neutrophil levels at any point during the infection (Shiomi et al., 2008, Algood et al., 2009). These findings suggest that IL-17A is necessary for neutrophil infiltration, likely through the activation of IL-8 homolog expression in epithelial cells, which then recruits neutrophils. Mizuno et al. investigated

the levels of IL-17A and IL-8 expression in patients with and without gastric ulcers. They found that patients with *H. pylori* infection and gastric ulcers had higher levels of IL-17A and IL-8 in their gastric mucosa compared to uninfected patients without ulcers. Histologically, they observed a significant correlation between the expression of IL-17A and IL-8 and increased infiltration of neutrophils in infected patients (Mizuno et al., 2005). IL-21 is a cytokine that is produced by Th17 cells, T follicular helper (Tfh) cells, and to a lesser extent, NK T cells or Th1 cells. In mice, the absence of IL-21 has significant effects on *H. pylori*-induced gastritis compared to mice with normal IL-21 levels. This is likely because IL-21 has various activities in different tissues. After 3 months of infection, mice lacking IL-21 do not control *H. pylori* colonization as effectively and the bacteria are more frequently found in the gastric glands. Additionally, these mice show less inflammation compared to *H. pylori*-infected mice with normal IL-21 levels. These findings suggest that IL-21 plays a crucial role in maintaining both the Th1 and Th17 responses during *H. pylori* infection in the gastric mucosa (Carbo et al., 2014). According to a study that examined the levels of various cytokines and their connection to clinicopathological features GC, it was discovered that the presence of IL-17A (measured using immunohistochemistry) was linked to reduced survival rates (Kim et al., 2017). A study conducted on AGS cells, a gastric cancer cell line, provides evidence that IL-17A may have pro-carcinogenic effects. The study found that IL-17A can enhance the infiltration and invasion of gastric cancer cells. The results suggest that this effect may be attributed to the increased expression and activity of matrix metalloproteinase 2 (MMP-2) and MMP-9, as well as the decreased expression of TIMP-1 and TIMP-2, which are involved in regulating invasiveness (Wang et al., 2014). It has been observed that that *H. pylori*-specific T lymphocytes produce large amounts of IL-17 in patients with gastric adenocarcinoma (Amedei et al., 2014, Della Bella et al., 2022, Capitani et al., 2019). In a recent study, it was reported that *H. pylori* can drive the production of IL-17A by gastric T cells obtained from *H. pylori* patients with gastric

intestinal metaplasia and gastric dysplasia. Additionally, the levels of IL-17A in the blood were significantly higher in *H. pylori*-infected patients with intestinal metaplasia/dysplasia compared to controls. The findings suggest that assessing serum levels of IL-17A could be beneficial in the management of patients with *H. pylori* infection and potentially in predicting the risk of developing gastric cancer (Della Bella et al., 2023a).

#### 1.7.2.2.3 Regulatory T cells (Tregs)

The cytokine TGF-β is known to promote the differentiation of naive helper T cells into Tregs, which are a subset of T cells that regulate the immune system. Tregs are characterized by the expression of CD4, high levels of CD25, and a transcription factor called FOXP3 (Hori et al., 2003a, Fontenot et al., 2003). These cells are essential in maintaining self-tolerance and preventing autoimmune diseases in both humans and mice. There are two types of Tregs: natural Tregs (nTreg) and inducible Tregs (iTreg). nTregs are created during normal T-cell development in the thymus and act to suppress self-reactive T cells in peripheral tissues. iTregs are developed in peripheral lymphoid organs from naive T cells after antigen exposure (Curotto de Lafaille and Lafaille, 2009).

Tregs control the immune response through various mechanisms, including the production of immunosuppressive cytokines like IL-10 (Annacker et al., 2003), TGF-β (Read et al., 2000), and IL-35 (Collison et al., 2007), as well as cytolysis of effector cells through granzyme-A , granzyme-B-dependent and perforin-dependent pathways(Gondek et al., 2005). Tregs can also inhibit the proliferative response via the IL-2 receptor, cyclic AMP-mediated inhibition, and CD39 and/or CD73-generated immunomodulation via the A2 adenosine receptor. Finally, Tregs can interact with DCs to modify their function and maturation (Vignali et al., 2008).

Following infection with *H. pylori*, different cells such as gastric fibroblasts, FOXP3+ Tregs, macrophages, and DCs exhibit an increased secretion of TGF-β. A recent study has provided evidence highlighting the significance of TGF-β derived from DCs in the expansion of Tregs. This study suggested that following *H. pylori* infection, DCs migrate to lymphoid tissue outside of the stomach and release TGF-β. This leads to the activation of Tregs, which in turn affects the overall immune response in the body. As a result, there may be a decrease in inflammatory Th1 cytokines and an increase in *H. pylori* colonization (Owyang et al., 2020).

Tregs, which are associated with decreased gastritis, are found to reduce the pathological outcomes of *H. pylori* infection in mice and humans (Raghavan et al., 2003, Raghavan and Quiding-Jarbrink, 2012). However, this reduction in gastritis leads to increased levels of *H. pylori* colonization. Studies using mouse models have shown that depleting Tregs leads to higher levels of IFN-γ and increased inflammation (Raghavan et al., 2004). Counter to this, however, a recent study has reported that Treg and Th22 cells could be implicated in the development of *H. pylori* chronic gastritis (Yao et al., 2023). This study reported an elevation in IL-22$^+$ CD4$^+$ and FOXP3$^+$ CD4$^+$ T cells percentage in the peripheral blood during *H. pylori* infection, and a positive association between *IL22* and *FOXP3* mRNA levels and the degree of *H. pylori* colonization degree and the severity of gastritis (Yao et al., 2023). Furthermore, co-culturing *H. pylori*-stimulated gastric epithelial cells with human CD4$^+$CD25$^{high}$ cells has been found to suppress IL-8 expression, suggesting that Tregs may indeed modulate gastric inflammation in humans (Robinson et al., 2007).

Higher numbers of CD4$^+$ CD25$^{high}$ Treg cells have been observed in both the gastric and duodenal mucosa of *H. pylori*-infected patients compared to uninfected healthy controls, and in the stomach of *H. pylori*-infected individuals with gastric adenocarcinoma. The recruitment of Tregs to the *H. pylori* colonized mucosa is believed to be facilitated by the GEC response

through CCL20/CCR6-mediated chemotaxis. It has been observed that CCL20 is upregulated in gastric biopsies of *H. pylori*-infected patients, and this upregulation of CCL20 in GECs is dependent on signaling via the c*ag* type IV secretion system (T4SS) (Cook et al., 2014).

Tregs can target not only T cells but also GECs by producing IL-10 and TGF-β. While IL-10 expression may have a protective effect during inflammation, it can be detrimental once cancer develops. In fact, an increase in IL-10 levels in the serum is seen as an unfavorable prognostic marker for gastric cancer (Szaflarska et al., 2009). IL-10 in the tumor microenvironment can be produced by various cell types. One possible way that IL-10 can stimulate GECs in the tumor is by activating c-Met-STAT3. GEC lines responded to cancer-associated macrophages in a way that depended on IL-10, inducing proliferation and migration while also suppressing apoptosis (Chen et al., 2019). TGF-β is synthesized by Tregs and other immune cells and may have a dual role in tumor development, acting as a suppressor in the early stages but as a promoter in later stages (Batlle and Massagué, 2019).

To study the impact of TGF-β signaling in *H. pylori* infection, researchers created mice that had overexpression of a mutant form of the TGF-β RII receptor in their stomachs (pS2-dnRII). When these mice and their normal littermates were infected with *H. pylori*, the mice without TGF-β signaling developed gastric adenocarcinoma with a high level of cell proliferation in their epithelial cells, while the normal littermates did not. This suggests that TGF-β has a protective effect in suppressing the development of cancer (Hahm et al., 2002).

Increased levels of TGF-β in the serum and gastric tissue are associated with advanced cancer (Nakamura et al., 1998, Ebert et al., 2000), and expression appears to be higher in the tissues of intestinal type GC than diffuse type GC (Pak et al., 2016). In laboratory experiments conducted on gastric epithelial cell lines (BGC823 and MKN-45), it was observed that treatment with

TGF-β resulted in a notable decrease in the expression of E-cadherin while increasing the expression of proteins related to epithelial-mesenchymal transition, namely snail and vimentin. Interestingly, before TGF-β signaling, MFAP2 is activated and facilitates this process. While Tregs may have a role in reducing the inflammatory response to *H. pylori*, they could be harmful in cases of GC as Tregs and their cytokines can activate pathways that promote cancer development in the tumor microenvironment (Algood, 2020).

Despite the robust immune reaction described above, *H. pylori* has developed evasion mechanisms, including the modulation of T-cell responses and induction of T-cell exhaustion, resulting in chronic infection and the development of *H. pylori*-associated diseases.

Numerous studies have reported that *H. pylori* utilizes several factors, such as CagA, VacA, and gamma-glutamyl transpeptidase (GGT) protein to block or inhibit T-cell activity. The *H. pylori* CagA protein was believed to have a paralyzing effect on T cell proliferation. This belief was confirmed when a recombinant fragment of the *H. pylori* CagA antigen showed anti-proliferative activity (Rudnicka et al., 1998). In a separate study, researchers utilized *H. pylori* mutant strains lacking CagA and VacA proteins to demonstrate that the 120-to 128-kDa CagA protein is accountable for suppressing the proliferation of T cells driven by phytohemagglutinin (PHA). The cytoplasmic fraction obtained from the *H. pylori cagA* knockout mutant exhibited significantly reduced anti-proliferative effects compared to the cytoplasmic fractions from the original *H. pylori* strain containing both CagA and VacA proteins, or the *H. pylori* strain lacking VacA but still containing CagA protein (Paziak-Domanska et al., 2000).

VacA, a protein secreted by *H. pylori*, causes vacuolation in epithelial cells. *H. pylori* also disrupts the tight junctions between GECs, allowing VacA to enter the lamina propria. VacA enters T cells by binding to the β2 integrin subunit of the lymphocyte function-associated antigen 1 (LFA-1) receptor

(Sewald et al., 2008). Once inside the T cells, VacA inhibits their proliferation and activation through various mechanisms. One of these mechanisms is interrupting the signaling of IL-2, which is essential for lymphocyte activation and proliferation. VacA also blocks the transcription of IL-2 by inhibiting the nuclear translocation of the transcription factor nuclear factor of activated T cells (NFAT), which is necessary for the activation of the IL-2 promoter. (Gebert et al., 2003). Additionally, VacA inhibits T-cell responses by blocking the presentation of antigens through MHC class II and by inhibiting T-cell activation. VacA interferes with the processing of antigens by B cells by affecting endosomal trafficking. Molinari et al. demonstrated that VacA disrupts the proteolytic processing of antigens and inhibits the Ii-dependent antigen presentation pathway that relies on newly synthesized class II MHC molecules in endosomes (Molinari et al., 1998).

The enzyme GGT is essential for the survival and pathogenicity of *H. pylori*. *H. pylori* GGT (HpGGT), is a membrane protein attached to the outer membrane of the bacteria. HpGGT is found in all strains and is highly conserved (Rossi et al., 2012). HpGGT has direct effects on immune cells. One study found that this enzyme can reduce T-cell proliferation (Schmees et al., 2007) by causing cell cycle arrest at the G1 phase through interference with the Ras-dependent signaling pathway (Schmees et al., 2007). This dampening of T cell proliferation is believed to contribute to the immunosuppression that helps *H. pylori* persist in the body. Another study discovered that HpGGT induces the expression of microRNA-155 (miR-155) in T lymphoblast T cells and peripheral blood mononuclear cells (Fassi Fehri et al., 2010). This induction relies on the activation of the cyclic adenosine monophosphate cascade and the presence of the master regulator of regulatory T-cell development, FOXP3 (Hori et al., 2003b). Regulatory T cells have immunosuppressive activity and are often found in the *H. pylori*-infected gastric mucosa, promoting increased colonization of *H. pylori* and tumor infiltration (Ohue and Nishikawa, 2019).

Supporting the role of HpGGT in promoting regulatory T cell development, mice infected with GGT-isogenic *H. pylori* mutant strains had lower counts of regulatory T cells compared to mice infected with wild-type *H. pylori* (Oertli et al., 2013). Finally, *H. pylori* utilizes the T4SS to transfer the effector protein CagA and fragments of the cell wall to enhance the expression of B7-H1, a co-inhibitory molecule, while simultaneously downregulating the expression of T cell co-stimulatory molecules, B7-H2 and B7-H3, on GECs, which collectively impact the balance of Treg and Th17 cells and promote the persistence of bacteria (Lina et al., 2015, Lina et al., 2013, Lina et al., 2019). This information provides insight into how *H. pylori* can manipulate the immune response by interfering with T cell activation and proliferation.

## 1.8 Cytokines are produced in response to *H. pylori* infection.

The mechanisms used by *H. pylori* to initiate and maintain the local immune response are complicated, but there is evidence that cytokines secreted during both innate and adaptive responses can lead to the development of ulcer disease, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue (Caruso et al., 2007). A hallmark of the infection of the gastric mucosa with *H. pylori* is characterized by increased production of several proinflammatory cytokines, most of which are produced by gastric epithelial cells and others by the infiltrating B and T cells (Genta et al., 1993). IL-8 is one of the earliest cytokines produced by GECs during *H. pylori* infection (Crabtree et al., 1994, Crabtree and Lindley, 1994, Genta et al., 1993, Matsushima et al., 1988, Oppenheim et al., 1991). These findings have been confirmed in both *in vivo and in vitro studie*s. The elevation in IL-8 levels was associated with *cagA*$^{+}$ strains (Crabtree et al., 1994) and increased numbers of neutrophils recruited to the infected gastric mucosa. The urease virulence factor induces the production of IL-6 and TNF-$\alpha$ by *H. pylori*-infected gastric epithelial cells. Increased IL-6 and TNF-$\alpha$ levels have been also found in *H. pylori* patients with chronic gastritis.(Crabtree et al., 1991) Gastric epithelial

cells induce the expression of intracellular proinflammatory cytokines IL-32 during *H. pylori* infection in a *cag* T4SS dependent manner. IL-32 stimulates activation of the transcription factor NF-ĸB and induces the production of different cytokines and chemokines, including IL-8, CXCL1, CXCL2, and TNF-α. Interestingly, increased IL-23 levels have also been reported in patients with gastritis and gastric cancer (Sakitani et al., 2012). Additional cytokines reported to be secreted by the infected gastric epithelium include IL-6, IL-1β, IL-1α, granulocyte-macrophage colony-stimulating factor (GM-CSF), and monocyte chemoattractant protein-1 (MCP-1) (Tanahashi et al., 2000). VacA also induces mast cells to secret various proinflammatory cytokines such as IL-6, IL-1ß, IL-10, and IL-13 in a dose-dependent manner without mast cell degranulation (Supajatura et al., 2002).

Both gastric epithelial cells and lamina propria mononuclear cells (LPMC) isolated from *H. pylori*-infected gastric mucosa showed increased levels of IL-18. The induction of IL-18 in gastric epithelial cells was influenced by both *cag* PAI and OipA, while the *cag* T4SS did not play a role in the induction of IL-18 in monocytes (Yamauchi et al., 2008).

## 1.9 Interleukin-16 Cytokine

Interleukin-16 (IL-16), which was initially described as lymphocyte chemoattractant factor (LCF), is a pro-inflammatory cytokine that induces chemotaxis of $CD4^+$ T lymphocytes, monocytes, and eosinophils (Cruikshank and Center, 1982). Besides its chemotaxis activity, IL-16 can induce expression of CD25, (Cruikshank et al., 1987) and prime T cell proliferation (Parada et al., 1998). IL-16 can also act as an immunomodulator, inhibiting antigen-induced T lymphocyte activation and proliferation (Lynch et al., 2003, Little et al., 2003, De Bie et al., 2002). IL-16 is secreted mainly by $CD8^+$ lymphocytes as a 67-kDa precursor protein (Baier et al., 1997a). Human IL-16 is secreted as a 631-amino acid precursor protein, pro-IL-16, which is then cleaved by the caspase-3 enzyme to release the biologically active C-terminal fragment, consisting of

121 amino acids (Center et al., 1997, Chupp et al., 1998, Zhang et al., 1998). Pro-IL-16 consists of three postsynaptic density proteins, disc-large, zonulin-1 (PDZ) domains that facilitate multimerization, and a CcN motif that induces nuclear localization. Caspase-3 splits precursor IL-16 between PDZ2 and PDZ3 into N-terminal pro-IL- 16 and C-terminal secreted/mature form of IL-16. While mature IL-16 is secreted after cell activation, the N terminus can be found in either the cytosol or the nucleus of the T cell to regulate cell growth (Muhlhahn et al., 1998). In the human genome, the *IL16* gene is located on chromosome 15, whilst in the mouse it is located on chromosome 7. This gene consists of six introns and seven exons in both human and mouse and encodes pro-IL-16 *(Baier et al., 1997b).*

IL-16 is generated by different immune and parenchymal cells, as well as $CD4^+$ and $CD8^+$ T cells (Bannert et al., 1999), eosinophils (Bellini et al., 1993, Bannert et al., 1999, Laberge et al., 1999), macrophage/dendritic cells (Laberge et al., 1999, Bellini et al., 1993), mast cells, fibroblasts (Franz et al., 1998), and bronchial epithelial cells (Bellini et al., 1993). Both $CD4^+$ and $CD8^+$ cells constitutively express pro-IL-16 mRNA (Laberge et al., 1995).
T lymphocytes require stimulation with antigen or mitogen to synthesize IL-16 (Center and Cruikshank, 1982). While in $CD8^+$ T cells caspase 3 constitutively cleaves pro-IL-16 to release a 121 amino acid C-terminal domain, activation of caspase 3 is necessary to cleave pro-IL-16 in $CD4^+$ T cells. The cleavage C- C-terminal fragment then accumulates and is released as a mature, bioactive form of IL-16 (

**Figure 8**)

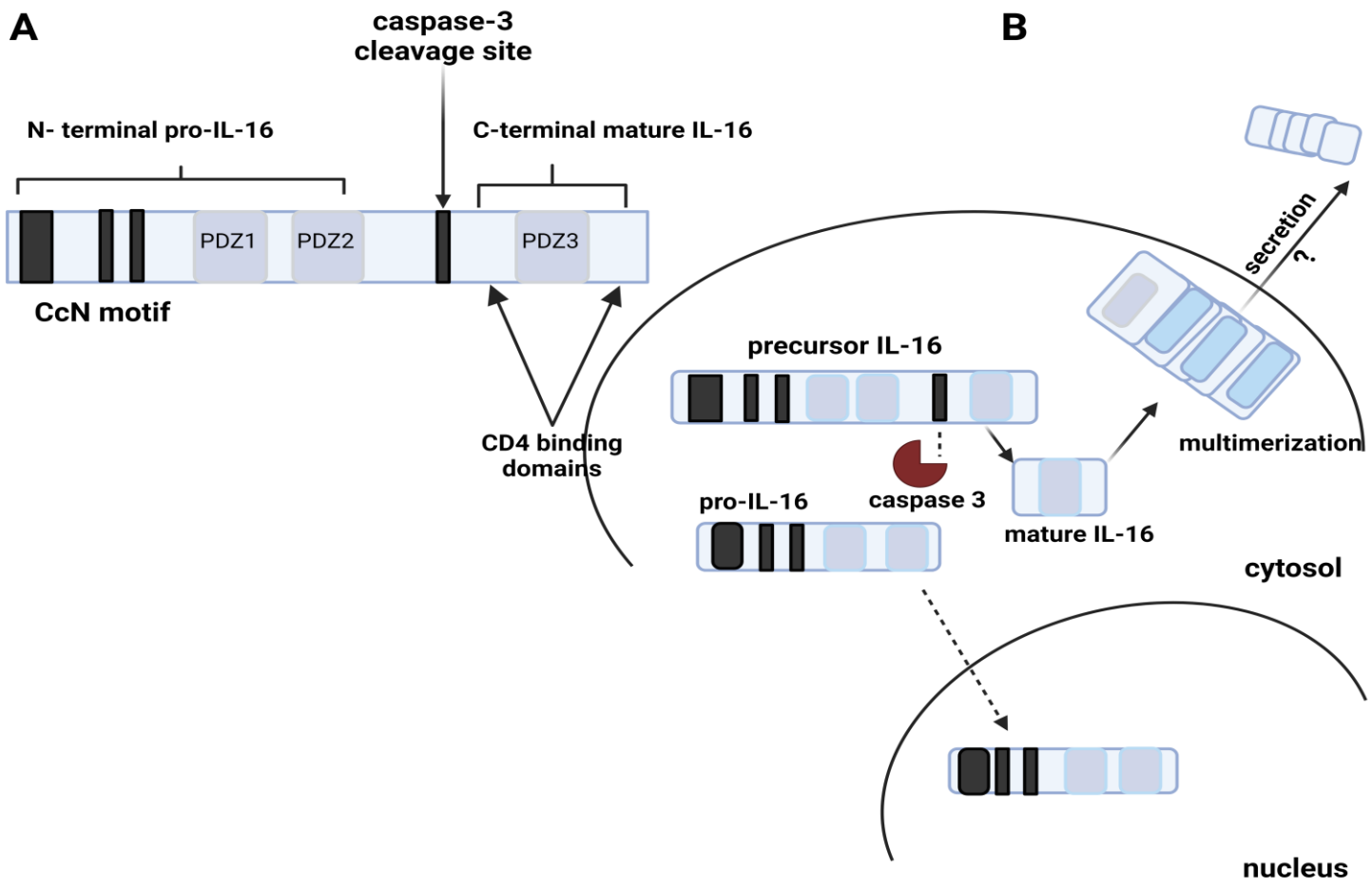

A
caspase-3
cleavage site
N- terminal pro-IL-16
C-terminal mature IL-16
PDZ1
PDZ2
PDZ3
CcN motif
CD4 binding
domains
B
secretion
?
precursor IL-16
multimerization
pro-IL-16
caspase 3
mature IL-16
cytosol
nucleus

**Figure 8. Structure and processing of IL-16.**

**A:** Schematic of the domain structure of IL-16. The N-terminal portion of pro-IL-16 consists of a CcN motif and two PDZ domains. The C-terminal portion of mature IL-16 is cleaved by caspase 3 and contains a PDZ domain as well as CD4 binding domains. **B:** The precursor form of IL-16 is processed into pro-IL-16 and mature IL-16 through caspase 3 cleavage. Pro-IL-16 can then move to the nucleus using a nuclear localization sequence (NLS). Mature IL-16 forms multimers, likely through interactions with PDZ domains, and is released from the cell through a mechanism that is not yet known. This figure was created with BioRender.com.

IL-16 secretion is a complicated process influenced by multiple factors, and the specific mechanisms vary based on the context and cell types involved. For example, activated CD8+ cells can produce IL-16 when exposed to histamine or serotonin, which act on the H2 receptor and S2 receptor respectively. Mast cells also accumulate pro-IL-16 mRNA and protein on a constitutive basis and release active IL-16 in response to stimulation by C5a and phorbol 12-myristate 13-acetate (Brkanac et al.) (Mathy et al., 2000). While IL-12 p40 homodimer, but not IL-12 p70, can stimulate the expression of IL-16 in microglia and macrophages (Jana and Pahan, 2009), the expression of IL-16 in primary bronchial epithelial cells can be induced by histamine, IL-1β, and TNF-α. Additionally, fibroblasts stimulated with IL-1β can also produce IL-16 (Arima et al., 1999). Eosinophils release IL-16 within 24 hours of being cultured with GM-CSF (Conti et al., 2002). IL-16 mRNA expression levels were increased in a dose-dependent manner by incubating IL-17 with rheumatoid arthritis- fibroblast-like synoviocytes (RA-FLS) and peripheral blood mononuclear cells. However, IL-17 did not have the same effect on IL-16 production in osteoarthritis- fibroblast-like synoviocytes (OA-FLS). Additionally, peptidoglycan, a selective TLR2 ligand, increased IL-16 production by RA-FLS in a dose-dependent manner, while LPS, a selective TLR4 ligand, did not have a similar stimulatory effect (Cho et al., 2008).

### 1.9.1 IL-16 Receptor

CD4 acts as a primary ligand for the secreted IL-16. Expression of CD4 is necessary for mediating IL-16 function. IL-16 selectively induces recruitment of CD4+ T lymphocytes via direct interaction with CD4 and upregulates IL-2 receptor and priming the responding cells for IL-2 or IL-15 dependent proliferation. Binding between CD4 and IL-16 leads to increases in intracellular calcium and inositol trisphosphate (Cruikshank et al., 1991), activation of p56lck (Ryan et al., 1995), and translocation of protein kinase C from the cytoplasm to the cell membrane (Parada et al., 1996). In $CD4^+$ macrophages, IL-16 activates the p38 MAPK and SAPK/JNK pathway in dose-dependent manner. However, IL-

16 was not able to activate the extracellular signal-regulated kinases ERK-1 and ERK-2 (Krautwald, 1998). Although it has been concluded that CD4 is the primary receptor for IL-16 in T cells, a study has shown that CD9 is also important for the IL–16–mediated chemotaxis and activation of the HMC-1 cell line (Qi et al., 2006a). In this study, anti-CD9 monoclonal antibodies blocked activity in the IL-16–responsive human mast cell line HMC-1 which lacks CD4, and inhibited the IL-16 mediated chemotactic and $Ca^{2+}$ mobilization responses of these cells. Such effects were not induced by monoclonal antibodies (mAbs) directed against CD4 or other tetraspanins (Qi et al., 2006b).

### 1.9.2 IL-16 acts as a chemoattraction factor.

Initially, IL-16 was described as a lymphocyte chemoattractant factor, however, IL-16 has now been found to recruit other cells that express CD4, such as monocytes, eosinophils, and dendritic cells (Cruikshank et al., 2000). IL-16 can act to regulate an immune response via direct effects on cell migration patterns. It has been demonstrated that IL-16 preferentially induces Th1 cell migration and that it promotes $CD4^+$ $CD25^+$ cell expansion in long-term cultures alongside IL-2. In addition to Th1 cells, IL-16 selectively induces migration of T regulatory cells. Thus, IL-16 may be involved in Treg cell expansion via the attraction of existing Treg cells as well as by de novo generation of $FOXP3^+$ cells and expansion of the inducible Treg cells (McFadden et al., 2007) **(Figure *9*).**

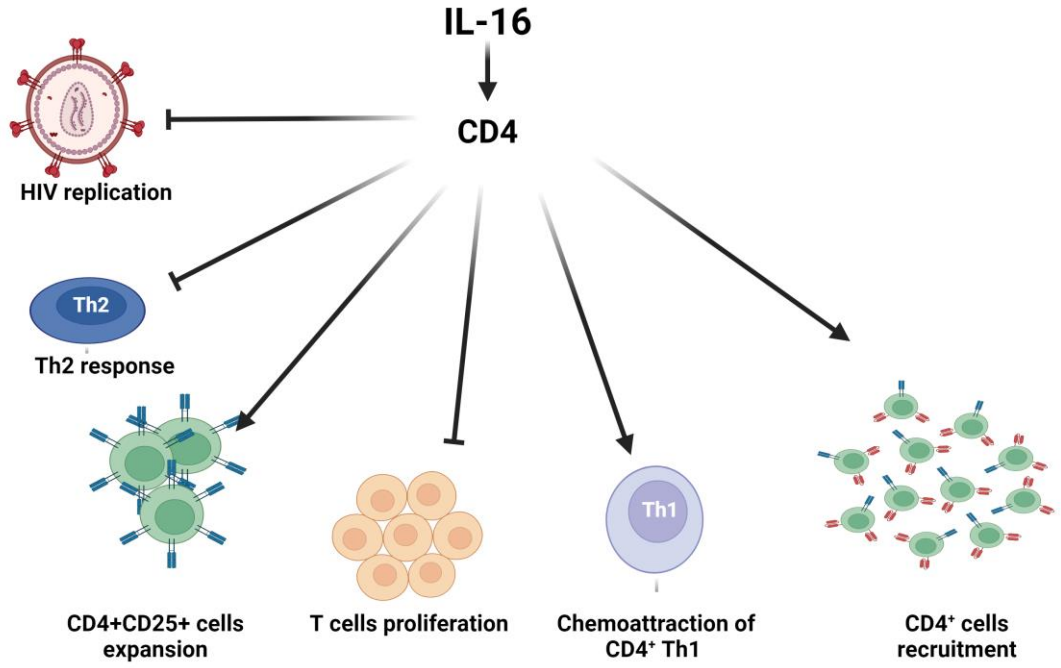



**Figure 9. A summary of how interleukin-16 affects T lymphocyte-mediated inflammation, activation, and proliferation**. Bioactive interleukin-16 (IL-16) binds to the CD4 receptor and promotes CD4 Th1 chemotaxis while reducing antigen-mediated Th2 inflammation. IL-16/CD4 binding stimulated T-cell chemoattraction. In addition, IL-16 inhibits T-cell proliferation while increasing $CD4^+$ and $CD25^+$ cell proliferation in IL-2-treated long-term cultures. IL-16 inhibits the transcription of the HIV-1 long terminal repeat using CD4-dependent signals that need the presence of the HIV core enhancer. This figure was created with BioRender.com.

### 1.9.3 IL-16 in inflammatory diseases

IL-16 expression was associated with the number of infiltrating $CD4^+$ T cells in the asthmatic epithelium of the airways, and with inflammatory processes in various inflammatory conditions such as multiple sclerosis (Skundric et al., 2006), systemic lupus erythematosus (Lard et al., 2002), rheumatoid arthritis

(Blaschke et al., 2001), colitis (Seegert et al., 2001), myocardial infarction (Schernthaner et al., 2017), and atopic dermatitis (Laberge et al., 1998).

In asthma, IL-16 secretion was identified from bronchial epithelial cells, and this was further enhanced by histamine. These findings indicate that histamine-producing mast cells might be involved in the development of inflammatory responses in the airway mucosa by stimulating the production of IL-16, a potent CD4+ chemoattractant factor (Bellini et al., 1993). Elevation of IL-16 was also found in mouse models of allergic asthma, in which infiltration of lymphocytes was significantly elevated compared with controls. This response was significantly suppressed after treatment with a monoclonal antibody to block IL-16 (Hessel et al., 1998). IL-16 is also involved in the inflammatory process of patients suffering from acute myocardial infarction and connects with inflammatory cell activation and clinical and biochemical markers. It might upregulate the proinflammatory response and recruitment of inflammatory cells into infarcted myocardium (Schernthaner et al., 2017). Inflammatory bowel disease (IBD) is generally classified into two chronic inflammatory diseases of the intestines: ulcerative colitis (UC) and Crohn's disease. Crohn's disease is distinguished from UC in the physical areas of the bowel involved (Keates et al., 2000). Inflammatory bowel disease is also characterized by the influx of CD4+ T cells and other mononuclear cells in inflamed mucosal regions. The inflamed mucosa of patients with inflammatory bowel disease shows significant increases in IL-16 levels compared to healthy individuals (Seegert et al., 2001).

IL-16 also plays a critical role in the development of delayed-type hypersensitivity (DTH) footpads, and the recruitment of white blood cells, including Th1 cells, which produce cytokines and chemokines mediating the DTH reaction. A study showed that biologically active IL-16 is produced after antigenic stimulation in the DTH footpads by infiltrating cells or epithelial cells. Treatment of sensitized mice with neutralizing monoclonal antibody against IL-16 before the antigenic stimulation significantly suppresses the DTH response (Yoshimoto et al., 2000).

Furthermore, the synovial fibroblasts from rheumatoid arthritis (RA) patients produce high levels of IL-16 in response to RA-IgG, and this explains the accumulation of T cells in articular tissues in RA (Pritchard et al., 2004). RA is one of the most common autoimmune disorders. It is a chronic, systemic inflammatory disease of unknown causes and affects joints' synovial membranes and articular structures. Patients with RA have a massive infiltration of immune cells to the synovial area of joints. This infiltration of inflammatory cells has led investigators to believe that chemotactic factors may be responsible for this local cellular recruitment. Consistent with this belief, a study of RA patients confirmed increased IL-16 levels in RA patient synovial fluid compared with controls (Franz et al., 1998). Stimulation of synovial fibroblasts with RA-IgG produces high levels of IL-16; this response might explain the accumulation of T cells in articular tissues in RA patients (Pritchard et al., 2004)

IL-16 is also closely involved in developing multiple sclerosis and other inflammatory diseases in the central nervous system as demonstrated by its elevated expression in the glial and recruiting immune cells (Hridi et al., 2021). A recent study focused on the impact of IL-16 on Influenza A virus (IAV) infection using wild-type and *IL16* knockout (KO) mice concluded that *IL16* deficiency upregulated Th1 and cytotoxic T lymphocyte response as well as DC maturation after IAV infection in association with the relieved lung injury and reduced viral load which provides new insight into the host regulation of T-cell immune responses during IAV infection (Jia et al., 2020). Another study has demonstrated that IL-16 promotes the host IAV by acting as a supporting factor for IAV infection, and prospected IL-16 as a potential therapeutic target for viral infection. This study showed that increased IL-16 serum levels in samples from IAV-infected children and mice, and *IL16* overexpression facilitate IAV replication by inhibiting IFN-γ and ISG production, which are critical for the host resistance to IAV infection and inhibiting IAV replication, while IL-16 deficiency suppresses the replication (Jia et al., 2021). Finally, a study found a positive correlation between the serum IL-16 level and the severity of the disease in *C. difficile*

infection, indicating that IL-16 and T cells play a role in the immunopathology and progression of *C. difficile*-mediated disease (Gotshal et al., 2021).

### 1.9.4 IL-16 in Cancer

IL-16 is not only linked to disease states in infectious diseases and chronic autoimmune conditions but also in tumorigenesis. Elevated serum IL-16 levels have also been associated with different types of cancers, such as multiple myeloma, gastric cancer, colorectal cancer (Gao et al., 2009), hepatocellular carcinoma (HCC) (Takeba et al., 2021), breast cancer, gastrointestinal cancer, uterine/ovarian cancer, and renal/bladder cancer (Kovacs, 2001). Furthermore, higher serum levels of IL-16 have also been correlated with progression stages of cancer (Kovacs, 2001), and poor prognosis depending on the type of tumor (Alexandrakis et al., 2004).

In a study by Comperat *et al.* (2010), the researchers investigated whether the presence of IL-16 in prostate cancer tissue could be used as a prognostic factor for survival in 304 patients. They discovered that IL-16 expression was strongly associated with the aggressiveness of the tumor and the likelihood of biochemical relapse. Additionally, higher levels of IL-16 were correlated with a high Gleason score and were identified as an independent factor for survival in patients with prostate cancer (Comperat et al., 2010).

According to a study conducted by Yellapa *et al.* (2013), it was found that *IL16*-expressing cells were more prevalent in patients with ovarian cancer compared to those with benign ovarian tumors and healthy individuals. Accordingly, the concentration of IL-16 in the serum was significantly higher in ovarian cancer patients compared to those with benign ovarian tumors and healthy controls (Yellapa et al., 2013). Furthermore, participants with benign, early-stage, and finally late-stage ovarian tumors showed monotonic increases in serum IL-16 levels (Yellapa et al., 2014). Another study also revealed that the expression of *IL16* in tumor tissues and the levels of IL-16 in the serum positively correlated with the extent of tumor vasculature, indicating a potential role of this

cytokine in angiogenesis (Yellapa et al., 2013). Furthermore, the authors observed a significant increase in serum IL-16 levels even before the detection of ovarian tumors in animal models (Yellapa et al., 2012).

According to Atanackovic *et al* (2012), *IL16* is found to be significantly more expressed in the bone marrow of myeloma patients as compared to healthy blood donors. The study also demonstrated that *IL16* and its receptors CD4 and/or CD9 are constitutively expressed in myeloma cell lines and primary tumor cells from myeloma patients, and these cells spontaneously release soluble IL-16. Silencing IL-16 resulted in a reduction in the proliferative activity of myeloma cells by approximately 80% compared to untreated cells (Atanackovic et al., 2012). Additionally, a monoclonal antibody that blocks IL-16 or its receptors had a great suppressive effect on the growth of the tumor cells. Likewise, *IL16* has also been found to be overexpressed in primary effusion lymphoma cell line BCBL-1 (Arguello et al., 2006).

In a study conducted by Yang *et al* (2017), the objective was to examine the expression level of serum IL-16 in patients with GC and determine the diagnostic and prognostic significance of IL-16. The findings of this study indicated that serum IL-16 levels were notably higher in GC patients compared to healthy individuals. Additionally, elevated levels of serum IL-16 were significantly associated with tumor recurrence and a poor prognosis. Furthermore, the study demonstrated that suppressing IL-16 expression significantly inhibited the migration and invasion of GC cells. These findings suggest that serum IL-16 levels may have diagnostic and prognostic value for patients with GC (Yang et al., 2017). Patients with nasopharyngeal carcinoma had higher serum levels of IL-16 compared to the control group. This suggests that IL-16 may be linked to an increased risk of nasopharyngeal carcinoma by promoting the production of IL-16 (Qin et al., 2014). Moreover, previous studies have consistently shown that IL-16 protein is linked to advanced stages of multiple myeloma and other malignant tumors, leading to poorer survival rates (Alexandrakis et al., 2004) (Kovacs, 2001). However, in Kovacs' study (2001), IL-

16 levels were found to be elevated alongside IL-12, which is known to have anti-tumor effects (Yuzhalin and Kutikhin, 2012). Thus, the specific role of IL-16 in these conditions has not been fully clarified in this study. A recent study also discovered an association between the plasma level of IL-16 and biomarkers, clinical stage, and T cell phenotypes in chronic lymphocytic leukemia (CLL) patients. The study found that patients with CLL had higher levels of IL-16 in their plasma compared to healthy individuals and patients with monoclonal B-cell lymphocytosis (MBL). Furthermore, the levels of IL-16 were particularly elevated in patients with advanced stages of CLL and were correlated with lymphocyte count. Additionally, the study suggested that IL-16 can directly stimulate the expression of *Foxp3*, resulting in the creation of cells with suppressive function and potentially leading to the recruitment of Th-1 cells and the induction of immunosuppressive phenotypes. Therefore, an abundance of IL-16 may potentially have a role in the later stages of CLL, when there is an increase in both Th-1-like and Treg cells (Wu and Wu, 2023). Finally, a comprehensive study using the proteomics approach has also identified IL-16 as an important cytokine overexpressed in human HCC tissues from both non-tumor and tumor regions, and IL-16 secretion may activate the ERK/cyclin D1 signaling pathway, thereby causing tumor growth (Takeba et al., 2021).

Although the exact mechanism for how IL-16 may promote carcinogenesis is still undiscovered, IL-16 stimulates the production of various tumor-related cytokines, such as TNF-$\alpha$, IL-1$\beta$, IL-15, and IL-6, which may, in turn, boost tumor cell proliferation (Gao et al., 2009).

### 1.9.5 IL-16 polymorphisms

There are indications that genetic variations in the *IL16* gene may play a role in cancer risk such as lung cancer (Wu et al., 2020), oral cancer (Shih et al., 2020), osteosarcoma (OS) (Tang et al., 2016b), breast cancer (Balasubramanian et al., 2006), colorectal cancer (CRC) (Gao et al., 2009), GC (Gao et al., 2009), renal cancer (Zhu et al., 2010) and nasopharyngeal carcinoma (Qin et al., 2014).

A study in a Chinese population reported that the rs11556218 T/G variant of the *IL16* gene was found to be significantly associated with susceptibility to CRC and GC in both male and female patients. Individuals carrying the G allele had a higher risk of developing CRC and GC compared to those carrying the T allele. However, in women carrying the T allele (rs4072111 C/T), there was a decreased risk of CRC and GC compared to individuals carrying the C allele. Additionally, in patients with CRC or GC, the serum levels of IL-16 were significantly higher than in healthy controls. However, there was no significant association observed between *IL16* variants and serum levels of IL-16 (Gao et al., 2009). Another study found that individuals with the rs4778889 CC and rs11556218 GG genotypes had a higher risk of developing non-cardia gastric cancer in a Chinese population (Zhang and Wang, 2013) In contrast, previous studies by (Azimzadeh et al., 2011, Zhu et al., 2010)suggested that the CC genotype of the rs4778889 polymorphism was associated with a decreased risk of colorectal cancer and renal cancer, respectively. Furthermore, Azimzadeh *et al*. (2011, 2012) conducted a series of studies that demonstrated a correlation between the CC genotype and C allele of the rs1131445 polymorphism, as well as the TG genotype of the rs11556218 polymorphism, with an increased risk of colorectal cancer in the Iranian population (Azimzadeh et al., 2011, Azimzadeh et al., 2012). Another study conducted by Kashfi *et al*. 2016 detected an association between *IL16* rs1131445 T/C and rs4072111 T/C polymorphisms and gastric cancer susceptibility in the Iranian population and showed that the *IL16* rs1131445 T/C and rs4072111 T/C variants may be useful markers for gastric cancer (Kashfi et al., 2016).

Moreover, (Batai et al., 2012) demonstrated a significant association between prostate cancer risk and the rs7175701 and rs11556218 polymorphisms. The TG and GG genotypes, as well as the G allele of the rs11556218 polymorphism, and the TT genotype of the rs4072111 polymorphism, have been linked to a significantly higher risk of developing hepatitis B virus-related HCC (Li et al., 2011).

Previous studies have found that the TG genotype and G allele of the rs11556218 polymorphism are linked to an increased risk of developing nasopharyngeal carcinoma, colorectal cancer, and gastric cancer (Gao et al., 2009) (Qin et al., 2014). Furthermore, the G allele of the rs11556218 polymorphism has been correlated with higher levels of serum IL-16 in patients with nasopharyngeal carcinoma (Qin et al., 2014). In a meta-analysis of seven studies involving 1678 cases and 1937 controls, the rs11556218 T/G polymorphism of the *IL16* gene was found to be significantly associated with an increased risk of cancer in Asian populations. However, the rs4778889 and rs4072111 polymorphisms showed no association with cancer risk (Zhao et al., 2014).

A study focused on the crucial roles of genetic polymorphisms of interleukins to the transition of *H. pylori*-induced gastric lesions in a Chinese population at high risk of GC has reported that the rs4778889 polymorphism, located at position -295 in the promoter region of *IL16*, maybe a functional polymorphism that regulates promoter activity (Wang et al., 2016). A previous study found that individuals with the rs4778889 CC genotype had a higher risk of non-cardia gastric cancer (Zhang and Wang, 2013), while a meta-analysis showed no significant association between the rs4778889 polymorphism and cancer risk in the Asian population (Zhao et al., 2014). Another study reported no significant association between the rs4778889 polymorphism and the risk of GC but did find an increased risk of chronic atrophic gastritis (CAG) for individuals carrying the rs4778889 C allele, suggesting that the rs4778889 polymorphism may influence the early stages of gastric lesions (Wang et al., 2016). In summary, measuring IL-16 levels in both serum and tumor tissue, as well as examining specific *IL16* gene polymorphisms, may serve as a valuable biomarker for predicting and diagnosing various types of malignant tumors.

### 1.9.6 IL-16 in *H. pylori* infection

The role of IL-16 cytokine has been extensively studied in various inflammatory conditions and cancer types. However, there have been only limited studies investigating the involvement of IL-16 cytokine in *H. pylori* infection.

The presence of cytokeratin 20 (CK-20) in gastric carcinoma has been observed, and the detection of CK-20 expression can be utilized for identifying disseminated carcinoma. TGF-α is a significant growth factor that promotes mucosal repair by stimulating the migration and proliferation of mucosal cells. The association between *H. pylori* infection and gastric cancer is well-established, but the underlying mechanisms remain unclear. Nakajima *et al.* (2001) investigated the impact of IL-16 on the expression of CK-20 and TGF-α in the AGS gastric epithelial cell line infected with *H. pylori*. The co-incubation of AGS cells with *H. pylori* and IL-16 resulted in a greater increase in the expression of CK-20 and TGF-α compared to *H. pylori* alone (Nakajima et al., 2001). Additionally, it was found that administering IL-16 increased the expression of murine double minute 2 (MDM2) protein and cell proliferation in human gastric carcinoma cell line AGS co-incubated with *H. pylori*. It is important to note that MDM2 overexpression is seen in several types of cancers and is known to bind to tumor suppressor protein p53 (Iwakuma and Lozano, 2003). Based on these results, the authors suggested that IL-16 may be a risk factor for *H. pylori*-induced gastric cancer (Nakajima et al., 2009).

In 2016, the same research team investigated the impact of *H. pylori* and IL-16 on cell proliferation and the expression of insulin-like growth factor-1 receptor (IGF-1R) in gastric epithelial cells. IGF-1R plays a crucial role in cell growth factor signaling, cell proliferation, and differentiation in intestinal metaplasia and gastric cancer. The study found that *H. pylori* infection in gastric mucosa leads to early-stage carcinogenesis, indicated by increased expression of IGF-1R. Additionally, IL-16 production by *H. pylori* acts as a trigger for the

expression of IGF-1R and contributes to the development of gastric cancer (Nakajima et al., 2016).

**Overall hypothesis:** Elevated IL-16 levels in *H. pylori*-infected patients may contribute to the infection's chronicity through recruitment and inhibition of $CD4^+$ T-cells, resulting in gastric cancer development.

Due to current gaps in knowledge, this thesis **aimed** to investigate the role of IL-16 in *H. pylori* infection and gastro-duodenal disease. This was achieved by quantifying IL-16 in human gastric tissue and plasma, investigating IL-16 expression by human monocytic cells, and the relationships between IL-16, other cytokines, *H. pylori* virulence factors, and disease status. It also aimed to understand differential cytokine expression concerning gastric cancer progression and their potential as biomarkers for the disease.

# Chapter 2.Plasma and Gastric Levels of IL-16 in Patients with or without *H. pylori* Infection.

## 2.1 Introduction

A marked immune response has been reported during *H. pylori* infection is characterized by the infiltration of different immune cells such as mast cells, macrophages, dendritic cells, neutrophils, B cells, and T cells. Surprisingly, this response is not sufficient to clear the infection leading to the establishment of a chronic infection (Robinson et al., 2007) **().**

$CD4^+$ T cells play a crucial role in the immune response triggered by *H. pylori* infection. Previous research has reported that *H. pylori-specific* $CD4^+$ T cells preferentially migrate and accumulate in the infected stomach. Stomach biopsies from individuals infected with *H. pylori* showed higher levels of $CD4^+$ T cells in the lamina propria compared to *H. pylori*-negative individuals. The *H. pylori*-infected gastric mucosa is infiltrated with $CD4^+$ T cells that are considered pathogenic Th17 (Pinchuk et al., 2013) or inhibitory Treg for effector Th1 cells (Lundgren et al., 2005). However, there is limited knowledge about the mechanisms involved in recruiting these $CD4^+$ T cells to the infected mucosa. Despite an increase in the number of $CD4^+$ T cells in the gastric lamina propria during *H. pylori* infection, these cells are less responsive and tend to polarize towards a Th1 response (Pellicano et al., 2007). Th1 cells produce IFN-γ, which is essential for the proinflammatory reactions induced by *H. pylori* infection. Previous studies have demonstrated that T cells exposed to *H. pylori* exhibit reduced rates of proliferation (Knipp et al., 1994, Pellicano et al., 2007), and when *H. pylori*-exposed gastric epithelial cells are incubated with isolated $CD4^+$ T cells, it leads to impaired proliferation and IL-2 production (Das et al., 2006). While some studies suggest that *H. pylori* may suppress human T cells, it is important to note that *H. pylori* does not directly contact lamina propria T cells. Instead, *H. pylori* resides in the gastric lumen and is separated from T cells by the epithelial barrier. Therefore, *H. pylori* utilizes the gastric epithelium to communicate with T cells in the lamina propria, influencing their function and differentiation.

During *H. pylori* infection, various cytokines/chemokines are generated to attract other cells (section 1.8). However, IL-16 is unique among cytokines as it specifically binds to CD4 as a receptor and selectively attracts $CD4^+$ cells (Berman et al., 1985, Cruikshank et al., 1994). IL-16 uses CD4 as a receptor and after binding, IL-16 induces intracellular signal transduction that stimulates $CD4^+$ T-cell proliferation and migration into inflamed areas. However, binding of IL-16 to its receptor, CD4, leads to inhibition of T-cell receptor (TCR)/CD3-mediated activation, and T-cell anergy (Wilson et al., 2004). Despite extensive research on IL-16 cytokine in various inflammatory conditions, its role in *H. pylori* infection has not been thoroughly investigated. IL-16 has been observed to be elevated in different inflammatory conditions (section 1.9.3), and in various *in vivo studie*s to be an immunomodulatory cytokine that plays a role in the recruitment and activation of $CD4^+$ T cells at inflammatory sites, particularly concerning asthma and autoimmune diseases (Cruikshank et al., 2000). Therefore, IL-16 may be accountable for attracting $CD4^+$ T cells and suppressing effector T cells, which play a crucial role in adaptive immunity. This, in turn, could facilitate the development of a persistent *H. pylori* infection **(Figure 10**).

Because of these findings, **it was hypothesized that:**

1. Higher concentrations of IL-16 are present in the plasma and gastric mucosa of *H. pylori*-positive patients, which affects the recruitment and activation of $CD4^+$ T cells during the infection.
2. IL-16 concentrations are higher in patients with infected more virulent *H. pylori* strains, and in those with more severe gastric inflammation and damage.
3. IL-16 concentrations are associated with those for other co-expressed inflammatory cytokines.
4. Lower IL-16 concentrations are present in the plasma of female patients, who tend to be at reduced risk of peptic ulcer disease and gastric cancer development.
5. Elevated systemic IL-16 levels are associated with smoking history.

6. Higher IL-16 concentrations are present in the serum of patients with gastric cancer.
7. plasma IL-16 concentrations are reduced following *H. pylori* eradication.

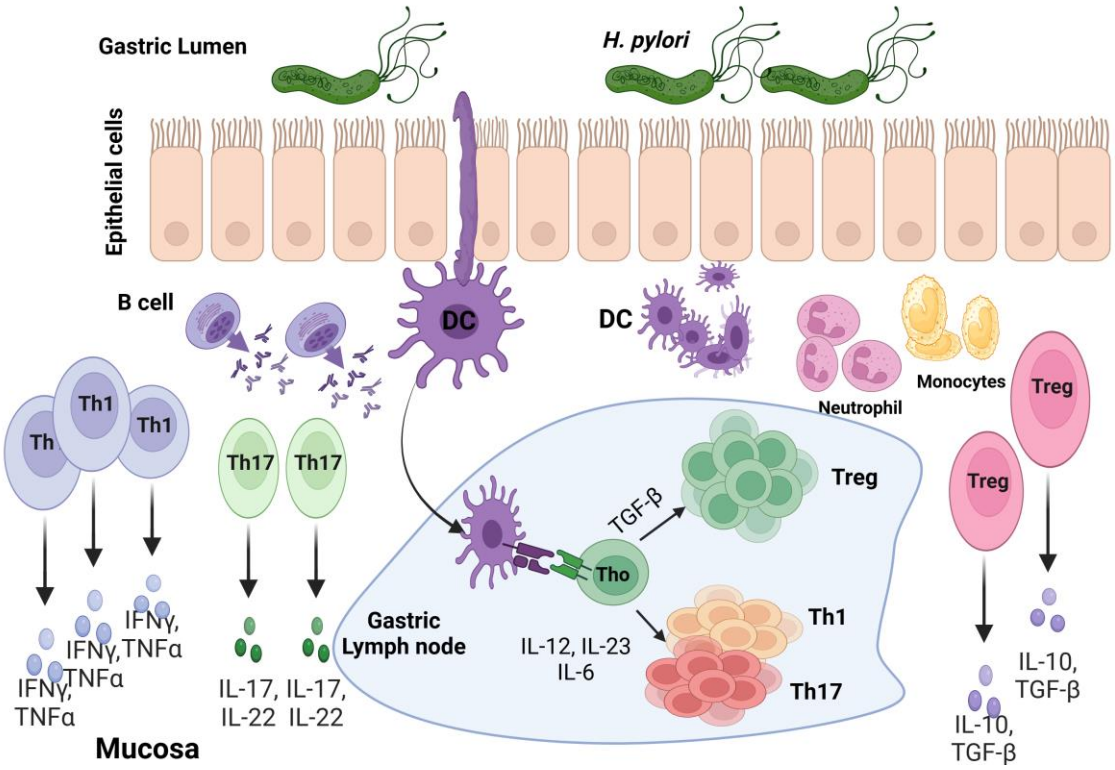



**Figure 10. A model for mucosal immune modulation during *H. pylori* colonization**

The colonization of the gastric epithelium by *H. pylori* leads to the accumulation of various immune cells, including macrophages, dendritic cells (DCs), neutrophils, B cells, and T cells. Resident mucosal DCs sample luminal *H. pylori*, and then migrate to stomach-draining lymph nodes. *H. pylori* induces DCs to undergo tolerogenic programming, skewing naïve T cells towards higher Th1, Th17, and Treg. This figure was created with BioRender.com.

## 2.2 Materials and Methods

### 2.2.1 Patients and clinical materials

Gastric biopsies and 10 ml peripheral blood were collected from patients undergoing routine upper-intestinal endoscopy at the Queens Medical Centre, Nottingham (with University of Nottingham Ethics approval), with informed written consent and approval from the Nottingham Research Ethical Committee 1 (REC Ref:12/EM/0446).

Patients who had been taking NSAIDs, proton pump inhibitors (PPT), antibiotics, or immunosuppressive therapies in the last 4 weeks were not included in the study and were not asked to provide samples. The use of probiotics and/or H2 receptor blockers did not lead to exclusion. None of the donors had any other health conditions that could affect immune regulation. The age of the participants ranged from 18 to 70, with an average age of 55. None of the donors had a clinically diagnosed allergy or asthma that required medical intervention, but 18% (9 out of 50) had a history of sub-clinical atopy, and 6% (3 out of 50) had a history of adult asthma. Information on age, gender, and relevant medical history, including peptic ulcer disease, was collected for all *H. pylori* participants who were enrolled in the study.

In addition to the urease breath test (UBT), a biopsy sample from the antrum was tested for *H. pylori* using the CLOTM Test. Biopsies from both the antrum and corpus were cultured in 1ml iso-sensitise medium with 15% glycerol within 3 hours. Another biopsy from the corpus was immediately stored in RNA Later for RNA stabilization and extraction. Two more biopsies from the antrum and corpus were placed in formalin and sent for histopathology examination. Blood samples were collected, and the plasma was separated and stored at -80°C. The *cagA* status of the *H. pylori* strain was determined using the CagA Enzyme-Linked Immuno-Sorbent Assay (ELISA) (Genesis) with plasma donated by the patients.

### 2.2.2 Eradication study clinical samples

The first blood sample (Month 0) was collected after patients were diagnosed with *H. pylori* infection. Then, first-line triple therapy was prescribed (Amoxicillin, PPI, clarithromycin/metronidazole; 7-day course) for 4-10 weeks. Enrolled participants returned 2 months after starting the treatment, and the UBT was performed to determine if the eradication therapy had been successful. If the UBT was negative, whole blood was then taken at specific time points (2, 6, 12, and 24 months after eradication) and labeled accordingly e.g., T001M0, T001M2, T001M6, T001M12, and T001M24. If UBT was positive, a second-line treatment regimen was prescribed until a negative UBT result was obtained. Individuals with eradication failure were analyzed as a separate control group. Whole blood was collected in vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) anticoagulant (Greiner). Patient Demographic Information is shown in (**Table *2***)

**Table 2. Patient demographic information.**

| | |
|---|---|
| **The number of infected** | **50** |
| **Male: Female** | **24:26** |
| **Mean age (years)** | **55.5(18-70)** |
| **Non-smoker** | **52% (26/50)** |
| **Ex-smoker** | **20% (10/50)** |
| **Smoker** | **28% (14/50)** |
| **Allergy/atophy** | **18% (9/50)** |
| **Adult asthma** | **6% (3/50)** |
| **Peptic ulcer disease** | **20% (10/50)** |
| **Multiple eradication attempts** | **14% (7/50)** |

## 2.2.3 Isolation and analysis of RNA

### 2.2.3.1 Preparation of RNA from gastric biopsies

Endoscopic gastric biopsies obtained from patients for RNA extraction were immediately transferred to 250µl of RNA later stabilization reagent (Qiagen, UK) and stored at -80˚C according to the manufacturer's recommended protocol. Biopsies samples were thawed on ice and transferred to 600µl of Buffer RLT and β-mercaptoethanol (Qiagen, UK), and homogenized for 30-50 seconds using a T8 ultra Turrax rotor-stator homogeniser (IKA, Werke & Co., Freiburg, Germany). The homogenized samples were transferred to QIA shredder columns (Qiagen, UK) and spun for 2 minutes at full speed. The lysate was centrifuged for 3 minutes at maximum speed, and the supernatant was transferred to a new microfuge tube. Total RNA was prepared using the RNeasy® Mini Kit (Qiagen, UK) according to the manufacturer's protocol. To elute the RNA, 50 µl of RNase-free water was added directly to the spin column membrane and centrifuged for 1 min at 11200x g The eluted RNA samples were stored at -80˚C.

### 2.2.3.2 Quantitation of total RNA

After the total RNA was extracted from the biopsy, the total RNA concentration (expressed as ng/µl) was measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All samples show satisfactory purity (A260/A280 1.95-2.06 and A260/A230 1.95-2.37).

### 2.2.3.3 cDNA synthesis

The RNA preparations were reverse-transcribed using a Superscript II reverse-transcription kit (Invitrogen). Reactions were carried out in a total volume of 18 µl consisting of Oligo(dT)$_{12-18}$ Primer (500 µg/mL), 10 mM of each dNTP mix, nuclease-free water, and 6µl of RNA at a concentration of 33.3 ng/µl. The mixture was incubated at 65°C in a PCR block for 5 min, then immediately in

chill ice for 2 min. The contents of the tubes were collected by brief centrifugation, and 4 µl of 5X First-Standard Buffer, 2 µl of 0.1 M DTT, and 1 µl RNaseOUT were added to each tube. The mixture was incubated at 42°C in a PCR block for 2 min, then 1 µl Superscript II was added to positive reaction only, and water to negative reactions. The reactions were incubated at 42°C for 50 min, followed by inactivation by heating at 70°C for 15 min in a PCR block. cDNAs were stored at -20°C until required.

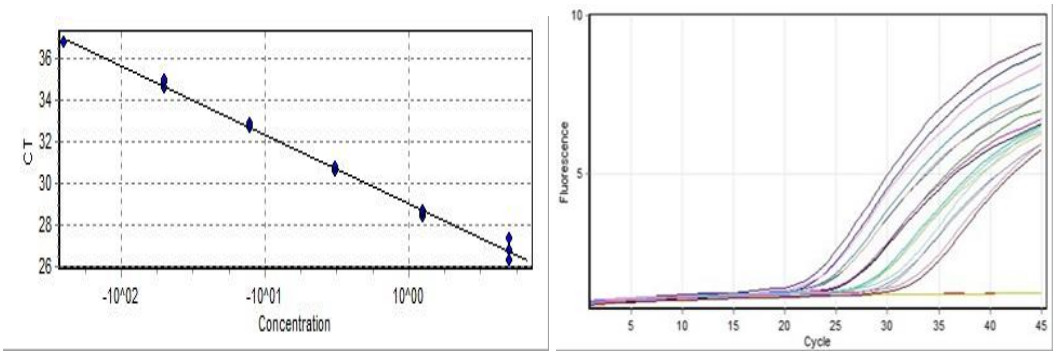
### 2.2.4 qPCR Assay validation and primer efficiency determination.

First, the qPCR primers were validated by generating standard curves for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* and *IL16* **(Figure 11; A-B)**. These curves were used to determine the efficiency of each assay and establish the threshold value for subsequent quantification. The PCR reaction efficiencies were 2.12 for *GAPDH* and 1.87 for *IL16*. In qPCR, an ideal reaction will result in a doubling of PCR products during each cycle, resulting in an efficiency value of 2.0. The efficiency, linearity, slope, and y-intercept of each qPCR assay are presented in **(Table 3).**

**Table 3. Efficiency and linearity of the qPCR assays**

| Target Gene | Efficiency (E) | Linearity ($R^2$) | Slope (M) | Intercept (B) |
|---|---|---|---|---|
| ***GAPDH*** | 2.12 | 0.995 | -3.317 | 29.0127 |
| ***IL16*** | 1.87 | 0.99702 | -3.66244 | 24.46471 |

**A. *GAPDH***




**B. *IL16***

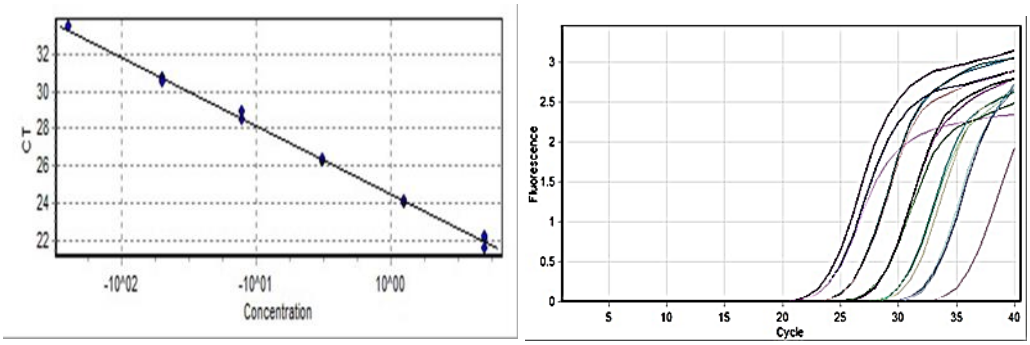



**Figure 11. A dilution series was used to determine the PCR efficiency and linearity of each specific qPCR assay.**

The qPCR efficiency and linear range of each qPCR primer assay were evaluated. A 7-fold serial 1:2 dilution of commercial total human reference cDNA was used as a standard. Figures A and B show the standard curves and raw amplification plots for *GAPDH* and *IL16*, respectively. The values for efficiency (E), slope (m), and linearity (R2) can be found in (**Table 3**).

### 2.2.5 Quantitative reverse transcriptase real-time PCR (RT-qPCR)

RT-qPCR was performed on a RotorGene 3000 instrument (Corbett Research) using RotorGene SYBR green PCR mixture (Qiagen; Cat No. 204074) and *IL16* primer from Sigma-Aldrich, and the reference gene, *GAPDH,* **(**

*Table 5*). The PCR cycling conditions can be found in **(Table 4**). The reactions were carried out in 20 $\mu$l volumes as instructed by the manufacturer or as previously described by(Robinson et al., 2008). To check for contamination, no-template controls were included using RNase/DNase-free water instead of template cDNA. A melt analysis was performed to confirm the specificity of the primer assays. To ensure the absence of genomic DNA contamination, a minus RT control was included, where water was used instead of reverse transcriptase, and it was run alongside the samples. A positive control was included using commercially available oligo(dT)-primed total human reference cDNA (Clontech; Cat No. 636692). Each sample was run in duplicate, and for comparison, pooled cDNA from *H. pylori*-negative donors was produced and tested in triplicate. The results of the samples and comparator were normalized to the housekeeping gene, *GAPDH*, which is expected to be stably expressed regardless of experimental conditions. PCR reaction efficiencies were calculated using standard curves generated from serially diluted cDNA, and all assay efficiencies ranged from >1.8 to <2.1. Data analysis was performed using the PfaffI method 360 to determine relative expression ratios.

**Table 4. RT-qPCR cycling conditions*: GAPDH* and *IL16*.**

| | | | |
|---|---|---|---|
| 95 °C | 15 minutes | Activate HotStart enzyme | |
| 95 °C<br>60 $^0$C<br>72 °C | 15 seconds<br>30 seconds<br>30 seconds (acquire SYBER signal) | Denature dsDNA.<br>Primer annealing.<br>Primer extension. | X45 cycles |
| 72°C | 10 minutes | Final extension | |
| 72-95°C | | Melting analysis | |

**Table 5. PCR primer sequences: *GAPDH* and *IL16*.**

| Gene | Primer | Sequence | Tm ($^0$C) | Length (bp) | Size (bp) |
|---|---|---|---|---|---|
| ***GAPDH*** | Forward | 5'-CCACATCGCTCAGACACCAT-3' | 58.62 | 20 | 114 |
| | Reverse | 5'-GGCAACAATATCCACTTTACCAGAGT-3' | 62.42 | 26 | |
| ***IL16*** | Forward | 5'-TTGGACACAGGGTTCTCGCTCA-3' | 71.3 | 22 | 148 |
| | Reverse | 5'-AGCAGGGAGATAACGGACTGAC–3' | 65.5 | 22 | |

## 2.3 Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 8 software. Individual data points are shown with the median. Statistically significant differences were determined by Kruskal-Wallis tests for three or more groups and Mann-Whitney U-tests for two groups analysis. Differences were considered significant at $p<0.05$*, $p<0.01$** and $p<0.001$***.

## 2.4 Results

### 2.4.1 Plasma IL-16 concentrations in *H. pylori*-infected patients compared to uninfected patients.

To investigate whether IL-16 production increases during *H. pylori* infection, the level of immune reactive IL-16 in plasma samples collected from *H. pylori*-infected patients (n=107) and non-infected patients (n=146) was measured using a human IL-16 ELISA kit. IL-16 was detected in all the plasma samples examined from *H. pylori*-infected patients and non-infected patients, with no significant differences between the two groups. The most notable feature was the wide range in concentrations, from 135.68 to 2788.456 pg/ml **(**

**Figure *12*).**

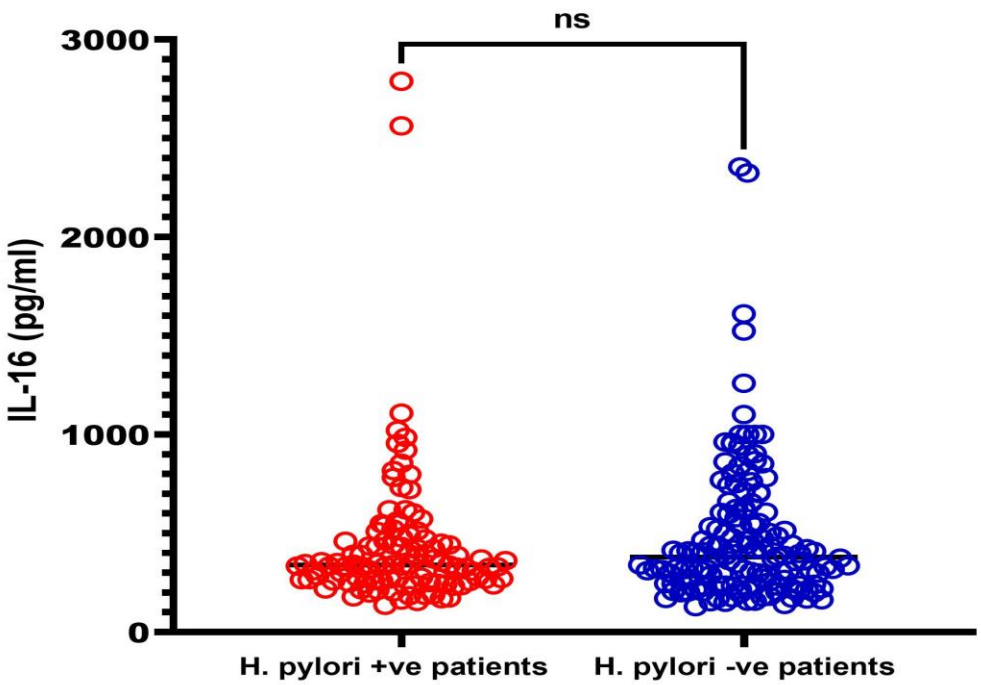



**Figure 12. IL-16 concentrations in plasma samples from *H. pylori-infected* and uninfected individuals.**

The level of IL-16 in each plasma sample was plotted and compared based on the *H. pylori* infection status. Plasma samples from *H. pylori-positive* patients (*H. pylori* +ve) (n =107) showed no significant difference in IL-16 level compared to *H. pylori*-negative individuals (*H. pylori* -ve) (n=146), using a Mann-Whitney U-test. The median IL-16 concentration for patients infected with *H. pylori* was 339.8 pg/ml, and 379.4 pg/ml for non-infected patients is displayed as horizontal lines. Ns= not significant.

### 2.4.2 IL-16 plasma levels in *H. pylori-associated* diseases.

Additionally, Since *H. pylori* infection is associated with different clinical outcomes ranging from asymptomatic gastritis to peptic ulceration and gastric cancer, it was important to investigate whether the plasma IL-16 concentrations varied according to the presence of *H. pylori* chronic gastritis (n=48), *H. pylori* peptic ulcer (n=61) or gastric cancer (n=9) (N.B. two of these patients were *H. pylori* negative), and a control group of healthy individuals, matched in age and gender to the gastric cancer group (n=26) Six of these controls were *H. pylori* positive). Analysis of the data revealed no significant difference between *H. pylori*-infected patients with chronic gastritis and *H. pylori*-infected patients with duodenal ulcers. However, the data showed a significant increase in circulating IL-16 levels in *H. pylori-infected* patients with chronic gastritis compared to IL-16 plasma concentrations in gastric cancer patients (p=0.0052), Also a significant increase in plasma IL-16C level was observed in *H. pylori*-infected patients with chronic peptic ulcer compared to IL-16 plasma level in patients with gastric cancer (p=0.0006) (

**Figure 13**).

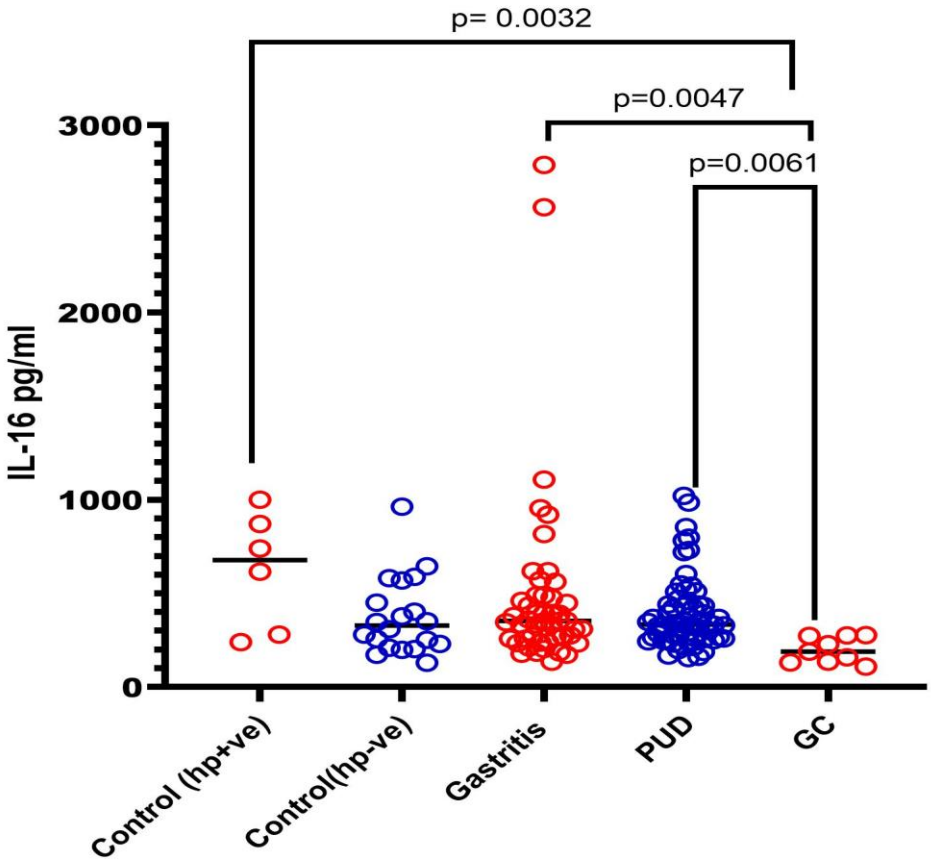



**Figure 13. Comparison of plasma IL-16 concentrations in *H. pylori*-associated diseases.**

The level of IL-16 in each plasma sample was plotted and compared based on the *H. pylori*-associated disorders such as gastritis, peptic ulcer disease (Frezzolini et al.), and gastric cancer (GC). The data showed no significant differences in IL-16 plasma level for patients with *H. pylori* chronic gastritis (n=48) compared to patients with *H. pylori* and a duodenal ulcer (n=61), and healthy controls (n=26) using a Kruskal-Wallis test. Significantly increased IL-16 levels were observed in samples from healthy controls and patients with *H. pylori* chronic gastritis, and PUD compared to IL-16 plasma concentration in GC patients. The IL-16 median level for healthy controls who are *H. pylori* positive (hp+ve) was 678.6 pg/ml while the IL-16 median level for healthy controls who are *H. pylori* negative (hp-ve) was 328 pg/ml, patients with chronic gastritis was 352.7 pg/ml, 334 pg/ml for patients with PUD, and 189.7pg/ml for cancer patients.

### 2.4.3 Plasma IL-16 levels in patients with *CagA*-positive and *CagA*-negative *H. pylori* infections.

*H. pylori* can be classified into *cagA*-positive and *cagA*-negative colonizing strains by PCR and according to the presence or absence of plasma anti-CagA IgG antibodies. *cagA*+ strains are linked to an increased risk for peptic ulcer diseases and gastric cancer, and the expression of *cagA* by the colonizing strains is also linked to increased inflammation and IL-8 production (Fazeli et al., 2016). The gastric mucosa of *H. pylori* patients infected with *cagA-positive* strains showed considerably higher mucosal levels of IL-1 $\beta$ and IL-8 than those infected with *cagA-negative* strains. Furthermore, those with *H. pylori* infection especially those with *cagA*-positive strains—have been shown to have higher serum levels of the inflammatory cytokine IL-18. Thus, the implications of strain *cagA* status in IL-16 responses were considered. To find out if *cagA* status was associated with increased IL-16 production in *H. pylori-infected* patients, plasma IL-16C levels in *H. pylori-infected* patients were measured by ELISA and compared according to the presence and absence of the plasma anti-CagA IgG antibody. The IL-16 median level for patients infected with *cagA*+ strains was 337.6 pg/ml, and 347.1 pg/ml for patients infected with *cagA*- strains. Also, no significant differences were found between patients infected with the *cagA*+ strain (n=62) compared to patients infected with a *cagA*- strain (n=39) (**Figure 14**).

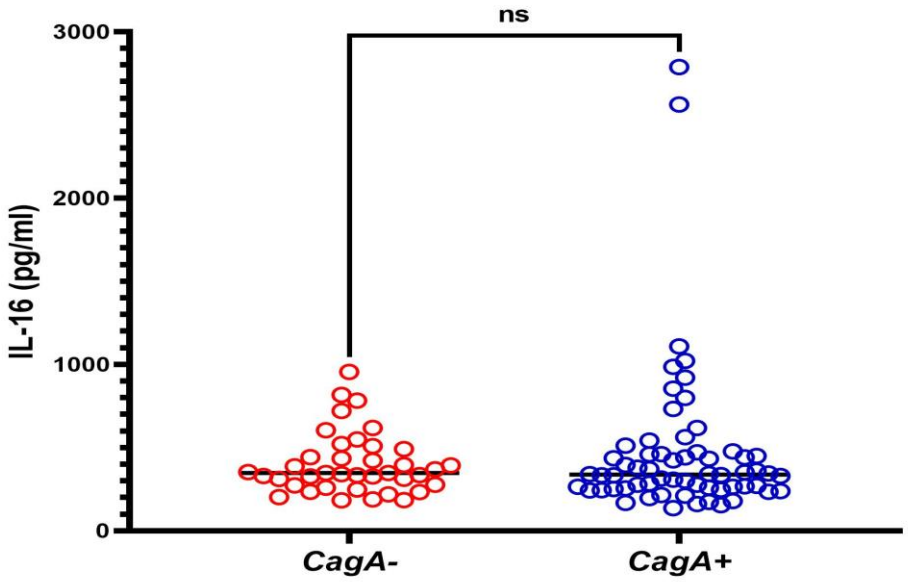



**Figure 14. Comparison of plasma IL-16 levels during *H. pylori* infection with *cagA* positive and negative strains.**
The level of IL-16 in each plasma sample was plotted and compared based on the presence and absence of anti-CagA IgG on the *H. pylori-infected* strain. The IL-16 median level for patients infected with *cagA*$^+$ strains was 337.6 pg/ml, and 347.1 pg/ml for patients infected with *cagA*$^-$ strains. No significant difference between patients infected with a *cagA*$^+$ strain (n=62) in compared to patients infected with a *cagA*$^-$ strain (n=39). The level of IL-16 in each plasma sample was plotted and compared based on the presence and absence of anti-CagA IgG in the plasma of infected patients using a Mann-Whitney U-test. ns= not significant.

### 2.4.4 The effect of gender and smoking on *H. pylori's* ability to induce IL-16 production.

Smoking can impact systemic immunity and inflammation in various ways, and IL-16 expression has been reported to be significantly affected by tobacco smoke in bronchoalveolar lavage fluid (BLF) and in isolated human blood CD4$^+$ and CD8$^+$ lymphocytes *in vitro* (Andersson et al., 2004). Previous studies investigating the impact of smoking on immune responses in the lungs and systemic level have yielded conflicting results, with some reporting elevated

levels of cytokines (Laan et al., 1999b, Andersson et al., 2004) and others reporting reduced levels (Shiels et al., 2014).

Because of a global pattern of male predominance for GC, oestrogen and oestrogen receptors have been thought to play a role in GC. Some researchers have argued that oestrogen is protective against GC, as men who receive estrogen therapy for prostate cancer, and women with delayed menopause have a lower risk of gastric cancer indicating the sex disparity in the rate of infection in GC patients (Lindblad et al., 2004).

In this study, further analysis of the data also showed that plasma IL-16C levels in *H. pylori*-positive patients were not influenced by gender or smoking. In the *H. pylori*-positive females (n=47), the IL-16 median levels were 329.2 pg/ml, and 354.1 pg/ml in positive males (n=38). This result suggests that gender does not affect plasma IL-16 levels during *H. pylori* infection **(Figure 15.B**). Differences between *H. pylori* positive and negative female patients were not statistically significant, and a similar result was found when comparing infected and uninfected male patients (data not shown). Plasma IL-16 levels amongst *H. pylori*-positive patients were analyzed according to whether they were from smokers (n=23), ex-smokers (n=11), and non-smokers (n=73). No significant differences were observed between the three groups **(Figure 15.A).**

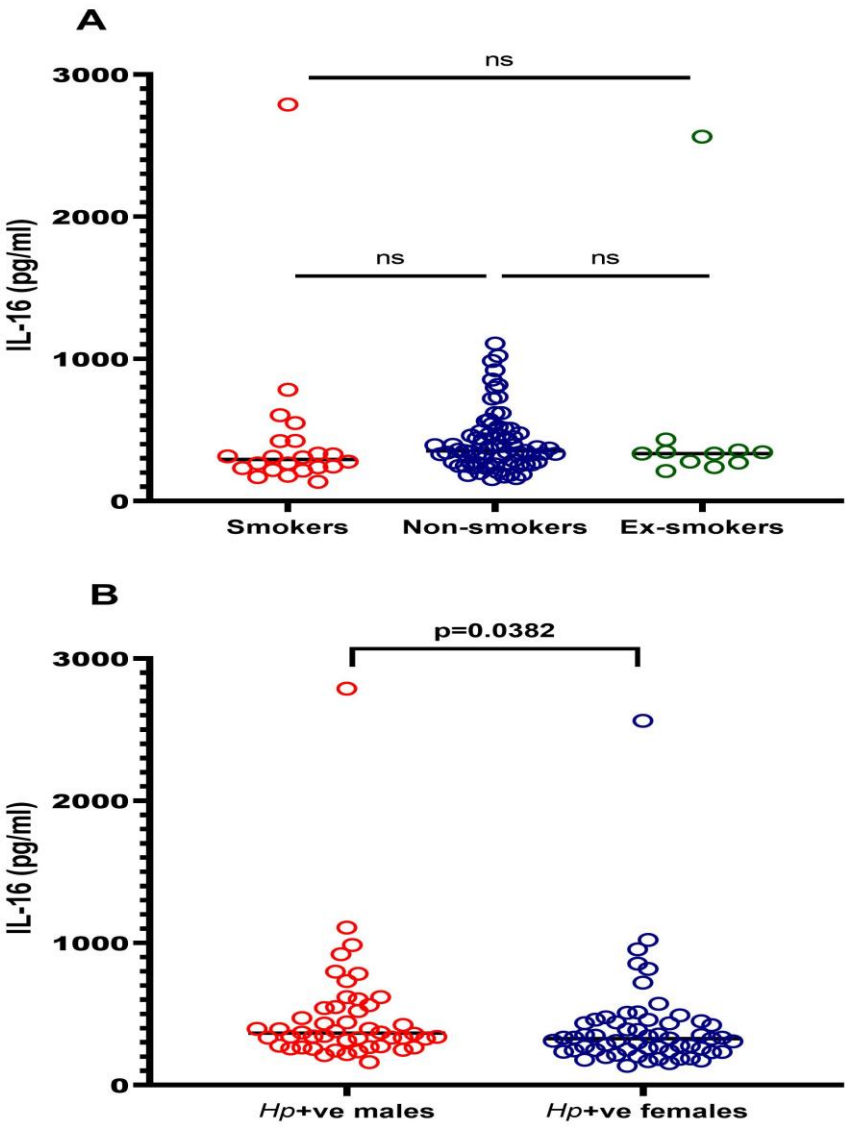



**Figure 15. The effect of gender and smoking on IL-16 plasma level during *H. pylori* infection.**

**A).** Plasma IL-16 from *H. pylori* smokers' patients (n=23), ex-smokers (n=11), and non-smokers (n=73) were also measured by ELISA, and each plasma sample was plotted and compared based on smoking status. Using a Kruskal-Wallis test, no significant differences were observed between the three groups. **B).** In *H. pylori*-positive (*Hp*+ve) females (n=59), the median level was 327.2 pg/ml, while 365.2 pg/ml in *H. pylori*-positive males (n=48). Using a Mann-Whitney U-test, a significant difference in plasma IL-16 levels between the male and female infected with *H. pylori,* but it was most likely not due to biological impact. Horizontal lines represent the median values for each group. ns= not significant.

### 2.4.5 Investigating the potential relationship of plasma IL-16 with gastric mucosal histopathological changes in *H. pylori*-infected patients.

Histopathological changes in the gastric mucosa during *H. pylori* infections, including metaplasia, atrophy, and dysplasia are considered precursors of gastric cancer. We aimed to examine the histopathological severity scores from gastric mucosal biopsies from *H. pylori*-infected patients in relation to the level of IL-16 cytokine. The severity of gastric pathology was graded using the Sydney scoring system on a scale of 0 to 3 (not present, mild, moderate, and severe) for parameters of chronic inflammation, atrophy, and intestinal metaplasia, in addition to the density of *H. pylori* in samples from the antrum and corpus of the stomach **(Figure *16*).** The data revealed no significant differences between colonization scores and plasma IL-16 levels in both antrum and corpus. Similarly, plasma IL-16 levels were not significantly different according to scores for chronic inflammation, atrophy, and intestinal metaplasia in the antrum as well as corpus (**Figure *17*** and **Figure *18***).

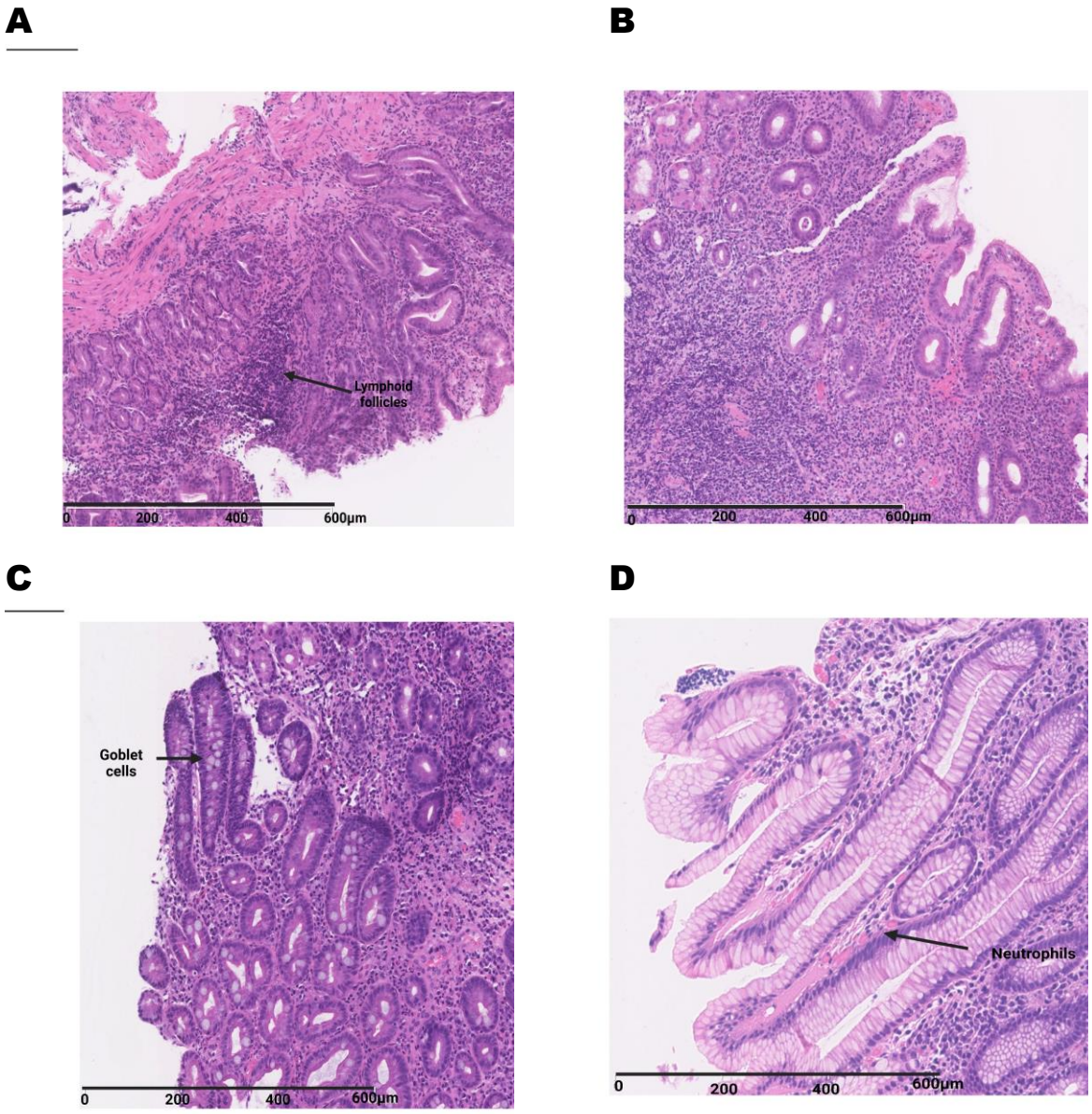



**Figure 16. Mucosa images (from corpus) from a representative patient diagnosed with gastritis, intestinal metaplasia, and atrophy**. **A)** Grade III inflammation in the corpus featuring lymphoid follicles. **B)** Grade II atrophy in the corpus, marked by increased spacing between the gastric glands due to some being lost. **C)** Grade II intestinal metaplasia, indicated by the presence of goblet cells. **D)** An example of corpus activity, showing neutrophils deep within the glands.

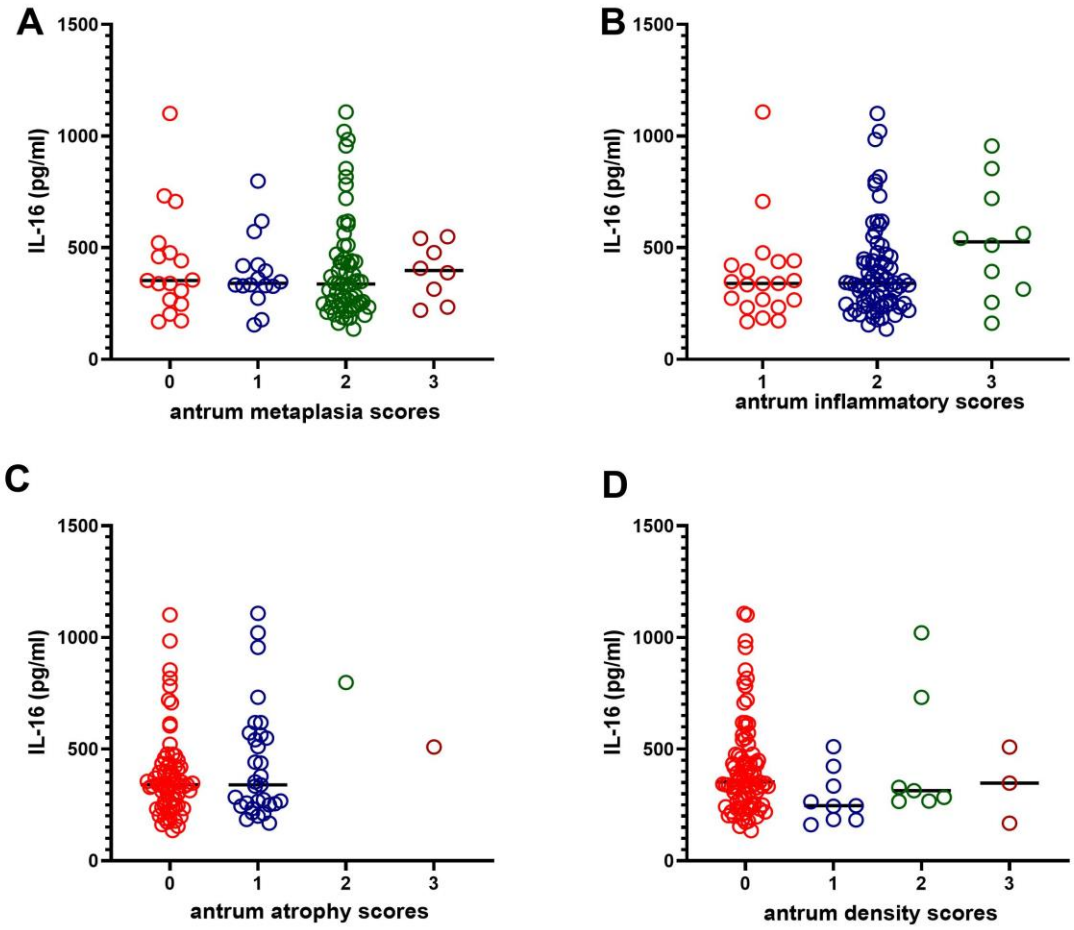



**Figure 17.Relation of IL-16 cytokine to histopathological changes in stomach antrum of *H. pylori*-positive patients.**

Pathology scores for stomach antrum of *H. pylori*-infected patients were assigned by using four parameters: *H. pylori* density, intestinal metaplasia, chronic inflammation, and antrum atrophy (A, B, C, D). Using a Kruskal-Wallis test, the data shows no relation between plasma IL-16 level and the pathological scores for the four parameters. The median for each score is displayed as a horizontal line. 0= no inflammation, 1= mild inflammation, 2= moderate inflammation, and 4 sever inflammation. No significant difference between IL-16 levels and the pathological scores for the four parameters.

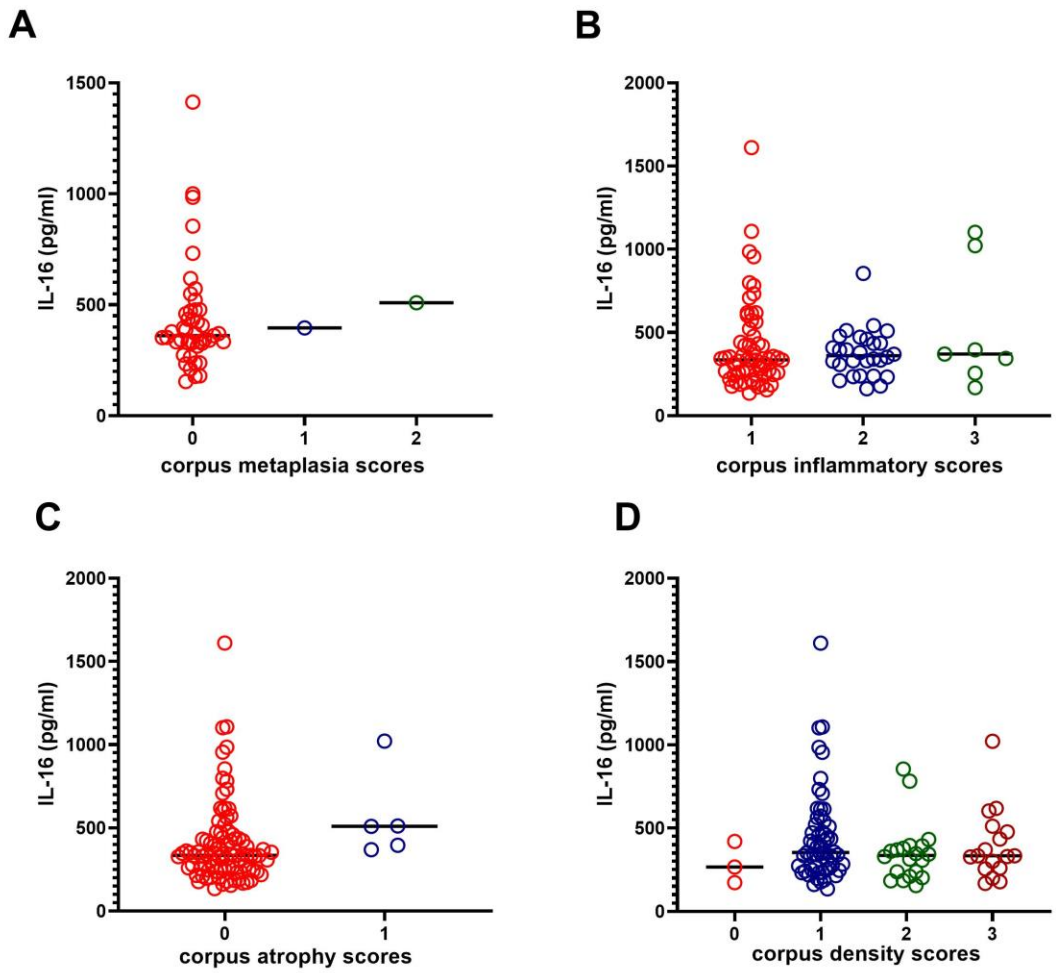



**Figure 18. Relation of IL-16 cytokine to histopathological changes in stomach corpus of *H. pylori*-positive patients.**

Pathology scores for stomach corpus of *H. pylori*-infected patients were assigned by using four parameters: *H. pylori* density, chronic inflammation, intestinal metaplasia, and corpus atrophy (D, A, C, B). The data shows no relation between plasma IL-16 level and the pathological scores for the four parameters using a Kruskal-Wallis test. The median for each score is displayed as a horizontal line. 0= no inflammation, 1= mild inflammation, 2= moderate inflammation, and 4 sever inflammation. No significant difference between IL-16 levels and the pathological scores for the four parameters.

### 2.4.6 Association between IL-16 plasma levels and oesophageal disorders.

To examine if there is a potential link between plasma IL-16 concentrations and oesophageal diseases in patients with either *H. pylori* positive or negative status, participants were categorized into different categories based on their specific esophageal condition: no oesophageal disorder (non) gastroesophageal reflux disease (GERD), Barrett's oesophagus (BO), and an oesophageal ulcer (OU). The data shows no relation between plasma IL-16 levels and the oesophageal disorders **(Figure 19).**

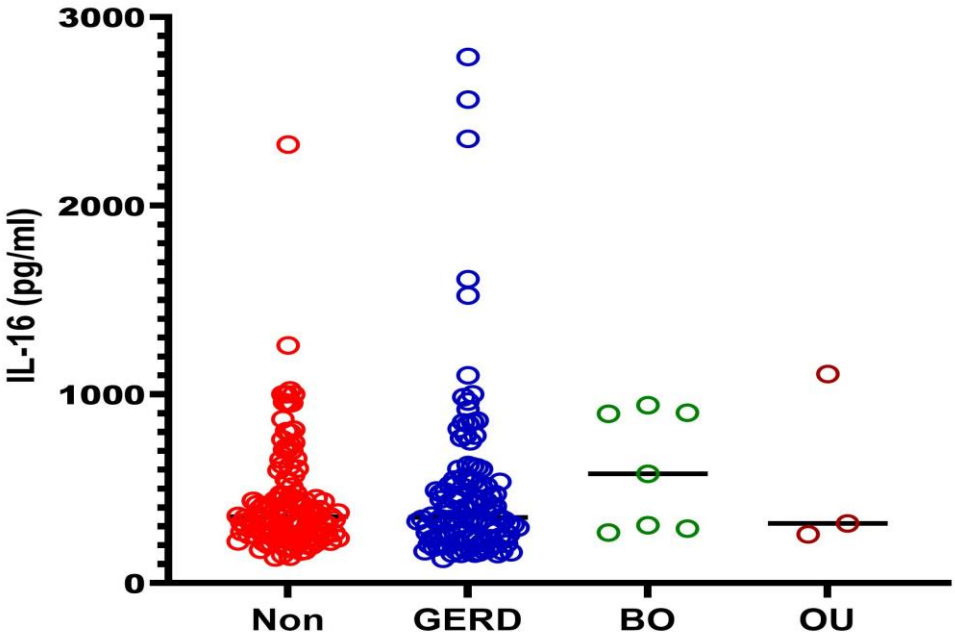



**Figure 19. Comparison of plasma IL-16 concentrations and oesophageal diseases.**

The level of IL-16 in each plasma sample was plotted and compared based on esophageal diseases such as gastroesophageal reflux disease (GERD), Barrett's oesophagus (BO), oesophageal ulcer (OU), and patients without the oesophageal disorder (non). The data showed no relation between IL-16 plasma level for patients with no esophageal diseases (n=125), GERD (n=117), BE (n=7), and OU

(n=3) using a Kruskal-Wallis test. The IL-16 median level for patients with no oesophageal diseases was 352 pg/ml, 347.9 pg/ml for patients with GERD, 579.7 pg/ml for patients with BO, and 315.9 pg/ml for patients with OU.

### 2.4.7 The impact of age on the circulating levels of IL-16 in patients who are positive or negative for *H. pylori*.

Participants were classified based on their age into three different groups (17-36, 41-60, and 61-82 age groups). In *H. pylori*-positive participants, the median IL-16 plasma levels were 379.1 pg/ml in the age group 17–36 years, 334.582 pg/ml in the age group 41–60 years, and 347.092 pg/ml in the age group 61–82 years **(Figure *20*).** In *H. pylori*-negative participants, the median IL-16 plasma levels were 386.303 pg/ml in the age group 17–36 years, 365.992 pg/ml in the age group 41–60 years, and 419.66 pg/ml in the age group 61–82 years (**Figure *20*Error! Reference source not found.**). There was no significant difference in circulating levels of IL-16 between different age groups among both *H. pylori*-negative as well as positive patients. Additionally, no significant difference in plasma IL-16 levels within the same age group was observed between *H. pylori* positive and negative patients. Thus, IL-16 plasma levels appear to be unaffected by age.

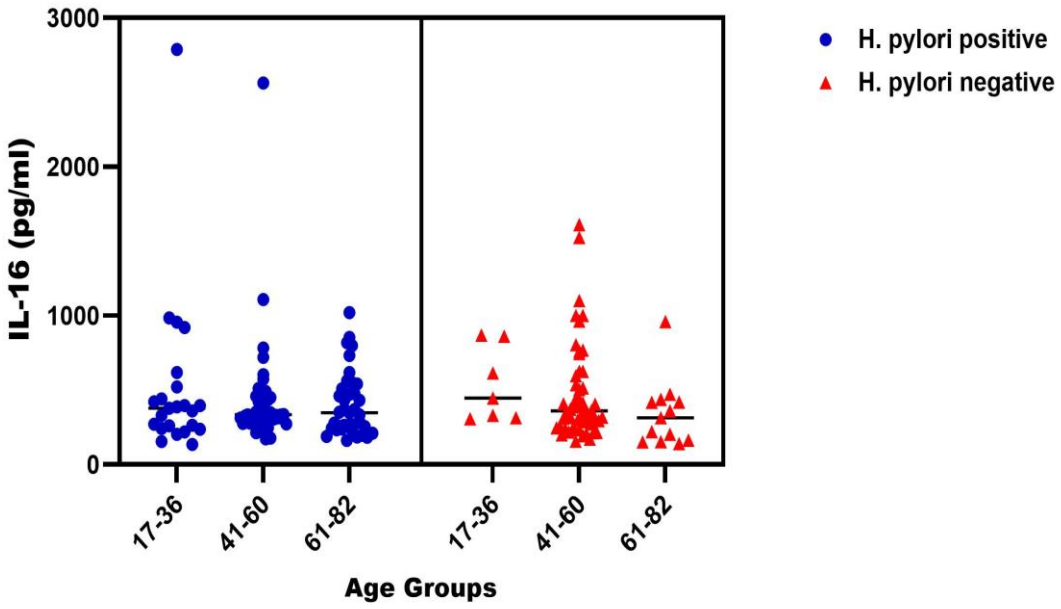



**Figure 20. Effect of age on IL-16 plasma levels among H. pylori-infected and non-infected patients.**

The concentration of IL-16 in each plasma sample was graphed and analyzed to compare different age groups and the presence or absence of *H. pylori* infection. Using a Kruskal-Wallis test, there were no notable variations in the levels of IL-16 in the plasma across different age groups, regardless of whether patients were infected with *H. pylori* or not. Furthermore, there were no notable differences in plasma IL-16 levels within the same age group between patients who tested positive or negative for *H. pylori*.

### 2.4.8 Correlations between circulating levels of IL-16 and known pro- and anti-inflammatory cytokines in *H. pylori* patients.

Increased production of IL-10 in biopsy and serum samples from individuals with *H. pylori* infection, with cytokine production being associated with the severity of chronic inflammation (Siregar et al., 2016) (Arachchi et al., 2017). IL-17 can also prompt the secretion of IL-16 in RA patients. Elevation of IL-17 expression is observed in the gastric mucosa of *H. pylori*-positive patients (Arachchi et al.,

2017). A recent study reported that the levels of IL-17A in the serum are notably higher in *H. pylori* patients with gastric intestinal metaplasia and dysplasia, in comparison to *H. pylori* patients with non-atrophic gastritis and healthy individuals (Della Bella et al., 2023b). Elevated serum IL-8 concentration was also reported in *H. pylori*-positive patients with chronic gastritis compared to *H. pylori*-negative patients with chronic gastritis. The relationship between gastric cancer and *H. pylori* is closely intertwined, with elevated levels of IL-8 serving as an indicator of an unfavorable prognosis (Lee et al., 2013). Thus, it was important to investigate the correlations between circulating levels of IL-16 and these cytokines. IL-8, IL-10, IL-16, and IL-17A levels were measured in plasma samples from 47 *H. pylori* positive patients, and 29 *H. pylori* negative patients using commercial ELISA kits from R&D Systems **(Figure 21).** For statistical analysis, the Mann–Whitney U-test was used, and the Spearman rank correlation coefficient was calculated. The data revealed that in *H.* pylori-*positive* patients, there was no correlation between plasma IL-16 levels and plasma levels of IL-8, IL-10, and IL-17A (**Figure 22 A, B, & C)**

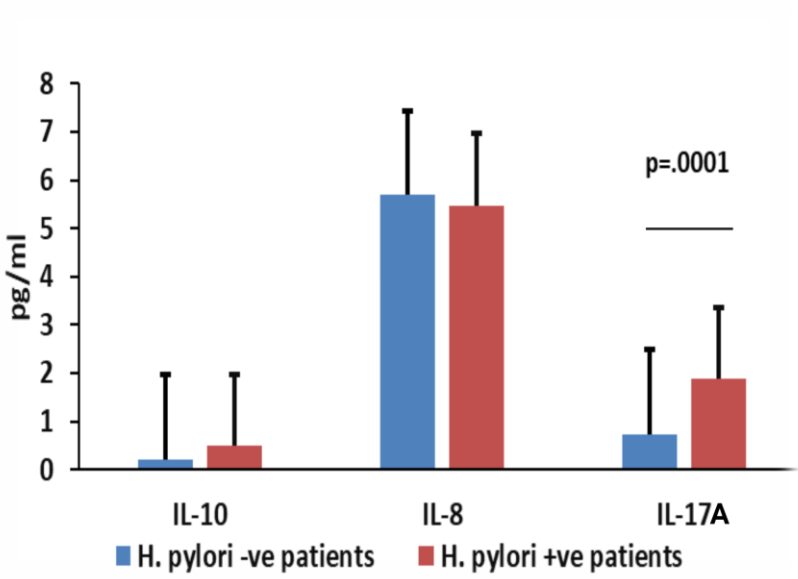



**Figure 21. Plasma levels of IL-8, IL-10, and IL-17A in *H. pylori*-infected vs non-infected patients.**

Plasma samples from 47 *H. pylori*-positive (+ve) patients and 29 *H. pylori*-negative (-ve) patients were analyzed to measure the levels of IL-8, IL-10, and IL-17. The Mann-Whitney U-test was employed for statistical analysis. The results revealed no significant difference in plasma levels of IL-8 and IL-10 between *H. pylori*-infected and non-infected patients. However, a significant increase in plasma levels of IL-17 was observed in *H. pylori*-infected patients compared to non-infected patients (p=.0001). The median level of IL-17A in *H. pylori*-infected patients was 1.180 pg/ml, and 0.73 pg/ml for non-infected patients. Concentrations of cytokines in the plasma were measured by ELISA.

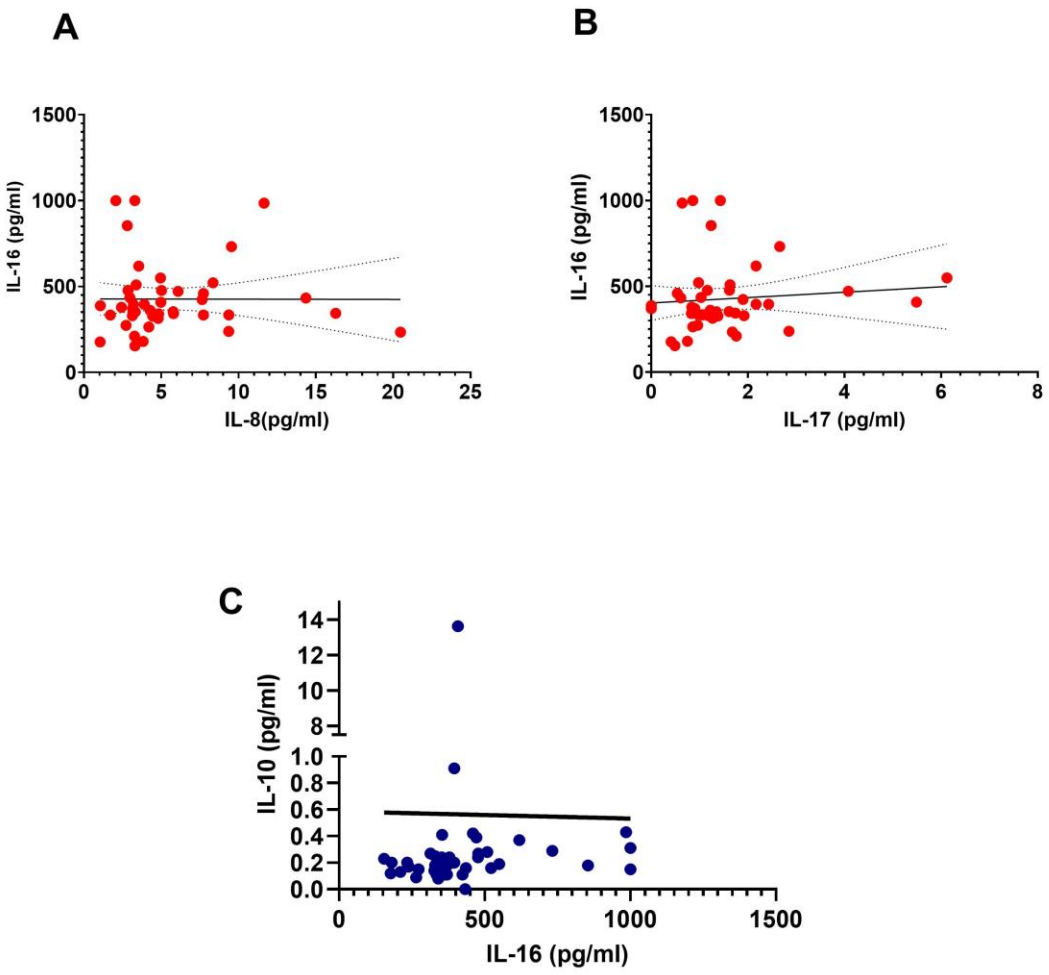



**Figure 22. Correlation between circulating levels of IL-16 and known cytokines in *H. pylori* patients.**

**A).** Correlation between IL-16 and IL-8 (P= 0.9843 and R squared=9.299). **B)** Correlation between IL-16 and IL-17 (P= 0.5480 and R squared= 0.008). **C)** Correlation between IL-16 and IL-10 (P= 0.9722 and R squared= 2.9). Simple linear regression was employed for statistical analysis. The results revealed no correlation was found between IL-16 and other mediators that are important in promoting inflammatory responses.

### 2.4.9 Impact of eradication therapy on IL-16 plasma level during *H. pylori* infection.

This study was done to remove some of the variation between individuals, by comparing the IL-16 concentrations before and 2 years after *H. pylori* eradication (Jonathan Richard White, 2017). The analysis of plasma IL-16 concentrations was carried out on 21 patients who donated blood samples at 5 time points over 24 months following treatment for *H. pylori*. The data were categorized based on IL-16 concentrations over 24 months following eradication. In **Figure 23A**, one patient exhibited a decrease in IL-16 levels post-eradication. In contrast, **Figure 23B** depicted two participants with elevated IL-16 plasma levels after the eradication period. **Figure 23C** illustrated that most participants experienced fluctuating IL-16 concentrations throughout the 24 months post-eradication of *H. pylori*. It was concluded that the plasma IL-16 concentration did not change significantly post-*H. pylori* eradication (**Figure 23**).

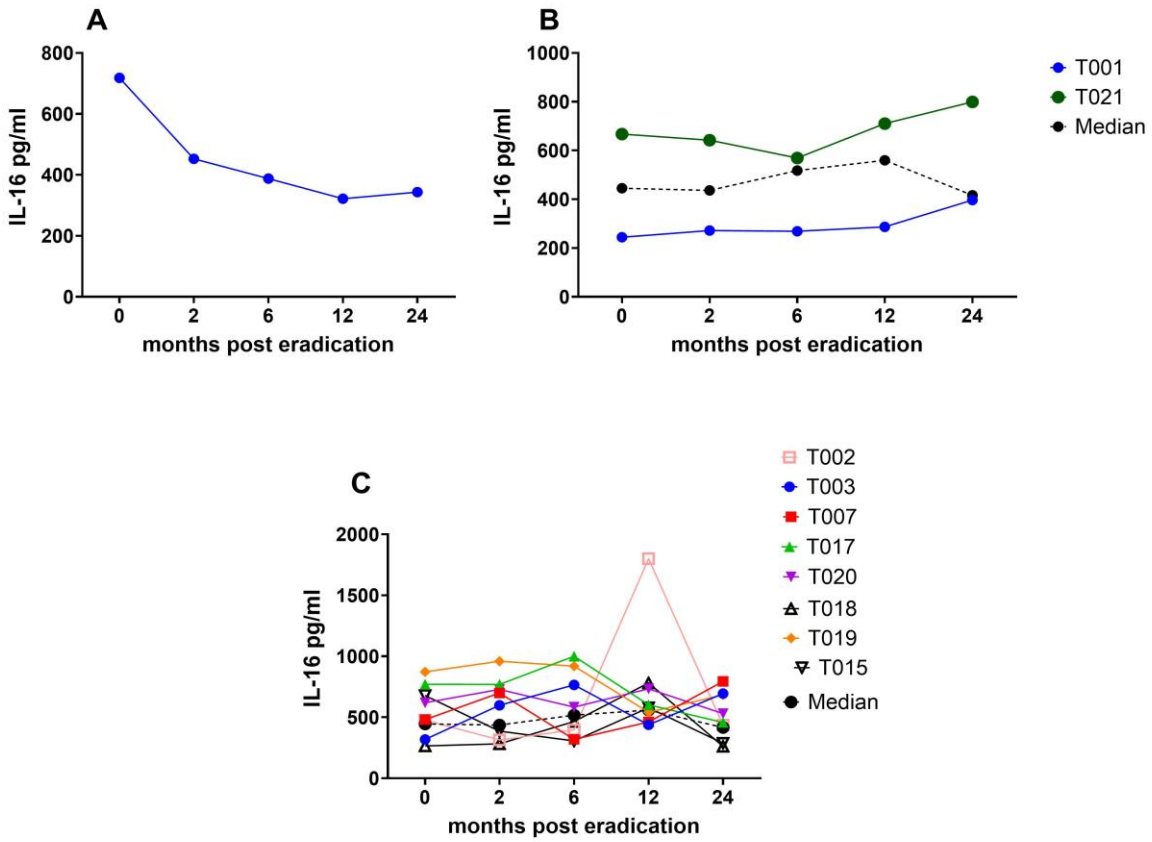



**Figure 23. IL-16 plasma concentrations from patients treated for *H. pylori* infection.**

After diagnosis and treatment at Month 0, patients donated further blood samples at Months 2, 6, 12, and 24. The linked lines represent samples donated by the same patient over time. **A)** represent patients who experienced a reduction in IL-16 levels after eradication. **B)** patients with increased IL-16 concentrations following H. pylori elimination. **C)** patients with fluctuating IL-16 concentrations post-eradication. Statistical analysis between Month 0 and Months 2-24 was conducted using a Wilcoxon signed rank test. The dotted line depicts the median at each time point. The plasma IL-16 concentration did not change significantly post-H. pylori eradication

### 2.4.10 Gastric *IL16* mRNA expression in *H. pylori*-infected gastric mucosa.

The immune response to *H. pylori* infection in the gastric mucosa typically involves the production of various inflammatory and anti-inflammatory cytokines

by different types of immune cells, including Th1, Th2, Th17, macrophages, monocytes, mast cells, and neutrophils. Most of these cytokines, such as IL-1, IL-2, IL-6, IL-10, IL-17, IL-23, TNF-α, TGF-β, IFN-γ, CXCL-12, and CXCL-4, are released both locally at the infection site and into the bloodstream (Niu et al., 2020, Jan et al., 2020). Nevertheless, certain studies have indicated an increase in levels of MMP-7, MMP-9 (Cheng et al., 2012, Siregar et al., 2016), IL-8, and IL-6 specifically at the site of infection, without any corresponding elevation in systemic levels (Di Bonaventura et al., 2007).

Therefore, in addition to examining the systemic levels of IL-16, it was crucial to assess the levels of *IL16* in the mucosa. Gastric mRNA expression was quantified, using RT-qPCR, comparing between 29 *H. pylori*-infected and 14 uninfected patients, using *GAPDH* as the reference gene. The results showed no significant differences in *IL16* mRNA expression between the two groups (**Figure 24 A)**. Additionally, to examine if the mRNA expression of gastric *IL16* differed based on the presence of *H. pylori* chronic gastritis (n=8), *H. pylori* peptic ulcer (n=9), or gastric cancer (n=11). Analysis of the data revealed no significant difference between *H. pylori*-infected patients with chronic gastritis and *H. pylori*-infected patients with peptic ulcer as well as infected patients with gastric cancer **(**Error! Reference source not found. **Figure 24B)**.

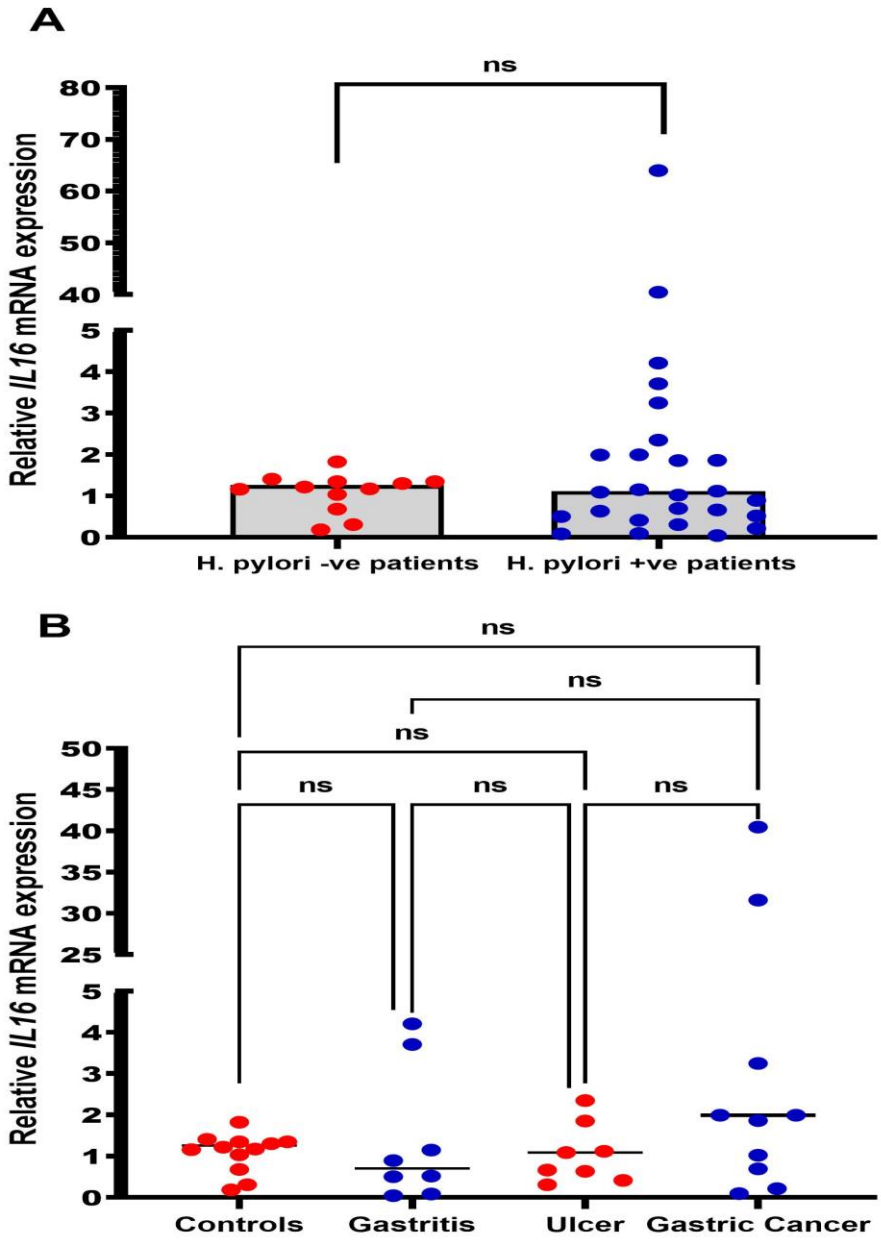



***Figure 24*. The relative expression of *IL16* mRNA in mucosa infected with *H. pylori* and its associated diseases.**

Gastric biopsy samples were collected from 14 *H. pylori* (-ve) negative and 29 positive (+ve) patients and analyzed for the *IL16* mRNA expression by real-time RT-PCR. No significant (ns) differences in *IL16* mRNA expression between the two groups. Mann-Whitney U-test. **(B)** No significant difference between *H. pylori*-infected patients with chronic gastritis and *H. pylori*-infected patients with

peptic ulcers and infected patients with gastric cancer, as well as non-infected patients using a Kruskal-Wallis test. ns=not significant.

### 2.4.11 Correlations of circulating levels of IL-16 with gastric protein levels and gastric mRNA expression in *H. pylori*-positive patients.

The data showed no association between circulating levels of IL-16 and gastric IL-16 protein levels. Also, no correlation was observed between systemic IL-16 levels and gastric *IL16* mRNA expression in *H. pylori*-positive patients.

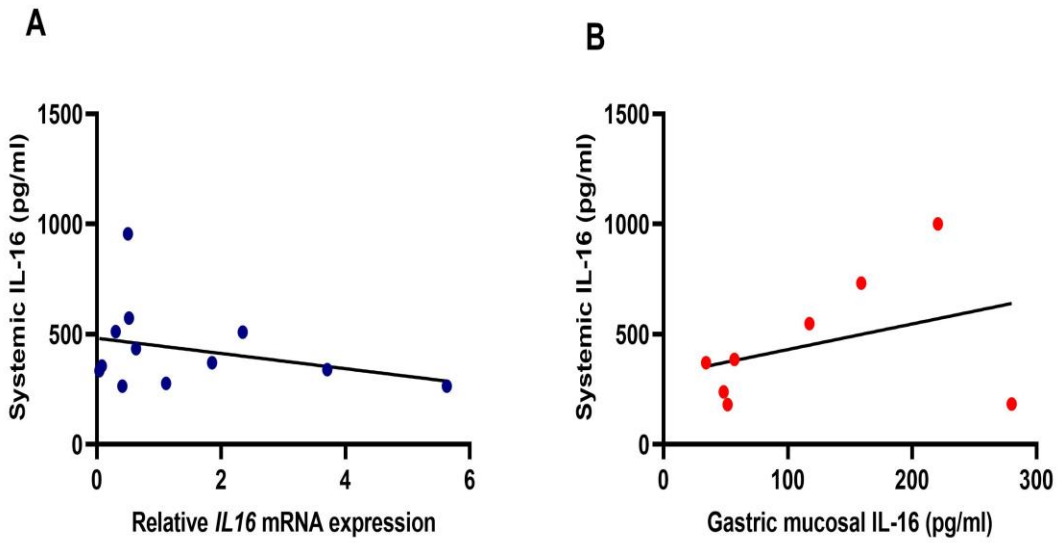



**Figure 25. Correlation of systemic levels of IL-16 with gastric protein level and gastric *IL16* mRNA expression in *H. pylori*-infected patients.**
Using the linear regression test, there was no observed correlation between plasma IL-16 levels and *IL16* mRNA expression (P= 0.3 and R squared=0.13) **(A),** as well as no reported correlation between plasma IL-16 levels and gastric mucosal IL-16 levels, (P=0.4 and R squared=0.09) **(B).**

### 2.4.12 Correlation between ELISA and Meso Scale Discovery (MSD) assays.

I used the more sensitive and precise MSD assays to compare the IL-16 concentrations obtained through ELISA and MSD methods. IL-16 levels were

analyzed in identical sets of samples from the eradication study. A significant correlation was observed between the results of these two assays, strengthening the reliability of the previously presented data from the IL-16 ELISA assays.

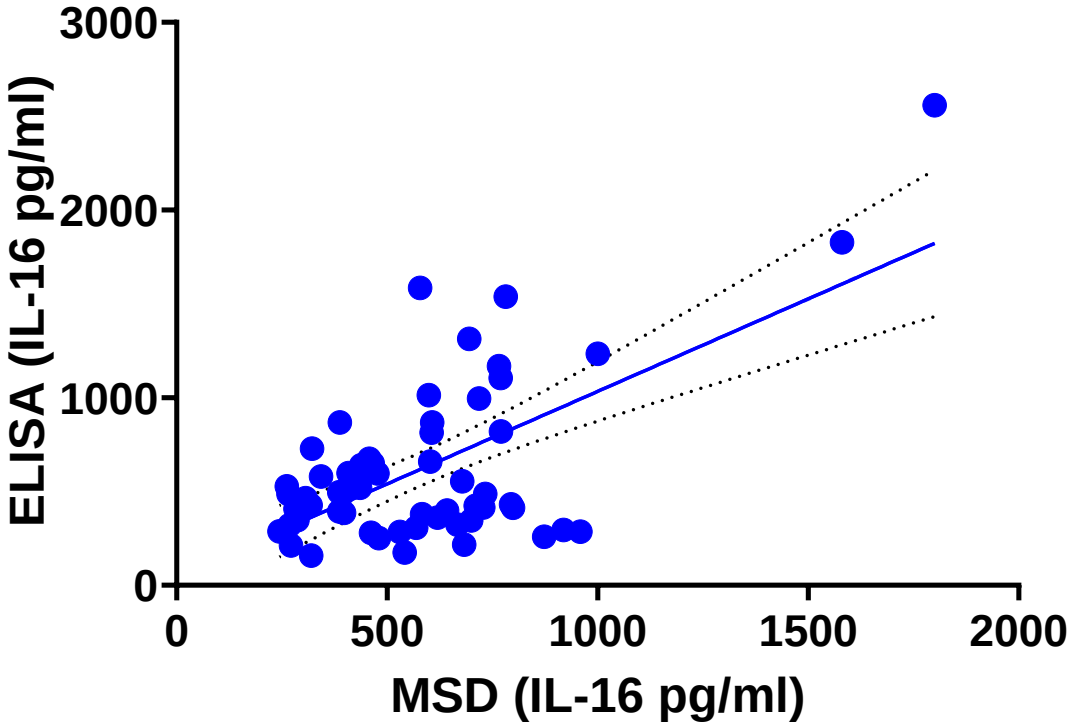



**Figure 26. Correlation between ELISA and MSD results of IL-16 concentrations.**

Using the linear regression test, there was a significant correlation between plasma IL-16 concentration obtained by using ELISA and IL-16 concentrations obtained by MSD. (P= 0.04 and R squared=0. 0.26).

## 2.5 Discussion

*H. pylori* infection is a chronic stomach infection that can lead to gastritis, peptic ulcers, and gastric adenocarcinoma (Bakhti et al., 2019) (Morgan et al., 2022). In the stomach, *H. pylori* triggers a strong immune response characterized by the infiltration of numerous immune cells such as macrophages, mast cells,

dendritic cells neutrophils, B cells, and T cells. However, this immune response is unable to eliminate the infection, suggesting that *H. pylori* can evade or manipulate the host immune system to persist (Robinson et al., 2007).

$CD4^+$ T cells play a crucial role in the immune response against *H. pylori*, with studies showing that these cells specifically accumulate in the infected stomach. Despite the increase in $CD4^+$ T cell numbers during *H. pylori* infection, these cells are less responsive and biased towards a Th1 response. Th1 cells produce IFN-γ which contributes to proinflammatory reactions. There are other subsets of CD4+ T cells found in the infected mucosa including Th17 and Treg cells (Lundgren et al., 2005). Th17 cells are linked to inflammation and the development of cancer, as they are produced during both *H. pylori* infection and gastric cancer (Della Bella et al., 2023a). Treg cells, on the other hand, express the FOXP3 transcription factor, can suppress immune responses and thus, play a role in the persistence of *H. pylori*. The mechanisms responsible for the trafficking of these cells to the infected mucosa are not well understood.

IL-16, initially known as lymphocyte chemoattractant factor, is a cytokine that uses CD4 as a receptor and selectively attracts $CD4^+$ cells but renders them unresponsive to antigens. IL-16 has been shown to favorably induce the migration of Th1 cells and expansion of FOXP-3(+) T cells. It is being shown that at the inflammatory site, IL-16 is involved in Treg cell expansion through the promoting of a migratory response from existing Treg cells, as well as by the promoting of *de novo* synthesis of FOXP3(+) cells (McFadden et al., 2007). Furthermore, IL-16 enhances Th1 responses and inhibits Th2 responses by modulation of cytokine activity (Wilson et al., 2004). Although extensive research has been conducted on this cytokine concerning various inflammatory and autoimmune diseases, as well as cancer **(section 1.9.3 and 1.9.4**), it has not been extensively studied in *H. pylori* infection.

Therefore, this study aimed to investigate IL-16 production during *H. pylori* infection which might be involved in sustaining the infection through

upregulation of the proinflammatory response, recruitment of inflammatory cells into infected gastric mucosa, the accumulation of CD4+ T cells, and impairment of T cell responses observed in *H. pylori* patients.

To our knowledge, no study has yet measured the systemic levels of IL-16 during *H. pylori* infection and its relationship to *H. pylori*-associated diseases. The main objective of this study was to investigate the levels of IL-16 protein and mRNA expression during *H. pylori* infection, and to determine if there is any association between IL-16 plasma levels and *H. pylori* diseases, oesophageal diseases, smoking history, age, and gender using ELISA and RT-PCR.

Because it was difficult to collect an adequate number of gastric biopsy samples, most of the work was based on a large collection of plasma samples. Patients who were regularly taking NSAIDs and/or had clinically diagnosed immune disorders e.g. allergy or asthma, were excluded from the study as increased serum and plasma levels of IL-16 have been reported in various inflammatory conditions.

No discernible differences in IL-16 levels, either in plasma or gastric mucosal tissue, were detected between *H. pylori*-positive and negative patients, and healthy subjects. IL-16 was detected in every plasma sample, with a very wide variation in concentrations ranging from 135 to 2788 pg/ml. These results align with a previous study that investigated the natural variation of cytokines in eosinophils obtained from healthy individuals. IL-16 was found in eosinophil lysates, with concentrations varying profoundly between donors, from as low as 274 pg/mg lysate to as high as 13,300 pg/mg lysate (Ma et al., 2019). Furthermore, a study has examined the effects of Gut Balance ™, containing four probiotics and two prebiotics, and acacia gum (potential prebiotic) on plasma cytokines levels found a large variation (~100–300%) in IL-16 concentration between and within the subject. A rise in baseline IL-16 concentrations was noted in both sets of participants undergoing treatment. Nevertheless, the use of Gut Balance appeared to reduce the extent of this increase in IL-16 by half compared to acacia gum, suggesting the impact of prebiotics and probiotics on systemic

IL-16 levels (West et al., 2012). An investigation examined the impact of Resveratrol (RSV)-microbiota on the levels of inflammatory cytokines, specifically IL-16. The study found that the presence of IL-16 was elevated in the intestines of mice fed a high-fat diet (HFD) compared to those on a standard diet (SD). However, treatment with RSV-microbiota modified this expression, leading to levels similar to those observed in the SD-fed mice (Wang et al., 2020). Thus, the inclusion of probiotics and prebiotics in the participants' regimen in this study may account for the fluctuations observed in plasma IL-16 levels.

Another recent study utilized Mendelian randomization (MR) analysis to examine the interaction between gastric microbiota, inflammatory factors, and acute respiratory distress syndrome (ARDS) suggesting a potential positive relationship between the *Victivallis* genus and elevation IL-16 level, demonstrating a link between gastric microbiota, cytokine production (Ma et al., 2023). It is important to note that the current study lacks information regarding the gastric microbiota of the participants recruited.

Another possible explanation for the observed variation in IL-16 levels in plasma could be a genetic variation. Variations in the DNA sequence of the *IL16* gene can result in changes in cytokine production and/or activity, potentially affecting an individual's susceptibility to various conditions. It has been observed that the rs4778889 polymorphism (T/C at position -295) in the promoter region of the *IL16* gene may affect gene expression and lead to increased concentrations of IL-16 (Nakayama et al., 2000). Moreover, higher systemic levels of IL-16 are linked to the TG/GG genotypes of rs11556218 and the T/G polymorphism of *IL16* rs11556218, which in turn increases the risk of osteosarcoma and nasopharyngeal carcinoma in the Chinese population, respectively (Tang et al., 2016b, Qin et al., 2014).

Furthermore, a study performed on overweight adolescents found that a significantly increased concentration of IL-16 was detected in the plasma of overweight adolescents compared to those of normal weight, and the levels of

IL-16 were correlated with measures of obesity, such as weight, BMI, and waist circumference (Lichtenauer et al., 2015).

The levels of IL-16, both locally and systemically, could be affected by specific medications such as allergy drugs and dexamethasone. However, individuals with allergies, asthma, or autoimmune conditions were excluded from the current study. A positive association between serum Interleukin-16 and total IgE has been documented (Wu et al., 2011). An analysis of total IgE concentrations in the entire patient group (regardless of Hp status) was conducted to elucidate the observed differences in plasma IL-16 levels, but no correlation with IL-16 was found (data is not shown). This analysis did not account for the significant variability observed in IL-16 concentrations.

Thus, the observed variations in IL-16 plasma levels in this study may be attributed to the lack of data on *IL16* polymorphisms and participants' obesity-related measurements, consumption of prebiotics and probiotics, and information regarding gastric microbiota in recruited individuals.

Relationships between the *cagA* gene and the production of IL-16 cytokine were also investigated. There were no discernible differences between the plasma of patients infected with a *cagA*+ strain and those infected with a *cagA*-stain. This implies that in patients with *H. pylori* infection, *cagA* status does not affect systemic IL-16 levels. Our results corroborate earlier studies that did not discover any connection between the expression of cytokines by human gastric epithelial cell lines and the stomach mucosa, such as IL-6, IL-8, IL-10, and TNF-$\alpha$, and *H. pylori* virulence factors like *cagA*. (Wen et al., 2007, Kim et al., 2000, Audibert et al., 2000, Kranzer et al., 2005).

I also examined the levels of IL-16 in the plasma of individuals who were infected with *H. pylori* and categorized them according to their smoking status (smokers, ex-smokers, and non-smokers). The findings indicated that the IL-16 levels in the three groups did not differ significantly from one another. Nevertheless, the current results are in line with earlier research that compared

blood levels of soluble IL-16 protein in smokers and non-smokers and likewise showed no appreciable differences in this protein (Andersson et al., 2016).

This study also examined the gender and age disparity in the plasma IL-16 levels in *H. pylori*-positive patients. The data showed that plasma IL-16 levels in *H. pylori*-positive patients were not influenced by gender. There was a tiny but statistically significant variation in the levels of plasma IL-16 between the *H. pylori*-infected male and female, a difference that is unlikely to have any biological significance. Also, differences between *H. pylori* positive and negative female patients were not statistically significant, and a similar result was found when comparing infected and uninfected male patients (data not shown). The findings are consistent with a previous study, which investigated the impact of various factors such as different anticoagulants, age, and gender on the levels of immunomodulators, and showed that there were no significant differences in the levels of IL-16, both in serum and plasma, between men and women in different age groups of healthy individuals (Krishnan et al., 2014).

Furthermore, when examining the histological changes associated with *H. pylori* infection and the levels of IL-16 cytokine, the results showed no significant differences in colonization scores and plasma IL-16 levels in both the antrum and corpus. Likewise, plasma IL-16 levels did not show significant differences based on scores for chronic inflammation, atrophy, and intestinal metaplasia in both the antrum and corpus. This result is consistent with previous research showing no meaningful relationships between the degree of intestinal metaplasia, atrophy, neutrophil infiltration, or chronic inflammation and blood levels of IL-10, Matrix metalloproteinases-7 (MMP-7), or MMP-9 (Siregar et al., 2016).

In this study, it was observed that the levels of circulatory IL-16 were significantly higher in healthy controls and *H. pylori*-infected patients with chronic gastritis and peptic ulcer disease compared to gastric cancer patients. Contradictory, One published study found that individuals with gastric cancer had higher levels of IL-16 in their serum compared to healthy individuals (Yang

et al., 2017). Although I used the same ELISA kit (R&D system) that was used by the Yang et al study to measure IL-16, the patient selection criteria were different from the current study. I specifically did not include patients who were currently taking antibiotics, NSAIDs, or PPIs. These medications have the potential to alter immune responses and influence cytokine production. Additionally, they can impact the composition of the host's microbiota, which could indirectly affect cytokine secretion (Morikawa et al., 1996a, Bailly et al., 1993) (Kiecka and Szczepanik, 2023) (Raaijmakers et al., 2022) (Chen et al., 2021) (Maseda and Ricciotti, 2020). Furthermore, the research indicated increased systemic levels of IL-16 in patients with GC but did not specify the particular types of stomach cancers studied. It is possible that the stomach cancer types in their research may not have encompassed those specifically linked to *H. pylori* and examined in the present study. Gastric carcinomas can be categorized into various subtypes, such as gastric adenocarcinomas. Additionally, lymphomas and mesenchymal tumors can arise in the stomach. However, it is important to note that *H. pylori* is specifically associated with gastric adenocarcinoma and gastric MALT lymphoma. The current study used EDTA plasm while Yang et al used serum samples to measure IL-16. In a study from 2018, it was discovered that the levels of IL-16 significantly rise in EDTA plasma, to a lesser extent in citrate plasma, and show no increase in serum. This suggests that the release of IL-16 by granulocytes is more pronounced in EDTA compared to citrate and is absent in serum where white blood cells may be confined within the blood clot (Kofanova et al., 2018). Additionally, a recent report examined the interplay between the *IL16* rs11556218 genotype and *H. pylori* infection status. The findings indicated that individuals infected with *H. pylori* and possessing the *IL16* rs11556218 TT genotype had a notably elevated odds ratio (7.90) for GC risk, a risk that significantly decreased to 2.5 among those infected with *H. pylori* and having the TT genotype. Although this study did not examine the systemic levels of IL-16 in patients with GC and controls (Fu et al., 2024), previous Chinese reports reported that rs11556218 TG/GG genotypes were linked to higher

systemic levels of IL-16 as compared to TT genotype (Qin et al., 2014, Tang et al., 2016a). Demonstrating the impact of *IL16* genotype on GC progression in individuals with *H. pylori* infection, as well as its influence on plasma levels. Another factor that should be considered to explain the difference between the findings of the current study and the research that indicated increased systemic levels of IL-16 in patients with GC is ethnicity. The previous study was conducted in a Chinese population, where higher morbidity and mortality rates compared to other global regions (Yan et al., 2023), while the current population comprises individuals from Western regions.

I also examined the potential link between plasma IL-16 concentrations and esophageal diseases (GERD, BE, and EU) in patients with either *H. pylori* positive or negative status. There was no association between plasma IL-16 concentrations and esophageal diseases.

To support the hypothesis that IL-16 acts as a mediator promoting inflammation, it was important to investigate the correlation between IL-10, IL-8, and IL-17 cytokines and IL-16 concentration in plasma, as these cytokines are reported to be elevated in *H. pylori*-infected patients, and their production is associated with chronic inflammation (Siregar et al., 2016) (Arachchi et al., 2017) (Della Bella et al., 2023b). First, the concentrations of IL-8, IL-10, and IL-17 were measured in plasma samples from *H. pylori* positive and negative patients. No significant association was noticed between *H. pylori* infection and the secretion of IL-8 cytokine, but at the same time, pointed out that *H. pylori*-positive patients tend to have an increased secretion of IL-10 as compared with non-infected subjects, and a significant upregulation of IL-17 plasma cytokine levels upon *H. pylori* infection was also observed. Moreover, the results showed no correlation between the plasma levels of IL-16 and the levels of IL-8, IL-10, and IL-17 in *H. pylori*-positive patients. Contrary to previous findings in RA disease, where positive correlations were observed between circulating levels of IL-16 and other inflammatory mediators (TNF-α, IL-6, and sIL-2R), supporting the proinflammatory role of IL-16 in RA (Kaufmann et al., 2001), this report found that

serum IL-16 and other known inflammatory cytokines (TNF-$\alpha$, IL-6, sIL-2R) levels were significantly upregulated in RA patients compared to non-RA controls. However, in this study, no significant differences were reported between serum IL-16, IL-8, and IL-10 levels in *H. pylori*-infected patients compared with controls. A positive correlation between IL-16 and IL-17 plasma levels had been anticipated, as IL-16 protein expression levels were upregulated when IL-17 was incubated with rheumatoid arthritis-fibroblast-like synoviocytes (RA-FLS) and peripheral blood mononuclear cells. However, IL-17 did not have the same effect on IL-16 production in osteoarthritis fibroblast-like synoviocytes (OA-FLS) (Cho et al., 2008).

To exclude variability between individuals, the IL-16 plasma concentrations before and during 2 years after receiving *H. pylori* successful eradication therapy were compared using paired data analyses. Blood samples were collected from 21 patients who tested positive for *H. pylori* at the time of diagnosis and were collected at regular intervals for 2 years after the eradication treatment. The results showed that there were no significant changes in IL-16 levels over time following the eradication treatment. Additionally, there were no statistical differences in the paired data between the different time points (0, 6, 12, and 24 months). It is worth noting that again there was considerable variation between individuals and within the sets of samples from the same patient over time.

IL-16 levels were also analyzed in the same sets of plasma samples from the eradication study by using more sensitive and accurate assays of MSD. The same trend was obtained from the eradication study as with the ELISA assay, and a significant correlation was reported between the results of these two assays.

In summary, the present study showed for the first time that *H. pylori* infection is unlikely to influence systemic as well as gastric IL-16 levels due to the lack of significant correlation found between *H. pylori* infection and high IL-16 levels in both plasma and gastric mucosa. The gender, age, smoking history, and *cagA* status of *H. pylori*-positive patients did not have any impact on IL-16

plasma levels. Additionally, there was no association observed between plasma IL-16 levels and other cytokines (IL-17, IL-6, and IL-10) or histological changes related to *H. pylori* infection. Surprisingly, and contrary to the published literature, patients with gastric cancer had lower concentrations of serum IL-16 compared to those with gastritis and peptic ulcer disease. Receiving eradication therapy for *H. pylori* did not affect the concentration of circulating IL-16 cytokine. All this evidence indicates that *H. pylori* infection may not impact the mucosal or systemic levels of IL-16.

Our study has several notable strengths. Firstly, we conducted a thorough examination of the impact of *H. pylori* on changes in both circulating and mucosal IL-16 levels. We utilized ELISA and RT-PCR techniques to ensure a comprehensive investigation, and the ELISA results were also validated using MSD assays. Additionally, our study included a large number of plasma samples and considered various parameters that could potentially influence plasma IL-16 levels. There are limitations to this study, including the absence of data on IL-16 polymorphisms, consumption of prebiotics and probiotics, information regarding gastric microbiota in recruited individuals, and participants' measurements related to obesity, which have been shown to affect systemic levels of IL-16. As mentioned earlier, the pandemic had a significant impact on the collection of samples, resulting in a limited number of gastric biopsy samples.

For the next chapter, I investigated the impact of *H. pylori* on the production of IL-16 and other cytokines by monocytes and dendritic cells. This was influenced by a prior study that demonstrated *H. pylori's* ability to stimulate gastric epithelial cells to produce IL-16 (Alzahrani, 2014), with monocytes being the primary source of this cytokine. Although there was no correlation between IL-16 and *H. pylori* at local or systemic levels, it was hypothesized that differences in the responses of $CD14^+$ monocytes could be masked by IL-16 produced by a variety of other cells in the tissue.

# Chapter 3. The Role of *H. pylori* Virulence Factors in Cytokines Production by Monocytic Cells and Dendritic Cells

## 3.1 Introduction

The interaction between bacterial pathogens and host cells initiates a range of intricate cellular signaling pathways that ultimately determine the outcome of the disease. These inflammatory signaling cascades can often be attributed to specific virulence factors within the pathogen, which interact with specific host cells that encounter the invading organism (Oghumu and Satoskar, 2014).

Most studies on pro-inflammatory immune responses to *H. pylori* infection have primarily relied on *in vitro* models utilizing the AGS gastric epithelial cell line. However, it is now evident that interactions between the pathogen and immune cells in the gastric mucosa, specifically DCs, and monocytes, play a crucial role (Oghumu and Satoskar, 2014). Myeloid antigen-presenting cells, including monocytes and DCs, are found in the gastric mucosa when infected with *H. pylori.* These cells play a role in both promoting and sustaining immune responses specific to *H. pylori*, as well as in inflammatory reactions. Monocytes are the first line of defense after *H. pylori* penetrates the epithelial cell layer. When monocytes cross the endothelial barrier, they can differentiate into tissue resident macrophages or DCs (Randolph et al., 2008). DCs are found throughout various human tissues, including the gastric mucosa, and can also enter the gut epithelial monolayers to collect luminal bacteria (Rescigno et al., 2001). DC doesn't only present antigens to T cells but also shapes the T-cell response based on their activation level. Various pathogens can interact with DCs through TLRs, which affect the expression of surface proteins and the secretion of cytokines by DCs. These cytokines, in turn, regulate the outcome of T-cell activation following interaction with the primed DCs. For example, IL-12 drives a Th1 response (Trinchieri, 1998), whereas IL-10 may induce a Th2/Treg response. Therefore, it is important to define the mechanisms by which *H. pylori* modulates DC function. DCs recognize PAMPs expressed on *H. pylori* through TLRs (Kabisch et al., 2014).

This interaction activates signaling pathways in host cells that are important for initiating the immune response. DCs can engage with *H. pylori* through CD209 (DC-SIGN), which binds to *H. pylori* lipo-oligosaccharide (LOS) and encourages the secretion of IL-10 (Bergman et al., 2004). Furthermore, *H. pylori* triggers DCs to induce the expression of IL-17 in CD4 lymphocytes (Khamri et al., 2010). *H. pylori* promotes a strong activation and maturation of human immature DCs characterized by secretion of IL-6, IL-8, IL-10, and IL-12 cytokines in a dose-dependent manner, and expression of CD80, CD83, CD86, and HLA-DR (Fehlings et al., 2012). However, prolonged exposure to *H. pylori* can lead to exhaustion of dendritic cells and suppression of the Th1 immune response. *H. pylori*-infected DCs have been found to secrete TNFα, IL-6, and IL-10. Unlike LPS from *E coli*, which primarily signals through TLR4, the secretion of these cytokines in *H. pylori*-infected DCs is delayed, suggesting different activation mechanisms, possibly involving TLR8 and TLR9 signaling pathways. These findings highlight the significance of pathogen-derived factors in modulating the host immune response, ultimately impacting disease outcomes (Oghumu and Satoskar, 2014). Monocyte-derived dendritic cells (MoDCs) exhibit increased expression of high-affinity IgE receptor (FcεRI) and IL-10 cytokine, proposing that *H. pylori* directs regulatory dendritic cell differentiation to weaken the hostile immune environment (Leon et al., 2019). *H. pylori* can stimulate the release of IL-23 in cultured DCs. The secretion of IL-23 by DCs infected with *H. pylori* may have implications for the initiation and persistence of Th17 responses, which could impact the progression of gastritis during *H. pylori* infection (Oghumu and Satoskar, 2014)

Monocytes are powerful producers of cytokines that promote inflammation and are associated with gastric cancer development, such as TNF-α, IL-1β, CXCL-8, and IL-6. Other mediators that are secreted by monocytes, such as IL-12p70, induce the generation of type Th1 responses and promote tissue damage (Frauenlob et al., 2022). A previous study found that *H. pylori* stimulates

the production of IL-12p40, partially released as IL-23, while no IL-12p70 was detected. Furthermore, monocytes infected with *H. pylori* produced high levels of IL-1β and IL-6, but only minimal amounts of IL-10. Interestingly, infected cells produced significantly more IL-12p40, IL-23, IL-1β, IL-6, and IL-10 compared to uninfected control cells, and more IL-12p40 and IL-1β, but not as much IL-23, compared to cells stimulated with LPS. The production of MIF by monocytes was not affected by *H. pylori* or LOS (Fehlings et al., 2012). The bacterium *H. pylori* stimulate human monocytes to release chemokines IL-8, epithelial neutrophil activating peptides 78 (ENA-78), and MCP-1 through CD14 (Bliss et al., 1998).

Despite being less potent, *H. pylori* LOS may still have a significant impact on the development of *H. pylori* gastritis (Bliss et al., 1998). The ability of *H. pylori* LOS to induce the production of cytokines TNF-α, IL-10, and GM-CSF by human monocytes and macrophages is significantly lower compared to LPS from other species. However, *H. pylori* LOS does stimulate the production of CXC chemokines IL-8 and growth-related oncogene alpha (GRO-α), which may contribute to the ongoing recruitment of neutrophils to the *H. pylori*-infected gastric mucosa (Innocenti et al., 2001). A recent study has found that repeated stimulations with *H. pylori* did not enhance the response. However, monocytes that were primed with *H. pylori* exhibited a heightened response to stimulation with *E coli* LPS shortly after infection. This resulted in increased expression and secretion of the cytokines TNF-α, IL-6, IL-10, and IL-12 compared to unprimed and unstimulated cells (Frauenlob et al., 2022).

IL-6 and IL-10 are critical cytokines that have been reported to be elevated in both gastric tissues and systemic levels during *H. pylori* infection (Yang et al., 2024). IL-10, an anti-inflammatory cytokine, regulates the activation and functions of various innate and adaptive immune cells. It helps to decrease the release of inflammatory cytokines and prevent damage to the host tissue. IL-10 is secreted by different cells such as T and B lymphocytes, DCs, macrophages, mast cells, NK cells, eosinophils, and neutrophils (Iyer and Cheng, 2012).

*H. pylori* employs several tactics to evade the immune system in the gastric mucosa. One of these tactics involves increasing the production of regulatory cytokines, such as IL-10, which plays a crucial role in suppressing inflammatory responses and promoting the persistence of *H. pylori* in the gastric environment. IL-10 has been shown to inhibit various immune reactions, including antigen presentation, inflammatory cytokine production, and T-cell activation. A decrease in IL-10 production is associated with more severe gastric inflammation, which raises the risk of developing gastric malignancies during *H. pylori* infection (Amin et al., 2022). Moreover, IL-10 is essential for the generation of regulatory T cells (Hsu et al., 2015). These cells can secrete high levels of IL-10 and TGF- β. In the context of transplantation, allergy, or autoimmune disease, these cells possess a limited ability to reproduce and can hinder abnormal immune reactions. Nonetheless, their inhibitory impact is not always advantageous as it can also impede the immune response against tumor antigens or pathogens (Groux, 2003). In infected individuals, regulatory T cells specific to *H. pylori* have been found to suppress the memory T-cell response to the bacteria (Lundgren et al., 2003). This suppression may play a role in the immune system's inability to eliminate the *H. pylori* infection.

IL-6 is a multifunctional cytokine that has proinflammatory properties and is primarily produced by monocytes. It promotes B-cell maturation and T-cell differentiation, and it also works in synergy with TNF-a and IL-1 to induce a systemic inflammatory response. IL-6 plays a crucial role in the development of gastric cancer associated with *H. pylori* infection. It promotes gastric inflammation and stimulates the growth of gastric cells to restore stomach function. However, chronic gastritis and inflammation can lead to the development of stomach cancer (Kuhn et al., 2014). IL-6 has various cancer-promoting effects, many of which are mediated by the activation of the JAK-STAT3 signaling pathway. This pathway triggers the transcription of genes involved in cell proliferation, inhibition of apoptosis, progression of the cell

cycle, and modulation of the extracellular matrix (Huang et al., 2022, Kinoshita et al., 2013). IL-6 can stimulate the synthesis of vascular endothelial growth factor (VEGF) as well, which is involved in promoting angiogenesis (Niu et al., 2002). Upon infection with *H. pylori*, the secretion of IL-6 and other cytokines is increased. In patients with GC, levels of IL-6 are also elevated. The correlation between IL-6 levels and the risk of GC in various countries strongly suggests that this cytokine plays a significant role in the progression of this deadly disease (Yu et al., 2023).

As mentioned earlier, *H. pylori* has developed mechanisms to modify and evade the immune response to survive within the host gastric mucosa. This involves targeting dendritic cells and monocytes, the key players in orchestrating the immune response. *H. pylori can* partially avoid the innate immune defense mechanism of phagocytosis, which is carried out by monocytes (Yuan et al., 2009). Additionally, it has been shown *in vitro* that *H. pylori* uses different apoptotic mechanisms in infected monocytes (human peripheral blood monocytes, and monocytic cell lines THP-1 and U937) to avoid its clearance. *H. pylori* can escape from peripheral blood monocytes by upregulation of genes involved in early apoptosis and suppression of those involved in later stages of apoptosis, which may diminish the inflammatory response to chronic infection (Zhang et al., 2017). The expression of *cag PAI* genes is necessary for *H. pylori* to cause human monocytes to undergo apoptosis *in vitro,* and for immature dendritic cells (iDCs) to produce proinflammatory cytokines. This highlights the significance of the *cag PAI* for evasion of the innate immune recognition system through the reduction of iDC numbers and damage to the gastric mucosa during the persistence of local infection (Galgani et al., 2004).

In response to inflammation, monocytes can generate cell-signaling chemicals that start and intensify an inflammatory response. Monocytes spontaneously experience programmed cell death in the absence of an inflammatory trigger, such as bacterial LPS or differentiation factors (Mangan et

al., 1991, Mangan and Wahl, 1991). With a half-life of 1-2 days (Cline et al., 1978, Thomas et al., 1976), blood monocytes are eliminated from the circulation through this mechanism. Caspase-3 has been shown to play an important role in the execution of apoptosis (Ohta et al., 1997). Caspase-3, in addition to its role in programmed cell death, cleaves *pro*-IL-16 into the 121-aa, 17-kDa mature IL-16 (Zhang et al., 1998). IL-16 is expressed constitutively in freshly isolated $CD14^+$ blood monocytes and is released spontaneously during apoptosis. The release of IL-16 by monocytes is accompanied by caspase-3 activation, and both caspase-3 activation and IL-16 release can be inhibited by proinflammatory stimuli such as bacterial LPS (Elssner et al., 2004b). IL-16 stimulates the production of pro-inflammatory cytokines (IL-1, IL-6, and TNF-$\alpha$) or cytokines that lead to pro-inflammatory activity (IL-15) in $CD4^+$ $CD14^+$ monocytes, implying that IL-16 may play a role in initiating and/or maintaining an inflammatory response (Mathy et al., 2000). Additionally, IL-16 plays a role in the trafficking of DCs and can act as a significant chemotactic signal for both DCs and T cells (Kaser et al., 1999).

The cag type IV secretion system facilitates the transfer of bacterial effector molecules into the host's gastric epithelial cells. *CagE*, a structural component of this functional secretion system, plays a crucial role in this process; inactivating the *cagE* gene product prevents the delivery of *H. pylori* proteins into host cells (Noto and Peek, 2012). *CagE* gene has been shown to prompt the production of chemokines, such as IL-8, from infected host epithelial cells (Tummuru et al., 1995). Furthermore, the expression of *cagE* is required to fully induce the cytokines IL-1, IL-12, and TNF-$\alpha$ in iDCs, but not IL-10 (Guiney et al., 2003). Another study similarly demonstrated that *H. pylori* preferentially triggers the production of IL-12, as opposed to IL-6 or IL-10, in human dendritic cells (Guiney et al., 2003). In addition to the Cag proteins, *vacA* plays a significant role in *H. pylori*-induced gastritis. *VacA* is present in all *H. pylori* strains and consists of two variable regions, the s- and m-regions, with a strong correlation

between *vacA* type and bacterial virulence. A recent study traced *vacA* to myeloid cells in the gastric lamina propria and demonstrated that it suppressed IL-23 expression by dendritic cells while inducing IL-10 and TGF-β expression in macrophages. These findings support the notion that *H. pylori* uses *vacA* to create a tolerogenic environment, skewing T-cell responses toward Tregs, and promoting *H. pylori* persistence (Altobelli et al., 2019). Furthermore, *vacA* enhances the production of IL-8 and monocytes MCP-1 by U937 cells, a human monocytes-derived cell line (Hisatsune et al., 2008). *H. pylori* VacA has emerged as a critical factor in shaping the outcome of *H. pylori* infection, acting on both T cells and myeloid cells. It prevents T-cell activation and function while inducing tolerogenic activities in DCs (Gebert et al., 2003, Gerhard et al., 2005).

Secondary bile acids (BAs) are metabolites produced from gut microbial fermentation of primary BAs, mainly deoxycholic acid (DCA) and lithocholic acid (LCA) (Winston and Theriot, 2020), that can be found amongst other metabolic byproducts in the human bloodstream and tissue fluid. In the gut, they can affect the composition of the microbial communities. BAs interact with receptors found on macrophages, dendritic cells, myeloid-derived suppressor cells (MDSCs), Treg cells, Th17 cells, innate lymphoid cells (ILCs), CD4 cells, CD8 cells, B cells, and NKT cells to regulate their differentiation and function, maintaining gut and systemic homeostasis (Su et al., 2023). These receptors encompass various nuclear receptors such as the farnesoid X receptor (FXR), liver X receptor (LXR), pregnane X receptor (PXR), vitamin D receptor (VDR), retinoid-related orphan receptor gamma t (RORγt), and constitutive androstane receptor (CAR). Additionally, they include membrane receptors like G-protein bile acid receptor 1 (GPBAR1, also known as Takeda G protein-coupled receptor 5 or TGR5), sphingosine-1-phosphate receptor 2 (S1PR2), cholinergic receptors muscarinic 2 and 3 (CHRM2 and CHRM3), and MAS-related G-protein coupled receptor family member X4 (MRGPRX4), as reviewed by Biagioli et al. (Biagioli et al., 2021). BAs also act as signaling molecules and can stimulate multiple signaling

pathways. For instance, the interaction between bile acids and the bile acid receptor (BAR) in LPS-activated macrophages suppresses NF-κB transcription, reducing excessive pro-inflammatory cytokine expression. Moreover, bile acids' activation of the G-protein-coupled bile acid receptor 1 (GPBAR1) triggers cyclic adenosine monophosphate (cAMP) production. Consequently, bile acids disrupt the NF-κB signaling pathway either through direct interference or by competing with cAMP for the transcriptional region. Additionally, the secondary bile acid DCA inhibits the LPS-induced expression of pro-inflammatory cytokines IL-1, IL-6, and TNF-α in DCs. This suppression can be reversed by a deficiency in the DCA receptor TGR5. The inhibitory effects of TGR5 are mediated through the suppression of NF-κB via the TGR5–cAMP–PKA signaling pathway (Hu et al., 2021). Additionally, the activation of TGR5 by bile acids induces the differentiation of human monocytes into DCs that produce lower levels of IL-12 and TNF-α through the TGR5-cAMP pathway (Ichikawa et al., 2012). They can also inhibit the function of macrophages. It is known that macrophages can secrete IL-6 to promote B cell precursors to become antibody-producing cells and secrete IFN-γ and TNF-α to promote apoptosis of cancer cells (Yang et al., 2021b). DCA and LCA inhibit the secretion of IL-6, IFN-γ, and TNF-α, and induce the polarization of anti-cancer M1 macrophages to pro-cancer M2 macrophages (Yunna et al., 2020). Secondary BAs can also inhibit the function of DCs. It is known that DCs can secrete TNF-α to promote apoptosis of cancer cells and secrete IL-12 to activate Th1 cells to participate in anti-tumor immune response (Gardner et al., 2020). However, a study has demonstrated that DCA and LCA can inhibit DCs to secrete TNF-α and IL-12, (Fiorucci et al., 2021) and then play a role in promoting cancer (Yang and Qian, 2022). Taurolithocholic acid 3-sulfate (TLCA3S) a secondary bile acid conjugated with taurine, suppressed the expression of IL-6, IL-12, TNF-α, and IFN-β induced by LPS (Haselow et al., 2013).

Secondary BAs can prevent the apoptosis of cancer cells, induce the progression of cancer cell cycles, enhance the ability of metastasis and invasion of cancer cells, and promote the transformation of cells into cancer stem cells (CSCs). Moreover, secondary BAs induce cancer by regulating the function of immune cells (Yang and Qian, 2022).

This study aimed to investigate the effect of *cag* PAI-negative *H. pylori*, *cag* PAI-positive *H. pylori,* and isogenic virulence factor mutants on cytokine production by dendritic cells and monocytes *in vitro.* Human peripheral blood monocytes, monocyte-derived DCs, and THP-1 and KG-1 cell lines were co-cultured with bacterial strains. In addition to *H. pylori* stimulation, the study also investigated the potential effects of secondary BAs on the monocyte cytokine response. **It was hypothesized that:**

1. *H. pylori* stimulates IL-10, IL-6, and IL-16 production by monocytes and DCs through a mechanism dependent on a functional *cag* PAI.
2. Secondary bile acids such as DCA, LCA, and TLCA3S play a role in regulating cytokine production by monocytes

## 3.2 Materials and Methods

### 3.2.1 THP-1 cell line and culture

THP-1 is a human monocytic cell line isolated from an acute monocytic leukemia patient (European Collection of Authenticated Cell Ceultures). These non-adherent cells were maintained in culture in Roswell Park Memorial Institute medium (RPMI 1640, Invitrogen) culture medium supplemented with 10% of heat-inactivated fetal bovine serum (FBS)(Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 ˚C and 5% CO2 in a humidified atmosphere.

### 3.2.2 KG-1 cell line and culture.

The KG-1 cell line was originally isolated from the bone marrow of a patient with erythroleukemia that developed into acute myeloid leukemia. KG-1 cells were obtained from the European Collection of Cell Cultures (European Collection of Authenticated Cell Ceultures), and were maintained in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Paisley, UK), supplemented with 10% FBS at 37 ˚C and 5% CO2 in a humidified atmosphere.

### 3.2.3 *H. pylori* strains and culture conditions

Experiments were performed with *H. pylori* insertion mutants with inactivation of the *cagA* (60190ΔcagA), *cagE* (60190ΔcagE), or *vacA* (60190ΔvacA) genes were studied together with their parental wild type strain 60190 (ATCC 49503, *cagPAI, vacA*s1/m1). In parallel experiments, *H. pylori* strain Tx30a (ATCC 51932) expresses s2m2 *vacA* toxin but does not possess the *cagPAI* were also used.
*H. pylori* strains were cultured on Columbia blood agar plates supplemented with 5% horse blood (Oxoid, UK) and incubated for 48h in a microaerophilic 2-65 workstation (MACS VA500 (DW Scientific) at 37˚C and 5% $CO_2$ in a humidified atmosphere for 48 h. The strains were sub-cultured every 2 days.

### 3.2.4 Co-culture of *H. pylori* and THP-1 cells

Human monocytic cells were cultured in T75 flasks with 25ml of RPMI 1640 medium with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS at 37˚C in an atmosphere of 5% $CO_2$. Flasks containing 3-7 X $10^5$ cells/ml were poured into 25ml universal tubes and centrifuged at 200 x g for 3 minutes. The supernatant was removed, and the cells were washed with an antibiotic-free medium 3 times and resuspended in 10 ml medium. Cells were diluted to 1 X $10^6$ per ml in the required volume for the assay. 0.5ml of this suspension was added to the wells of 24 well plates and incubated until the bacteria had been prepared. The two plates of 24h *H. pylori* growth was resuspended in 3ml sterile PBS. This

bacterial suspension was adjusted to a final optical density 600 nm wavelength ($OD_{600}$) of 0.02, (1 x $10^9$ bacteria/ml and 50 µl added to each well of the 24 well plates of cells to give a final multiplicity of infection (MOI) of 100 bacteria per THP-1 cell. Plates were incubated for 24 hours at 37˚C in an atmosphere of 5% $CO_2$, before harvesting the culture supernatants.

### 3.2.5 Separation of peripheral blood mononuclear cells (PBMCs) from buffy coats.

Buffy coats from three healthy blood donors were obtained from NHS Blood and Transport, and PBMCs were isolated by density gradient centrifugation using Histopaque-1077 (Sigma- Aldrich, UK). Buffy coat bags were cut and drained into universal tubes and diluted (1:2) up to 50 ml with RPMI-1640 media. Then 15 ml of the diluted buffy coat was layered onto 10ml Histopaque-1077 in 50 ml falcon tubes. Samples were centrifuged for 20 min at (725 x g), low acceleration and deceleration, using an Allegra X- 15R centrifuge (Beckman Coulter). After the centrifugation, the PBMC layer was collected using sterile Pasteur pipettes and placed in a clean universal tube, washed with 25 ml of washing media for 3 min at (725 x g). The medium was poured off, and the pellet was resuspended in another 25 ml and washing media (RPMI complete medium [RPMI-1640] (Sigma-Aldrich, UK) and repeat the centrifugation two times. Then, the cells were washed twice with Easy Sep reagent and suspended in 10 ml of Easy Sep. The monocyte ($CD14^+$ cells) fraction was obtained by positive selection using human CD14 MicroBeads (Miltenyi Biotec, UK) and LS MACS columns (Miltenyi Biotec, UK), following the manufacturer's instructions.

### 3.2.6 Co-culture of $CD14^+$ cells with *H. pylori* and bile acid treatment

Isolated $CD14^+$ monocytes were cultured in different tubes at $10^6$ cells/ml in serum-free RPMI 1640 alone or under different conditions as shown in (**Table *6***). Treated $CD14^+$ cells were incubated for 24 hrs at $37^0$C. Then, cells were collected

for intracellular and surface staining, and supernatants were collected for ELISA. These doses of DCA were chosen from previous studies (Park et al., 2008, Jenkins et al., 2004). Taurolithocholic acid 3-sulfate (TLCA3S) was sourced from Sigma-Aldrich, product number: T0512. Deoxycholic acid (DCA) and Lithocholic acid (LCA) were sourced from Avanti, product numbers: 700197P and 700218P respectively.

**Table 6. Co-Culture of CD14$^+$ cells with *H. pylori* or bile acids.**

| Tube # | Conditions |
|---|---|
| 1 | Serum-free RPMI 1640 containing LPS (1 ng/ml) |
| 2 | Serum-free RPMI 1640 containing *Hp* Tx30a (1:100) |
| 3 | Serum-free RPMI 1640 containing *Hp* 60190 (1:100) |
| 4 | Serum-free RPMI 1640 containing *Hp* 60190 *cagA*- (1:100) |
| 6 | Serum-free RPMI 1640 containing *Hp* 60190 *vacA*- (1:100) |
| 7 | Serum-free RPMI 1640 containing *Hp* 60190 *cagE*- (1:100) |
| 8 | Serum-free RPMI 1640 containing *Hp* 60190 vacA- (1:100) |
| 9 | Serum-free RPMI 1640 containing 200 µM DCA |
| 10 | Serum-free RPMI 1640 containing 200 µM LCA |
| 11 | Serum-free RPMI 1640 containing 200 µM TLCA3S |
| 12 | Serum-free RPMI 1640 containing 200 µM DCA, and *Hp* 60190 (1:100) |
| 13 | Serum-free RPMI 1640 containing 200 µM LCA, and *Hp* 60190 (1:100) |
| 14 | Serum-free RPMI 1640 containing 200 µM TLCA3S, and *Hp* 60190 (1:100) |

### 3.2.7 Surface and intracellular staining:

CD14+ cells were centrifuged and suspended in 1ml of RPMI 1640 medium in FACS tubes for flow cytometry panel staining. To analyze cytokines by flow cytometry, cells were stimulated with LPS or *H. pylori* for 1 hour at 37°C in a 5% $CO_2$ environment. After 1 hour, Brefeldin A (BFA) was added at a concentration of 10mg/ml to prevent intracellular protein transport and cytokine secretion. The

cells were then incubated overnight at 37°C in a 5% $CO_2$ environment. The next day, the cells were collected by centrifugation and washed once with sterile PBS at (725 x g), for 5 minutes. The cells were resuspended in 100 µl of viability buffer, which consisted of 100 µl of PBS with a 1:500 dilution of Zombie NIR viability dye (Biolegend), and incubated at room temperature for 15 minutes in the dark. The cells were washed twice with PBA buffer (PBS containing 5% FCS and 10 mM sodium azide). To prevent antibodies from binding non-specifically to the cells, 2 µl of FcR blocker (Biolegend) was added to the cells to minimize potential nonspecific antibody staining caused by IgG receptors and incubated at room temperature for 10 minutes in the absence of light. The cells were then stained for surface markers (**Table *7***) and incubated on ice for 30 minutes. Tubes that did not require cytokine staining were treated with 500µl of fixation buffer (Biolegend) for 30 minutes at room temperature in the absence of light, followed by three washes with PBA. Finally, the cells were resuspended in 500µl of PBA for analysis and kept at 4°C. For intracellular cytokine staining, the tubes were fixed and washed twice with PBA buffer, followed by one wash with 1ml of 1x permeabilization buffer (eBioscience). Then, the cells were incubated with staining antibodies in the dark at room temperature for 2 hours. After incubation, the cells were washed twice with permeabilization buffer and suspended in 0.5ml of PBA staining buffer. The suspension was stored at 4°C without exposure to light for further analysis. Fluorescence acquisition was performed using the SONY ID7000 spectral analyzer (Beckmann-Coulter®), and 200,000 events were collected for each sample.

**Table 7. Flow Cytometry Specific Antibodies used.**

| Antibodies and reagents used. | Catalog no. | Clone | Quantity |
|---|---|---|---|
| **Anti-Human IL6 BV421** | BD, 563279 | 1138814 | 50 tests, 250 µl, 5 µl per test |
| **Anti-Human IL16 PE** | Biolegend, 519106 | 14.1 | 50 µg/ml 5 µl per test |
| **Mouse Anti-Human CD14 BV570** | Biolegend, 301831 | M5E2 | 150 µg/ml 5 µl per test |
| **Anti-Human IL10 PE/Dazzle** | Biolegend, 506811 | JES3-19F1 | 50 µg/ml 5 µl per test |
| **Viability stain Zombie NIR** | Biolegend, 423105 | - | diluted 100 µl per test |
| **Trustain FC blocker** | Biolegend, 422302 | - | 5 µl per test |
| **Rat IgG isotype control BV421** | Biolegend, 400429 | RTK2071 | 5 µl per test |
| **Rat IgG1 isotype control PE** | Biolegend, 400407 | BRG1 | 5 µl per test |
| **Rat IgG2a isotype PE/Dazzle** | Biolegend, 400557 | RTK2758 | 0.2 mg/ml |

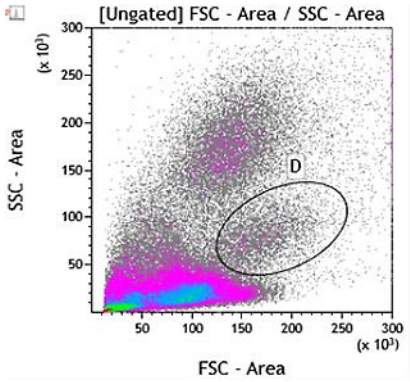
### 3.2.8 Flow cytometry analysis

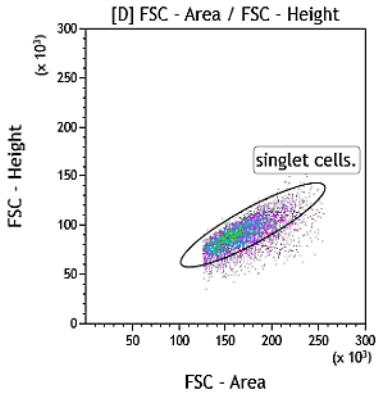
Data acquisition for multi-color flow cytometry was performed using an ID7000 spectral analyzer. Information on 100,000 events was collected and analyzed with Kaluza software (Beckman Coulter), ensuring appropriate isotype controls were considered. Samples were gated using forward and side scatter under the 488nm laser to exclude dead cells and debris.

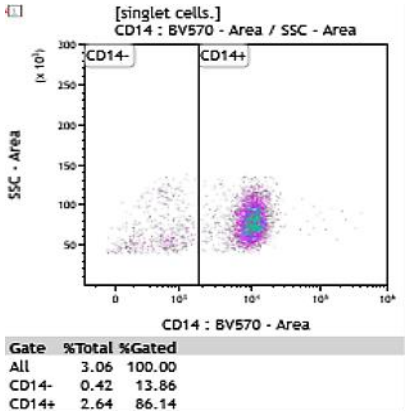
### 3.2.9 Gating strategy for $CD14^+$ cells

Flow cytometry gating parameters were determined based on isotype control antibodies. Figures showing a representative gating strategy from one experiment with human peripheral blood monocytes are shown in **Figure 28 A, B, C, and D**

**A**

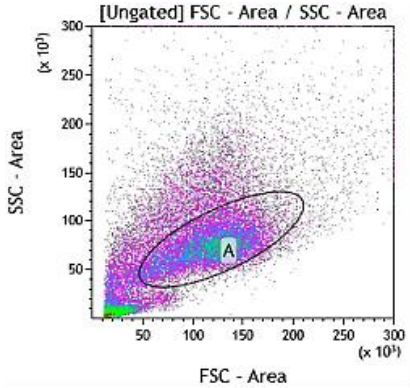


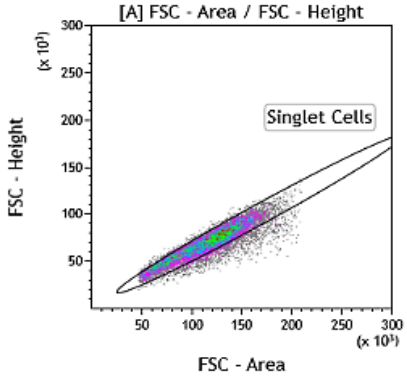



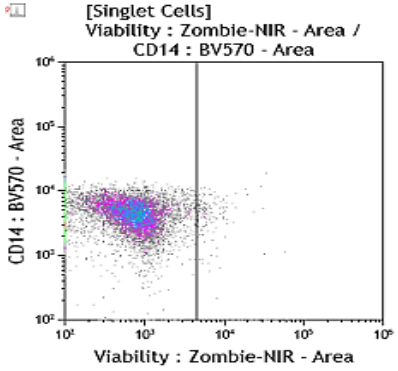




| Gate | %Total | %Gated |
|---|---|---|
| All | 3.06 | 100.00 |
| CD14- | 0.42 | 13.86 |
| CD14+ | 2.64 | 86.14 |

**B**



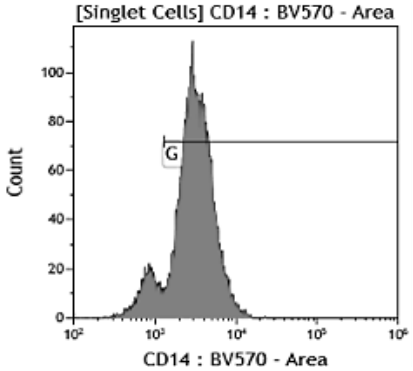
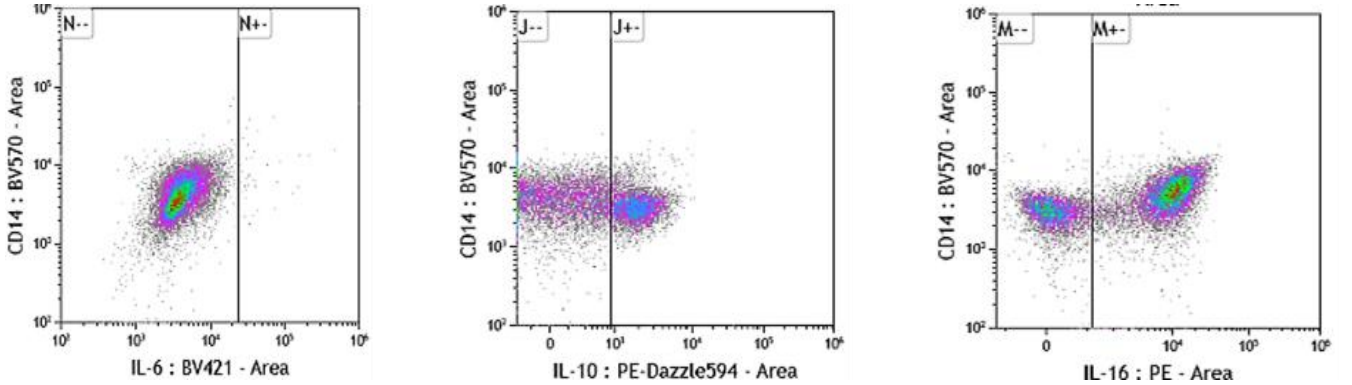




**Figure 27. Purity of isolated monocytes.**

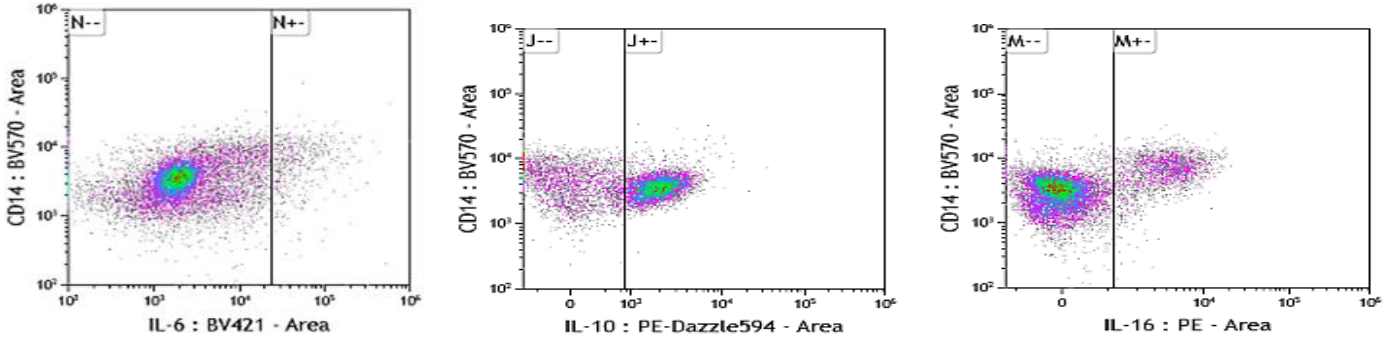
$CD14^+$ Monocytes were isolated from peripheral blood mononuclear cells (PBMCs) using CD14 positive selection. After isolation, monocyte purity was analyzed by flow cytometry. **(A) From left to right**; monocytes gated on PBMCs before separation according to forward and side scatter; debris and doublet exclusion; $CD14^+$ cells-stained cells. **(B)** monocytes after separation from PBMCs; debris and doublet exclusion; dead cells were excluded by viability dye; $CD14^+$ cells-stained cells. % $CD14^+$ cells after separation 88.23%.

**A**


N--
N+-
CD14 : BV570 - Area
IL-6 : BV421 - Area
J--
J+-
CD14 : BV570 - Area
IL-10 : PE-Dazzle594 - Area
M--
M+-
CD14 : BV570 - Area
IL-16 : PE - Area

Lps

**B**


N--
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CD14 : BV570 - Area
IL-6 : BV421 - Area
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CD14 : BV570 - Area
IL-10 : PE-Dazzle594 - Area
M--
M+-
CD14 : BV570 - Area
IL-16 : PE - Area

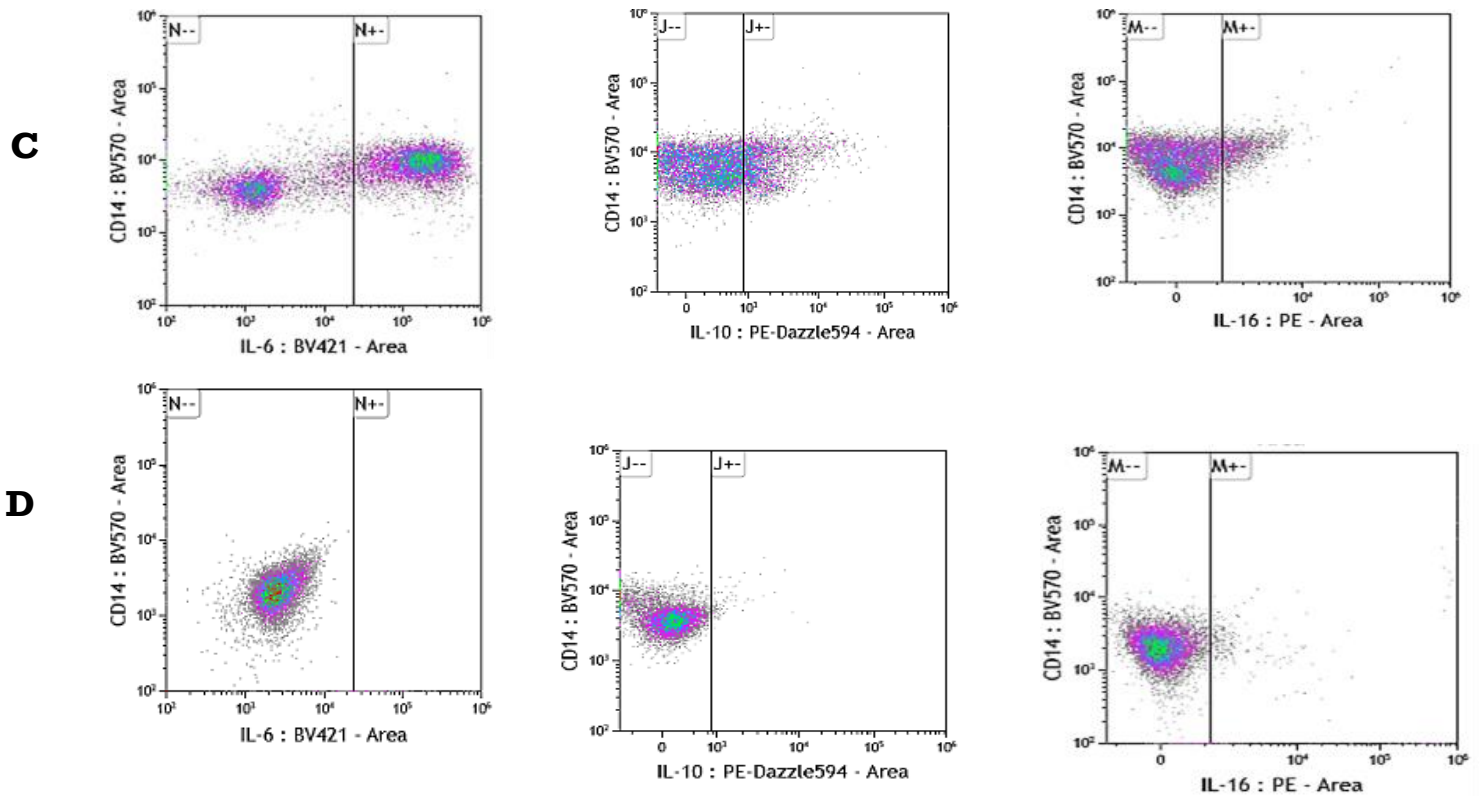


**Figure 28. Representative gating strategy for flow cytometry analyses.**

Isolated CD14$^+$ cells were stained with anti-human fluorochrome-conjugated antibodies to identify IL-6, IL-10, and IL-16 cytokines within the CD14$^+$ cell population. **From left to right; (A)** cytokine plots for unstimulated CD14+ cells, **(B)** cytokine plots for CD14+ cells stimulated with LPS, **(C)** cytokine plots for CD14+ cells stimulated with the Tx30a strain of H. pylori, and **(D)** cytokine plots for LPS-stimulated CD14+ cells using isotype control antibodies.

### 3.2.10 ELISA

The cytokine concentrations in culture supernatants of THP-1 and KG-1 cells and $CD14^+$ cells were measured using commercial microtiter plate ELISAs, following the manufacturers' instructions. All assays were based on a quantitative sandwich enzyme immunoassay technique; IL-6, IL-10, and IL-16 secretion were measured using ELISA kits from Invitrogen (88-7066 for IL-6; 2-200 pg/mL and 88-7106 for IL-10; 2-300 pg/mL assay rang), and R&D SYSTEMS (DY316 for IL-16; 15.6–1000 pg/mL assay range), respectively. An aliquot of 50 µl samples was measured in duplicate for all samples. Within and between assays, variations were less than 5 and 8%, respectively. Optical density readings were made using a BioTek EL800 microtiter plate reader (Labtech International), at a wavelength of 450nm, subtracting readings at a reference wavelength of 595nm

## 3.3 Results

### 3.3.1 Effect of wild-type *H. pylori* strains Tx30a and 60190 and 60190-derived isogenic mutants on IL-16, IL-6, and IL-10 production by THP-1 cells using ELISA.

To understand the involvement of innate immune cells in the persistence of *H. pylori* infection, we investigated which bacterial virulence factors are responsible for the induction of the inflammatory mediators IL-16, IL-6, and IL-10, during *H. pylori* infection of human monocytes. As monocytes are one of the first immune cells recruited to the *H. pylori*-infected gastric mucosa, these were the focus of this study. In initial studies, the THP-1 human monocytic cell line was infected with different *H. pylori* strains at a MOI of 1:100. These included the wild-type Tx30a strain, which expresses the inactive s2m2 type of VacA toxin but does not have the *cag*PAI, and the wild-type 60190 strain (*cag*PAI positive, toxic s1i1 type of VacA). Isogenic mutants of the 60190 strain that had mutations in these virulence factor genes, including *cagA* (60190ΔcagA), *cagE* (60190ΔcagE), and *vacA* (60190ΔvacA) were also used. Sterile culture medium was used as a negative control, while 1ng/ml of *E. coli* LPS Serotype (055:B50), Thermo Fisher Scientific) used as a positive control. After 24 hours of incubation, supernatants were collected, and cytokines levels were measured by ELISA. Comparisons of the effects of the wild-type Tx30a versus 60190 strain, and 60190 strain with its respective isogenic mutants, were made from four independent repeat experiments, and these paired data were analysed using the Friedman test.

As shown in **Figure 29 A**, the negative control cells produced an appreciable amount of IL-16 as the human monocytes constitutively expressed it. The levels of IL-16 produced in response to the Tx30a strain and LPS were similar, with mean values of 227 and 224 pg/ml, respectively. There were no significant differences observed between the wild-type 60190 strain and its isogenic

mutants. However, the *vacA* null mutant strain induced 25% lower IL-16 concentrations compared to the 60190 wild-type strain, with mean values of 199 and 267 pg/ml, respectively. Conversely, the *cagE* mutant strain produced 21% more IL-16 (mean=325 pg/ml) compared to the 60190 wild-type strain and other isogenic mutants. From **Figure 29 B**, it can also be observed that THP-1 cells cultured with LPS, 60190 Δ *cagE*, and 60190 Δ *vacA H. pylori* strains produced comparable amounts of IL-6, with mean values of 90 pg/ml for LPS and 99 pg/ml for both 60190 Δ *cagE* and 60190 Δ *vacA* strains. Interestingly, THP-1 cells infected with the Tx30a strain showed the highest IL-6 production (mean of 205 pg/ml), which is significantly different from the control cells (P=0.04). Furthermore, the 60190 *cagE*- and 60190 *vacA*- *H. pylori* mutants increased IL-6 production by THP-1 cells by 35% compared to the levels produced by THP-1 cells infected with the wild-type 60190 strain.

The production of IL-10 cytokines by THP-1 cells exhibited a comparable pattern of responses to that observed for IL-6 when stimulated by *H. pylori*. The Tx30a strain induced the highest level of IL-10 cytokine, which was significantly different from the control cells (P=0.007). There was no significant difference in the quantity of IL-10 produced by the 60190 strain or its isogenic mutants. The *cagE* mutant strain showed a slight increase in the amount of IL-10 compared to the wild type (mean = 7 pg/ml and 11 pg/ml respectively).

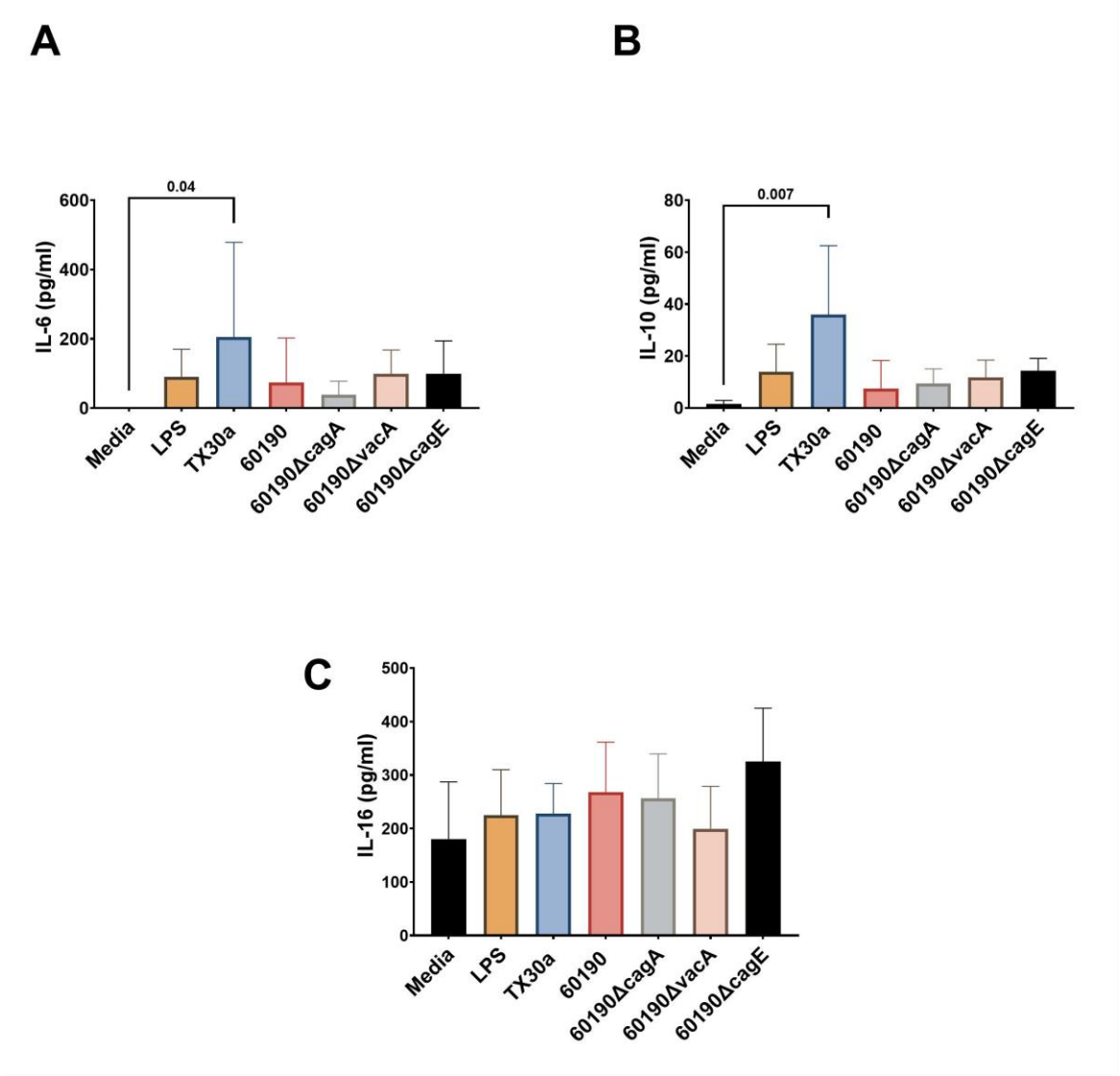


**Figure 29. Effect of wild-type *H. pylori* strains Tx30a and wild-type 60190 and its isogenic mutants on IL-16, IL-6, and IL-10 cytokines production by THP-1 cells.**

The human THP-1 monocytic cell line was cultured in the presence of wild-type Tx30a or the presence of wild-type 60190 or *the cagA, cagE, or vacA* isogenic *H. pylori* strain a multiplicity of infection (MOI) of 100:1 for 24 h. Sterile culture medium served as a negative control and LPS as a positive control. Cytokines levels by ELISA. The graphs on the left represent the cytokines levels as pg/ml from four independently repeated experiments, with data from each experiment linked with a line. The graphs on the right side represent the means ± standard deviations from four independent experiments. Using the Friedman test, P >0.05. No significant difference.

### 3.3.2 Effect of wild-type *H. pylori* strains Tx30a and 60190 and its isogenic mutants on IL-16, IL-6, and IL-10 production by KG-1 cells using an ELISA.

The KG-1 cell line was also infected with different *H. pylori* strains (MOI 1:100). These included the wild-type Tx30a strain. the wild-type 60190 strain, and isogenic mutants of the 60190 strain that had mutations in known virulence factors, including *cagA* (60190ΔcagA), *cagE* (60190ΔcagE), and *vacA* (60190ΔvacA) genes were also used. A sterile culture medium was used as a negative control while LPS (10 pg/ml) was used as a positive control. After 24 hours of incubation, supernatants were collected, and cytokines levels were measured by ELISA. Comparisons of the effects of the wild-type Tx30a versus 60190 strain, and 60190 strain with its respective isogenic mutants, were made from four independent repeat experiments, and these paired data were analysed using the Friedman test.

As shown in **Figure 30 A,** the negative control cells, similar to THP-1 cells, produced IL-16 (256 pg/ml). The level of IL-16 produced in response to Tx30a (mean=413.8 pg/ml), 60190 strain (mean=522.6 pg/ml), and its isogenic mutants were comparable. In **Figure 30 B & C**, the negative control cells did not produce IL-6 or IL-10. KG-1 cells cultured with LPS, Tx30a, 60190, 60190 Δ *cagA*, 60190 Δ *cagE*, and 60190 Δ *vacA*. *H. pylori* strains produce comparable amounts of IL-6 as well as IL-10. Moreover, it was observed that higher induction of IL-10 and IL-6 with the Tx30a strain, but this was observed in two experiments only. These data suggest that *H. pylori* and its virulence factors (*cagA, cagE, and vacA*) do not affect the induction of IL-16, IL-6, and IL-10 in KG-1 cells.

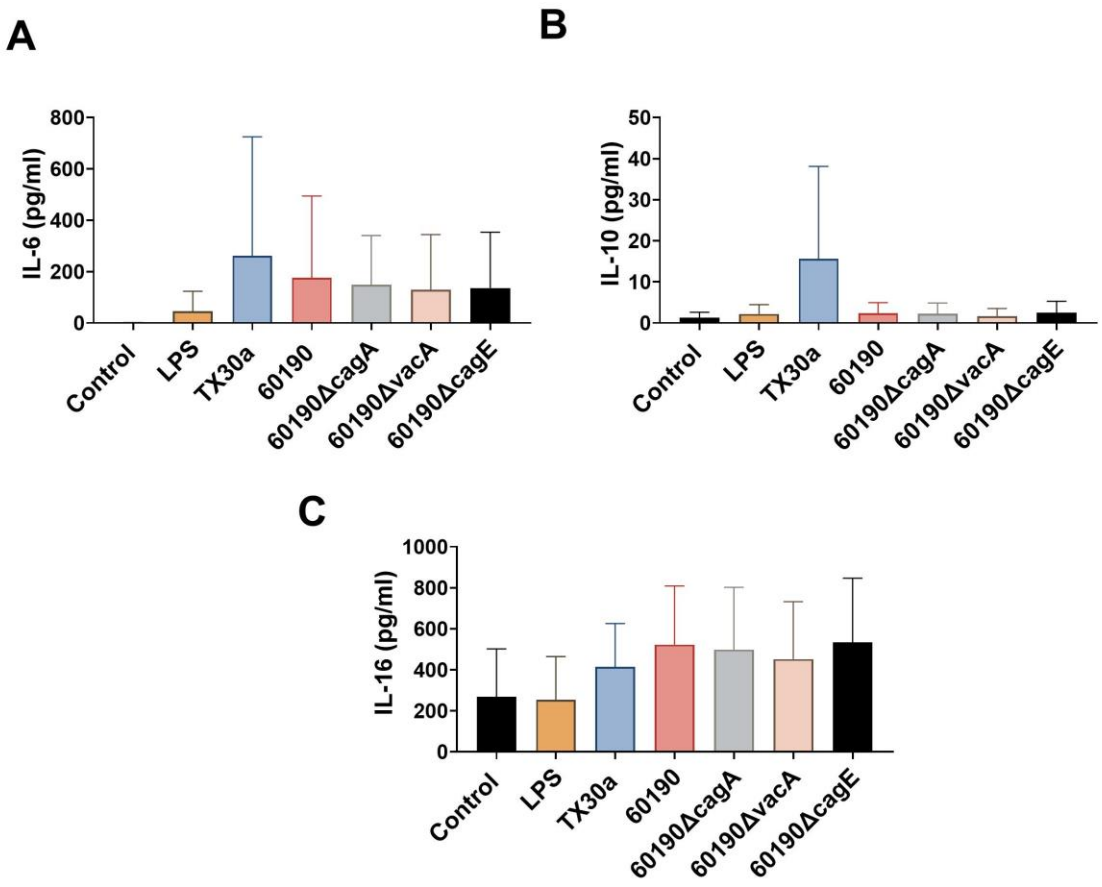


**Figure 30. Effect of wild-type *H. pylori* strains Tx30a and wild-type 60190 and its isogenic mutants on IL-16, IL-6, and IL-10 cytokines production by KG-1 cells.**

The human KG-1 cell line was cultured in the presence of wild-type Tx30a or the presence of wild-type 60190 or the *cagA, cagE, or vacA* isogenic *H. pylori* strain a multiplicity of infection (MOI) of 100:1 for 24 h. Sterile culture medium served as a negative control and LPS as a positive control. Cytokines levels by ELISA. The graphs on the left represent the cytokines levels as pg/ml from four independently repeated experiments, with data from each experiment linked with a line. The graphs on the right side represent the means ± standard deviations from four independent experiments. Using the Friedman test, P >0.05. No significant difference.

### 3.3.3 Effect of wild-type *H. pylori* strains Tx30a and 60190 and its isogenic mutants on IL-16, IL-6, and IL-10 production by $CD14^+$ cells using ELISA.

Human peripheral blood $CD14^+$ cells were isolated from buffy coats of four donors **Figure 27 A & B.** The isolated $CD14^+$ cells were infected with different *H. pylori* strains (MOI 1:100). These included the wild-type Tx30a strain, 60190 strain, and isogenic mutants of the 60190 strain that had mutations in known virulence factor genes, including *cagA* (60190 Δ cagA), *cagE* (60190 Δ cagE), and *vacA* (60190 Δ vacA) were also used. A sterile culture medium was used as a negative control, while *E. coli* LPS (10 pg/ml) was used as a positive control. After 24 hours of incubation, supernatants were collected, and cytokines levels were measured by ELISA. Comparisons of the effects of the wild-type Tx30a versus 60190 strain, and 60190 strain with its respective isogenic mutants, were made from four independent repeat experiments, and these paired data were analysed using the Friedman test.

As shown in **Figure 31 A**, the negative control cells, similar to THP-1, and KG-1 cells, produce a large amount of IL-16 (mean 288 pg/ml). The level of IL-16 produced in response to Tx30a, and 60190 strains is comparable with mean values of 439 and 579 pg/ml, respectively. There was no significant difference in the quantity of IL-16 produced by the 60190 strain or its isogenic mutants. As shown in **Figure *31* B & C**, it is evident that control $CD14^+$ cells did not secrete IL-6 or IL-10. The $CD14^+$ cells treated with LPS, Tx30a, the 60190 strain, and its isogenic mutants produced IL-6 and IL-10, but no significant differences were noted between the strains. Like THP-1 cells, $CD14^+$ cells infected with the Tx30a strain showed the highest IL-6 production (mean=98.2 pg/ml). These results imply that *cagA, cagE, and vacA* genes may not influence the *H. pylori*-induced production of IL-6, and IL-10 in human monocytes.

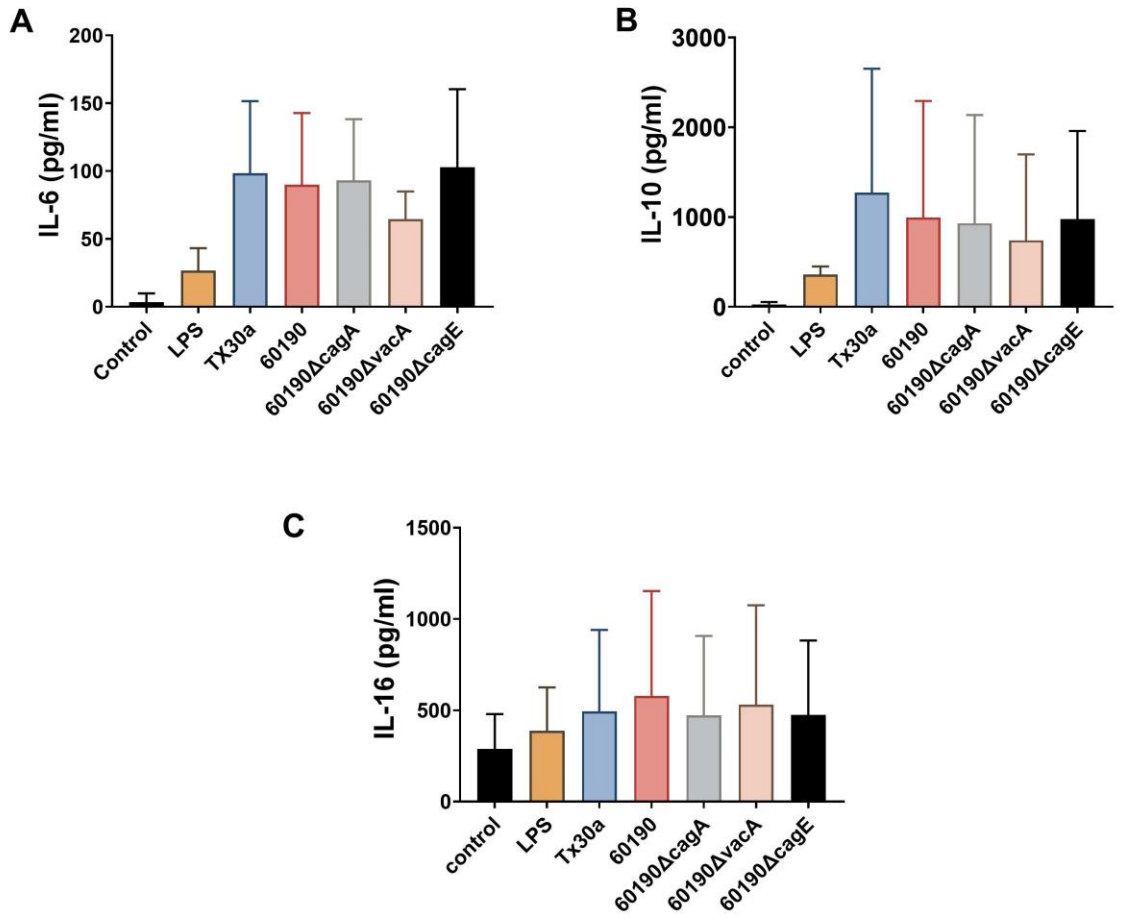


**Figure 31. Effect of wild-type *H. pylori* strains Tx30a and wild-type 60190 and its isogenic mutants on IL-16, IL-6, and IL-10 cytokines production by CD14$^+$ cells using ELISA.**

The isolated CD14$^+$ cells were cultured in the presence of wild-type Tx30a or the presence of wild-type 60190 or the *cagA, cagE, or vacA* isogenic *H. pylori* strain a multiplicity of infection (MOI) of 100:1 for 24 h. Sterile culture medium served as a negative control and LPS as a positive control. Cytokine levels were measured by ELISA. The graphs on the left represent the cytokines levels as pg/ml from four independently repeated experiments, with data from each experiment linked with a line. The graphs on the right side represent the means ± standard deviations from four independent experiments. Using the Friedman test, P >0.05. No significant

### 3.3.4 Effect of wild-type *H. pylori* strains Tx30a and 60190 and its isogenic mutants on IL-16, IL-6, and IL-10 production by $CD14^+$ human peripheral blood cells using flow cytometry.

For intracellular cytokines staining, consistent with the ELISA data, control $CD14^+$ cells did not show an increase in upregulation in the percentage of $IL\text{-}6^+CD14^+$ cells. Conversely, it was found that $CD14^+$ cells cultured with Tx30a, the 60190 strain, and its *cagA* and *cagE* isogenic mutants showed an increased percentage of $CD14^+IL\text{-}6^+$monocytes to comparable percent with no significant differences noted between the strains. For IL-10 and IL-16, it was found that elevation percentage of $CD14^+$ $IL\text{-}16^+$ (40%) and $CD14^+$ $IL\text{-}10^+$ in control cells (18%). Additionally, *H. pylori* strains downregulated the percentage of $IL\text{-}16^+$ cells to 3% compared to the initial control, (40%), While *vacA* mutant strain upregulated the percentage of $IL\text{-}16^+$ cells to 33%. gating strategy are shown in **Figure 28 A, B, C, and D.**

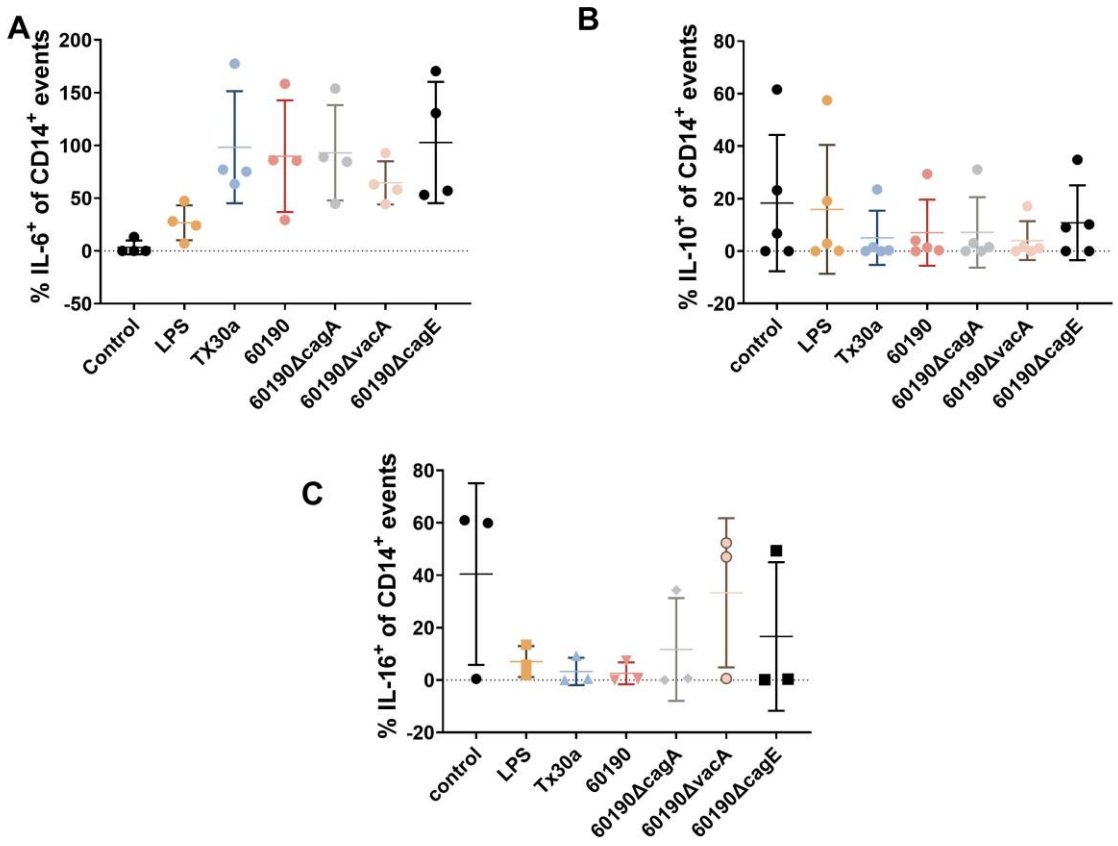


**Figure 32. Effect of wild-type *H. pylori* strains Tx30a and wild-type 60190 and its isogenic mutants on IL-16, IL-6, and IL-10 cytokines expression by CD14$^+$ cells using flow cytometry.**

Human peripheral blood CD14$^+$ cells were cultured in the presence of wild-type Tx30a or in the presence of wild-type 60190 or the *cagA, cagE*, *or vacA* isogenic *H. pylori* strain a multiplicity of infection (MOI) of 100:1 for 24 h. Sterile culture medium served as a negative control and LPS as a positive control. The frequencies of cytokine-positive cells were determined by flow cytometry. The graphs represent the percentage of cytokines of CD14$^+$ cells and the results are expressed as the means ± standard deviations from four independent experiments. Using the Friedman test, P >0.05.

### 3.3.5 Effect of bile acids on cytokines production by CD14$^+$ monocytes.

Since the stomach harbors various bacterial species, heightened sensitivity to bacterial PAMPs may lead to inflammation-induced tissue harm. Research has identified a range of bacteria naturally found in the gastric environment, like

*Streptococcus* and *Staphylococcus* (Yang et al., 2016), underscoring the importance of investigating how monocytes stimulated by *H. pylori* respond to antigens from these gastric bacteria. The diverse microbiota in the gastric region could elevate the chances of *H. pylori*-primed monocytes encountering other bacterial products and metabolites such as secondary bile acids, potentially triggering excessive activation. Evidence suggests that the absence of normal flora in *H. pylori*-infected mice decreased gastritis symptoms and delayed the onset of gastric neoplasms (Lofgren et al., 2011). Therefore, this research also examined changes in inflammatory cytokines following exposure to bile acids. $CD14^+$ monocytes were isolated from three different donors and exposed to 200 μM of three distinct bile acids (DCA, LCA, and TLCA3S) in the presence or absence of the 60190 *H. pylori* strain. Since secondary bile acids were dissolved in dimethyl sulfoxide (DMSO), $CD14^+$ cells were similarly treated with DMSO in the presence or absence of *H. pylori* to ascertain whether the cytokine release was a result of the bile acids' effects rather than the DMSO itself. Following a 24-hour incubation period, cytokines levels in the collected supernatants were measured using ELISA. The data showed that the presence of the secondary bile metabolites DCA, LCA, and TLCA3S inhibited IL-6 and IL-10 production by $CD14^+$ cells while adding the *H. pylori* to the secondary bile metabolites induced IL-6 and IL-10 production. $CD14^+$ cells treated with DMSO also inhibited the IL-6 and IL-10 cytokines secretion by $CD14^+$ indicating this effect cannot be unique for the bile metabolites.

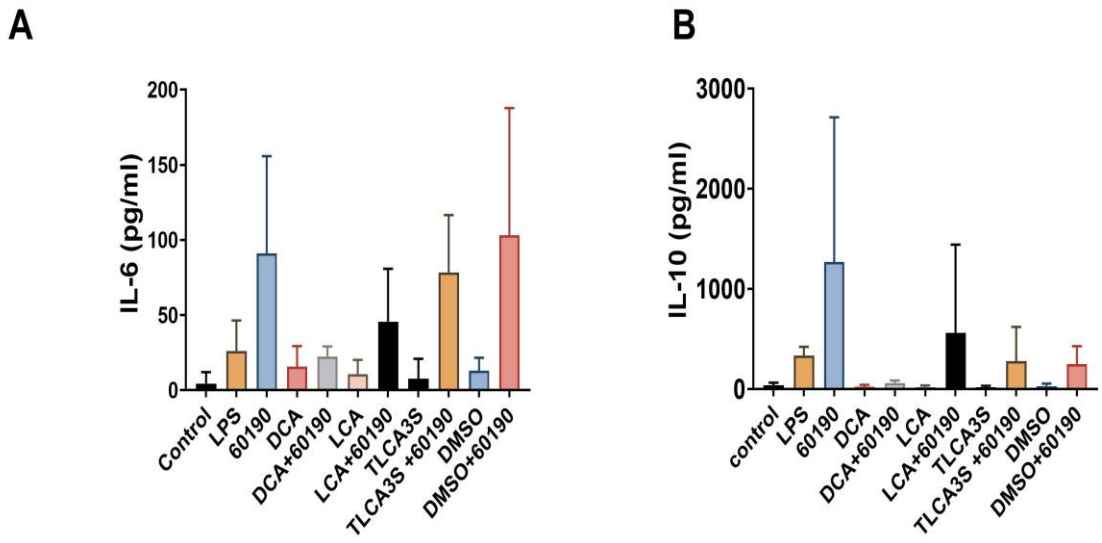


**Figure 33. The impact of bile metabolites on the production of cytokines by CD14$^+$ monocytes.**

The Isolated human peripheral blood CD14$^+$ monocytes were treated with DCA, LCA, TLCA3S, and DMSO (200 μM) in the presence or absence of *H. pylori* (60190 strain) a multiplicity of infection (MOI) of 100:1 for 24 h. Sterile culture medium served as a negative control and LPS as a positive control. Cytokine levels were measured by ELISA. The graphs on the left represent the cytokines levels as pg/ml from three independently repeated experiments, with data from each experiment linked with a line, and the graphs on the right side represent the means ± standard deviations from three independent experiments. Using the Friedman test, P >0.05. No significant. DMSO= dimethyl sulfoxide, DCA= deoxycholic acid, LCA= lithocholic acid (LCA), and TLCA3S= taurolithocholic acid-3 sulfate

## 3.4 Discussion

Several cytokines are increased in the stomach of *H. pylori*-infected individuals compared to uninfected controls such as IFN-γ, TNF-α, IL-1β, IL-18, IL-10, IL-8, IL-7, IL-12, and IL-6. These cytokines are thought to be secreted by gastric epithelial cells and infiltrating cells such as monocytes, macrophages, DCs, and lymphocytes (Alzahrani et al., 2014). Maintaining a balance between pro-inflammatory cytokines such as IL-6 and anti-inflammatory cytokines like IL-10 is critical in shaping the outcome of *H. pylori* infection. An imbalance in

these cytokines can result in persistent inflammation, tissue harm, and potentially the onset of *H. pylori*-related conditions like gastritis, peptic ulcers, and gastric cancer (Sugimoto et al., 2010)

Myeloid antigen-presenting cells play a role in initiating and maintaining immune responses against *H. pylori*, resulting in the infiltration of immune cells into the infected tissue. While previous studies have primarily focused on GECs during *H. pylori* infection, it is also likely that monocytes and DCs, which are antigen-presenting cells, contribute to the inflammatory processes induced by *H. pylori*. Therefore, this study has focused on cells of the monocytic lineage rather than GECs. Previous research has identified the ability of *H. pylori* to induce IL-6 and IL-10 secretion by human monocytes as well as DCs (Guiney et al., 2003, Kranzer et al., 2004). The current study is the first one that aimed to characterize the mechanisms utilized by *H. pylori* to induce cytokines production by monocytes and DCs using the THP-1 monocytic cell line, KG-1 dendritic cell line, and peripheral blood monocytes.

Culturing the THP-1 monocytic cell line with the *cag*PAI-negative strain Tx30a resulted in higher secretion levels of IL-6 and IL-10 compared to incubation with the *cag*PAI-positive strain 60190. Spontaneous production of IL-6 and IL-10 by THP-1 cells was not observed, suggesting that the THP-1 cells were not stimulated before incubation with the bacteria. The data also indicated that strain 60190 induced minimal secretion of IL-10 and IL-6 by THP-1 cells, with no significant difference observed between 60190 and its isogenic mutants. Although the sample size was small, there was no clear link found between the presence of *cagA, cagE* status, or *vacA* types and the levels of IL-6 and IL-10 secreted. This suggests that these factors are not crucial for IL-6 and IL-10 production in infected THP-1 cells. These findings align with previous data showing increased IL-12 and IL-8 production in THP-1 cells when exposed to *cag*-negative strains compared to *cag*-positive strains, and that *cag PAI* and *vacA* status were not essential for IL-12 and IL-8 production in the THP-1 monocytic cell model (de Jonge et al., 2001).

While Cag proteins play a significant role in inducing IL-6 secretion in GECs (Lu et al., 2005a), their relevance in IL-6 production by THP-1 cells appears to be minimal. This suggests that the mechanism of IL-6 production is different between gastric epithelial and monocytic cells. *H. pylori* strain Tx30a lacks the *cag* pathogenicity island yet induced high IL-6 levels in THP-1 cells, with the underlying cause remaining unknown. In the case of IL-16 cytokine, unstimulated THP-1 cells exhibit considerable IL-16 production due to their constitutive expression in human monocytes. Levels of IL-16 produced by strains Tx30a and 60190 were comparable. An isogenic mutant lacking *cagE* showed a 25% increase in IL-16 production in THP-1 cells compared to the wild type of strain, although this difference was not statistically significant. Conversely, in monocytes present in peripheral blood, the intracellular staining of IL-16 revealed that unstimulated $CD14^+$ cells produced a significant amount of IL-16. However, both LPS and the wild-type *H. pylori* strains suppressed IL-16 production by $CD14^+$ cells. The removal of the *vacA* gene from the 60190-strain restored IL-16 levels to the initial control level. This observation aligns with a previous study indicating that unstimulated $CD14^+$ cells release IL-16, while LPS stimulation hinders IL-16 release by inhibiting caspase-3 activity (Elssner et al., 2004a). IL-16 levels were also measured in monocyte-derived dendritic cells (MoDCs) following a 24-hour co-culture with *H. pylori*, it appears that *H. pylori* does not impact the production of IL-16 by MoDCs (data is not shown).

The KG-1 cell line was utilized as a dendritic cell-like model (Teobald et al., 2008). The cytokine levels were assessed in the supernatants following the co-culture of KG-1 cells with *H. pylori*. Among four experiments, two exhibited a similar response to the Tx30a strain, showing higher levels of IL-10 and IL-6 cytokines compared to the 60190 strain. In fact, IL-10 production was almost absent when the cells were exposed to *cag*-positive strains and their isogenic mutant strains. This result is comparable with a previous study that reported little IL-6 release, and no IL-10 secretion by human DCs after stimulation with *H. pylori (*Guiney et al., 2003). Additionally, co-culturing KG-1 cells with *cag* PAI-

negative and positive strains consistently led to the secretion of comparable levels of IL-16 with no difference between the strains.

This analysis also studied the impact of secondary bile metabolites (DCA, LCA, and TLCA3S) on cytokine production by peripheral blood monocytes in the presence or absence of *H. pylori*. The findings indicated that the secondary bile metabolites DCA, LCA, and TLCA3S suppressed the production of IL-6 and IL-10 by $CD14^+$ cells. The introduction of *H. pylori* along with the secondary bile metabolites stimulated the production of IL-6 and IL-10. This finding is in line with a previous report that has shown bile acids such as LCA mimic the LPS-induced expression of pro-inflammatory cytokines including IL-6 (Haselow et al., 2013). As bile metabolites were dissolved in DMSO, it was essential to discriminate if the observed response by $CD14^+$ cells was because of bile metabolites rather than the DMSO. The result showed that $CD14^+$ cells treated with DMSO also hindered the secretion of IL-6 and IL-10 cytokines by $CD14^+$, suggesting that this effect may not be exclusive to bile metabolites.

There are some limitations in this study. At the outset, it relied on continuous cell lines, which have the capacity for indefinite proliferation, are usually more resilient, and simpler to handle compared to primary cells. Another important advantage of using THP-1 cells is their uniform genetic background, which eliminates donor variability. Additionally, they are readily accessible and can be acquired without contamination from other blood components, unlike primary human monocytes, which are in limited supply. Several studies have demonstrated that THP-1 cells exhibit a more mature monocytic phenotype compared to other immortalized human monocyte cell lines, such as U937 cells (Schildberger et al., 2013).

However, a significant drawback of using immortalized cell lines is that they are genetically altered, which means they could have alterations in their signaling pathways and their physiological characteristics with extensive passaging. Next, primary cells ($CD14^+$ monocytes) were utilized due to their greater

relevance to biomedical research compared to continuous cell lines. However, these cells were taken from different human donors and might behave differently in case of immune responses because of genetic differences between the donors and potentially different viability of the isolated cell preparations. Buffy coats from the blood bank were used to isolate sufficient $CD14^+$ cells for the experiments, and these buffy coats were kept in the cold before being received. There may have been differences in the storage time for each buffy coat sample, which subsequently affected the viability of the isolated cells. Moreover, prior studies have indicated that certain NSAIDs and antibiotics can influence the production of cytokines such as IL-6 and IL-10 by monocytes and DCs (Bailly et al., 1993, Morikawa et al., 1996b, Raaijmakers et al., 2022). There is no available information regarding the medication being taken by the donors before blood donation. All these factors are potentially accounting for the noted variations in cytokine response following *H. pylori* infection although in each experiment, a consistent number of cells and bacteria were utilized to reduce variability in the results.

Additionally, in this research, $CD14^+$ monocytes were isolated from the buffy coats via positive magnetic sorting. Positive selection was used due to its ability to yield a greater number of monocytes at a higher purity compared to negative selection. The enhanced purity could be attributed to the heightened specificity of the anti-CD14 antibodies used. In contrast, negative selection leaves $CD14^+$ cells ''untouched' in the supernatant, while other cell types like NK cells, T cells, B cells, and granulocytes are trapped by antibody-conjugated beads and then eliminated through magnetic capture. Despite the higher purity of the positive selection method, it also has some disadvantages. A bead-conjugated antibody is used for positive selection and binds to the CD14 receptors present on the surfaces of monocytes. A compatible CD14 antibody clone was used as recommended by the manufacturer of the cell separation kit, but it is possible that the presence of the bound beads could have led to

difficulty in staining the $CD14^+$ cells for flow cytometry. The positive selection method is also linked to a reduction in monocyte-specific function, whereas negative magnetic sorting maintains monocyte-specific functions like migration, adhesion, and the response to inflammatory stimuli such as LPS (Hornschuh et al., 2022). Thus, using negatively selected $CD14^+$ monocytes may have improved the CD14 staining and the cytokine response. The secondary metabolites analyzed in this study were dissolved in DMSO. The findings indicated that these metabolites suppress the production of IL-10 and IL-6, and when *H. pylori* was introduced to these metabolites, it triggered the secretion of IL-10 and IL-6 by $CD14^+$ monocytes. A comparable outcome was noted when the monocytes were exposed to DMSO. Therefore, it is likely that these responses could be attributed to the DMSO. Thus, using negatively selected monocytes, and a different solvent to dissolve the bile metabolites could give us a clearer idea about the impact of bile metabolites and *H. pylori* on cytokines production by human monocytes. Finally, $CD14^+$ cells were obtained from just four donors; expanding the donor pool may provide a more comprehensive understanding.

The main weakness of this study is the variability in cytokine responses observed with $CD14^+$ cells, which makes it difficult to draw definitive conclusions. This study also presents several advantages, including the use of both cancer cell lines and primary cells. It incorporates various *H. pylori* strains alongside mutant strains. Additionally, cytokine measurements were conducted using two distinct techniques: ELISA and flow cytometry.

It was observed that the Tx30a strain exhibited a higher growth rate compared to the other strains used, which could account for the increased production of IL-10 and IL-6 observed in THP-1 cells stimulated with the Tx30a strain. Although the same bacterial CFU was introduced for all strains in all experiments, after coculturing THP-1 cells with H. pylori for 24 hours, the Tx30a strain may have grown more rapidly, influencing cytokine production. This

variation in growth rates among the strains could explain the differences in the results.

In summary, *H. pylori* and its virulence factors did not affect the secretion of cytokines by KG-1 cells. However, the Tx30a strain appeared to induce higher levels of IL-10 and IL-6 by THP-1 cells. Virulence factors did not impact on cytokine production by THP-1 cells, as also observed with $CD14^+$ monocytes. *H. pylori*-induced IL-6 and IL-10 production by $CD14^+$ monocytes, but mutations in the tested virulence factors did not affect cytokine secretion. IL-16 secretion remained unaffected by *H. pylori* or its virulence factors in all cell lines, and the isolated $CD14^+$ cells. The production of cytokine by $CD14^+$ cells was inhibited by secondary bile metabolites, although this effect was also induced in the DMSO controls.

# Chapter 4. Quantification of Peripheral Blood Cytokines in *H. pylori* Infection and Gastric Cancer

## 4.1 Introduction

Gastric cancer (GC) is the fifth most common cancer worldwide, constituting 5.6% of all new cancer cases (over 1 million) in 2020, and is the fourth leading cause of cancer-related deaths, accounting for 7.7% of such fatalities (Sung et al., 2021). From an anatomical perspective, gastric cancer is classified as cardia GC and non-cardia GC, which originates from areas other than cardia (Karimi et al., 2014). Chronic gastritis resulting from *H. pylori* infection is a primary risk factor in the development of non-cardia GC (Jonaitis et al., 2018, Sjomina et al., 2018, Goh et al., 2011).

Clinical symptoms of GC are sporadic and wide-ranging during the initial stages of the disease, leading to late-stage diagnoses for most patients (Zaanan et al., 2018). Although surgical resection and chemotherapy are effective treatments for GC, the overall prognosis remains grim, with a global five-year survival rate of less than 30% (Eom et al., 2022). Therefore, early and accurate diagnosis is necessary for prompt and effective treatment, ultimately improving the survival rates of individuals with GC.

The sequence of histological changes in gastric cancer, typically triggered by *H. pylori* infection (Leung et al., 2004), was originally outlined by Correa and colleagues in 1975. This series has since been thoroughly investigated and is commonly referred to as Correa's cascade of gastric carcinogenesis. It illustrates the histological progression from normal gastric mucosa to gastric carcinoma, encompassing stages such as normal gastric mucosa, non-atrophic gastritis (NAG), atrophic gastritis (AG), intestinal metaplasia (IM), dysplasia, and finally, GC (Correa and Piazuelo, 2012).

IM of the stomach is a histopathological precancerous condition and is recognized as a significant risk factor for the emergence of intestinal-type GC (Correa et al., 2010, Kinoshita et al., 2017). While it is generally believed that reversing IM is not feasible, researchers continue to explore the potential for

such a reversal (Hwang et al., 2018, Malfertheiner et al., 2017). There is no specific treatment for gastric IM (Lam and Lau, 2020). Eradicating *H. pylori* may stop the progression of *H. pylori*-associated IM, but research indicates that the reversal of IM following *H. pylori* eradication remains uncertain (Sanchez Cuen et al., 2016, Liu et al., 2016). Guidelines recommend regular monitoring of high-risk individuals and preventive measures as key management strategies for IM. Gastric IM is typically identified through upper endoscopy with biopsy, and various histologic classification systems exist to assess the likelihood of IM advancing to GC (Huang et al., 2019, Jencks et al., 2018). Scientists are still investigating alternative diagnostic techniques for detecting early-stage gastric abnormalities that could enhance screening initiatives and modify treatment plans for each patient (Leja et al., 2009, Suh et al., 2012, Gómez Zuleta et al., 2017)

Recent advancements in innovative diagnostic approaches enable researchers to detect diverse molecular changes in gastric IM, including gene polymorphisms, miRNA expression modifications, and shifts in microbiome composition. Certain of these changes exhibit significant links to IM and hold promise as diagnostic aids for screening, treatment, and prognostic evaluations. Nevertheless, the translation of these findings into practical application within clinical settings remains considerably constrained. Thus, Endoscopy is considered as the main method for GC diagnosis (Jonaitis et al., 2021). However, this procedure has some disadvantages. It is costly, invasive, depends on the proficiency of the clinician, and leads to anxiety and patient discomfort. On the other hand, using blood biomarkers for early GC diagnosis is desirable as sampling is simple, cost-effective, accurate, and less invasive. Therefore, it is essential to develop a test that incorporates multiple cytokines to enhance the early identification of individuals at risk of developing GC.

Cytokines are versatile regulators that play vital roles in controlling cell functions like growth, development, survival, and differentiation through autocrine or paracrine pathways (Lin and Leonard, 2019). The interaction

between *H. pylori* and gastric epithelial cells induces the release of cytokines like IL-8, IL-6, and IL-1β, attracting neutrophils and mononuclear cells and leading to chronic active gastritis (Israel and Peek, 2001). *H. pylori* also triggers the infiltration of dendritic cells, T cells, and B cells, along with the secretion of various inflammatory mediators such as MCP-1, TNF-α, IL-12, IL-10, TGF-β, and IFN-γ. These mediators can cause DNA damage, stimulate cell proliferation, and inhibit apoptosis, increasing the risk of oncogenic transformation. Additionally, inflammatory cytokines and chemokines elevate the expression of factors like hypoxia-inducible factor-1, vascular endothelial growth factor, L-selectin, cyclooxygenase-2, and matrix metalloproteinases, which contribute to carcinogenesis (Borrello et al., 2008).

The Tumor microenvironment (TME) is the cellular environment where tumors or cancer cells exist. This cellular environment includes neoplastic, mesenchymal, endothelial, immune, extracellular matrix, and fibroblast cells that all contribute to tumor progression, cytokines are of particular importance (Senthebane et al., 2017). Within the TME, key cytokines involved in cell communication include interleukins, interferons, the TNF superfamily, chemokines, and growth factors (Propper and Balkwill, 2022). Furthermore, cytokines can attract more immune cells into the TME and stimulate the production of immune checkpoint proteins, such as PD-1 or TIM-3, to assist tumor cells in evading the immune response (Engelhardt and Ransohoff, 2012) (Zhang et al., 2015). Furthermore, tumor-associated macrophages (TAMs) within the TME are heterogeneous and can be functionally reprogrammed, which is associated with poor prognosis in various cancers. Their role in tumor progression is multifaceted, promoting angiogenesis and cancer advancement through the secretion of cytokines, growth factors, and proteolytic enzymes. Macrophages are divided into M1 and M2 types, serving pro-inflammatory and anti-inflammatory functions respectively. In GC, M1 macrophages secrete cytokines like CXCL9, IL-1b, TNF-α, CXCL10, and IL-8, which encourage tumor

growth. Conversely, M2 macrophages produce anti-inflammatory cytokines such as IL-33, IL-10, and TGF-β (Oya et al., 2020). These cytokines, influenced by epigenetic factors, are crucial at various stages of cancer development (Reyes et al., 2024). Cancer cells can continuously generate cytokines. These cytokines can influence the cancer cells themselves in an autocrine fashion, or impact supportive tissues like fibroblasts and blood vessels to create a favorable environment for cancer proliferation. Additionally, cytokines may trigger normal cells, such as TAM (Mantovani, 1994) and endothelial cells (Negus and Balkwill, 1996), to release more cytokines that aid in the progression of malignancy. The array of cytokine profiles linked to different cancer types is vast and varied (Dunlop and Campbell, 2000).

Numerous cytokines circulate in the body, with limited analysis of protein expression levels. Since cytokines act within interconnected networks, there is significant redundancy in their functions. This redundancy, coupled with their intricate interactions, implies that it is improbable for a single protein to be solely responsible for cancer development. Thus, Comprehensive profiling of the plasma level of several cytokines in different GC prognosis stages (NAG, AG, IM, and GC) could provide insight into cytokines correlation with the disease progression and their potential as biomarkers for GC.

The Meso Scale Discovery (MSD) assay is a rapid, and useful technique that enables the simultaneous sensitive quantification of multiple analytes within a single sample of a very low volume, and within a short time. Thus, this approach provides a strong technical basis for investigating immune detection markers in tumors. This study is the first to utilize the MSD technique to assess differential cytokine and chemokine expression (IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-16, IL-17, IL-18, IL-23, CCL2, CCL3, CXCL10, INF-γ, and TNF-α) in plasma samples collected from patients at different stages of cancer progression (NAG, AG, IM, and GC), to evaluate patients with risk of CG, and the potential of these cytokines as diagnostic markers for GC.

## 4.2 Materials and Methods

### 4.2.1 Study participants

The study involved 320 patients who attended the Oncology Hospital at Centro Médico Nacional Siglo XXI, Instituto Mexicano del Seguro Social (IMSS), and the Instituto Nacional de Cancerología, Secretaría de Salud, in Mexico City between 2009 and 2012. Patients who were 18 years old or younger, had any autoimmune disease, diabetes, or a type of cancer other than GC, or had previously undergone treatment for gastric cancer were excluded from the study. Written informed consent was obtained from all participants before they were enrolled in the study. The Ethics Committees of both participating institutions, IMSS and Secretaría de Salud, approved the study. Clinical and pathological data were collected using a questionnaire from patients, who were also blood donors at the Central Blood Bank, Centro Médico Nacional Siglo XXI, IMSS. Characteristics of patients included in the study are shown in **Table 8**

**Table 8. Patient demographic information.**

| | Non-atrophic gastritis (NAG) | Atrophic gastritis (AG) | Intestinal metaplasia (IM) | Gastric Cancer (GC) |
|---|---|---|---|---|
| N= | 133 | 39 | 47 | 101 |
| % female | 60 % | 48.7% | 48.9% | 52.5% |
| Median age | 60 | 49 | 65 | 63 |
| Age range | 32-84 | 26-73 | 38-87 | 30-87 |
| % HP+ | 46% | 79.5% | 48.9% | 11.9% |

### 4.2.2 Blood Sample Collection

Patients were requested to provide a blood sample before undergoing surgery and before receiving any cancer treatment. Blood was drawn after an overnight fast into either EDTA tubes or plain tubes. The plasma or serum samples were then stored at −20 °C until analysis.

### 4.2.3 *H. pylori* Infection Status

The patient's sera were tested for antibodies against *H. pylori* whole-cell antigens and the CagA protein using a previously validated ELISA. A positive *H. pylori* infection was defined by the presence of antibodies to either the whole-cell antigens and/or the CagA protein.

### 4.2.4 Meso scale discovery (MSD) analysis of plasma cytokine concentrations

MSD assays (Rockville, MD, USA) are based on MULTI-ARRAY® technology with electrochemiluminescence detection. Human U-PLEX Biomarker Group 1 assay kits were used following the manufacturer's instructions. The plates were read on the MSD QuickPlex SQ120 instrument and data analysis was performed using MSD Discovery Workbench version 4.0 software. The following multiplex analytes were measured, using three different kits: interferon (IFN)-γ, interleukin (IL)-4, IL-6, IL-8, IL-10, IL-12p70, tumor necrosis factor-alpha (TNFα), IL-17A, IL-16, IL-18, IL-23, IL-1β, IP-10 (CXCL10), monocyte chemoattractant protein 1 (MCP1, CCL2), and Macrophage Inflammatory Protein (MIP)-1α (CCL3). The detection limits for each measured cytokine/chemokine are shown in **(Table 9).**

**Table 9. The lowest concentrations of the analytes were detected in patients' samples.**

| Cytokines and Chemokines | Detection Limits Concentration (pg/ml) |
|---|---|
| IL-1β | 0.959 |
| IL-4 | 0.194 |
| IL-6 | 0.552 |
| IL-8 | 0.461 |
| IL-10 | 0.402 |
| IL-12p70 | 0.963 |
| IL-13 | 16.3 |
| IL-16 | 7.26 |
| IL-17 | 5.01 |
| IL-18 | 1.81 |
| IL-23 | 3.36 |
| INF-γ | 5.6 |
| TNF-α | 1.44 |
| MCP-1 (CCL2) | 0.709 |
| MIP-1α (CCL3) | 10.9 |
| IP-10 (CXCL10) | 0.9 |

## 4.3 Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 10 software. Individual data points are shown with the median. To assess between-group differences, nonparametric or mixed One-Way ANOVA (Kruskal-Wallis tests) were used, while Mann-Whitney U-tests were used for two-group analysis. Differences were considered significant at $p<0.05$.

## 4.4 Results

### 4.4.1 Association of multiple plasma cytokines and chemokines with *H. pylori* infection.

To evaluate the impact of *H. pylori* infection on plasma levels of various cytokines and chemokines (including IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-16, IL-17, IL-18, IL-23, INF-γ, TNF-α CCL2, CCL3, and CXCL10,), plasma samples were collected from 131 *H. pylori*-infected patients and 189 non-infected patients. The concentrations of these cytokines and chemokines were measured using the MSD assay. No significant differences ($p > 0.05$) were observed in the plasma levels of IL-6, IL-8, IL-16, IL-18, CCL2, CCL3, and CXCL10 between the *H. pylori-infected* and uninfected patients. IL-4, IL-12p70, and IL-13 data were excluded from the study because their levels were below the detection range for all participants. IL-1β, IL-10, IL-17, TNF-α, and IFN-γ were also excluded from the analysis due to concentrations being below the detection limits for most patients **(Figure 34).**

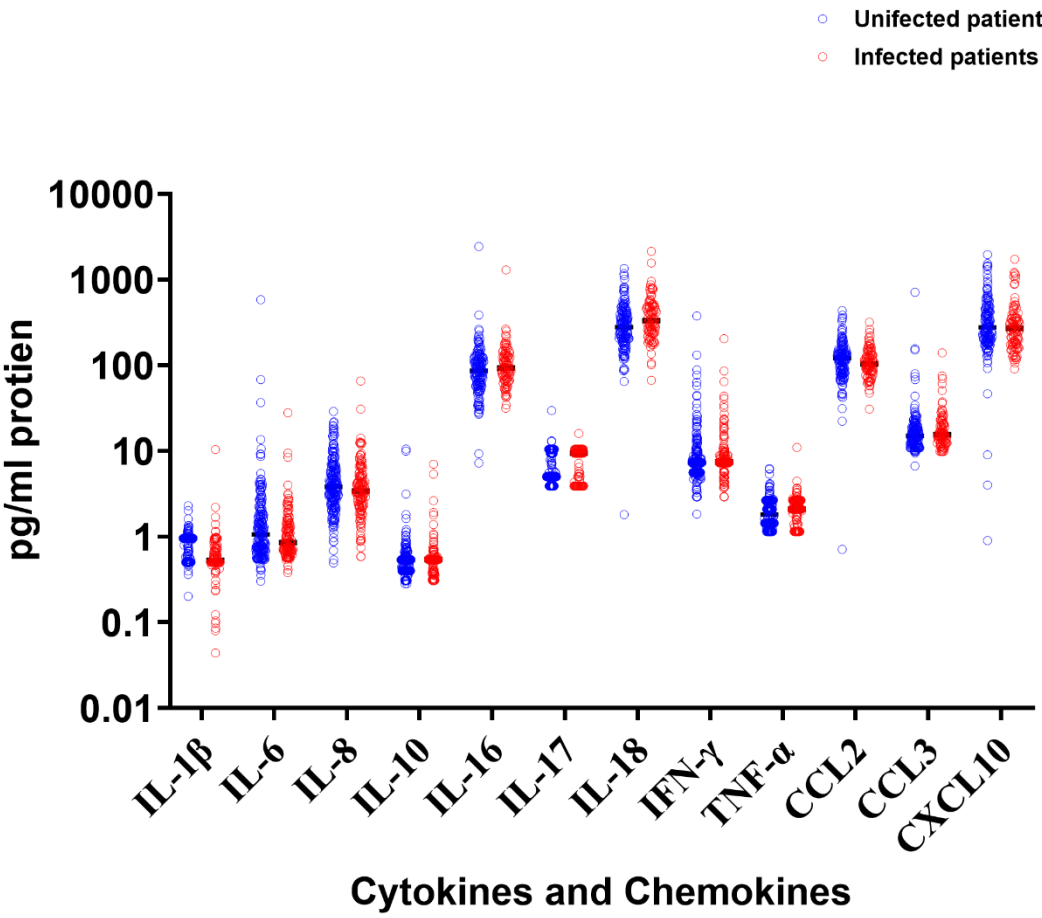


**Figure 34. Plasma cytokine and chemokines levels in *H. pylori*-infected patients vs uninfected patients.**

The levels of different cytokines and chemokines (including IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-16, IL-17, IL-18, IL-23, INF-γ, TNF-α CCL2, CCL3, and CXCL10,) in each plasma sample were plotted and compared using the Mann-Whitney test based on *H. pylori* infection status. The median plasma levels of each measured cytokines and chemokines from *H. pylori*-infected patients (n=131) showed no significant difference compared to the median plasma levels from *H. pylori*-uninfected patients (n=189), with p-values >0.05. IL = interleukin, CCL = CC chemokine ligand, CXCL= chemokine (C-X-C motif) ligand, TNF-α= tumour necrosis factor alpha; IFN-γ= interferon gamma.

### 4.4.2 Effect of gender on plasma cytokines and chemokines level

To investigate the influence of gender on various plasma cytokine and chemokine levels, the concentrations of cytokines and chemokines (including IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-16, IL-17, IL-18, IL-23, CCL2, CCL3, CXCL10, INF-γ, and TNF-α) were compared in samples from male (n=128) and female (n=192) patients, irrespective of their *H. pylori* status. As shown in **(Figure 35)**, male subjects exhibited significantly higher levels of CCL2 compared to female subjects (median of 137.7 pg/ml in males versus 108.6 pg/ml in females; $p = 0.015$; 1.3-fold increase), while their levels of CXCL10 were notably lower than those in females (median of 103.3 pg/ml in males versus 132.4 pg/ml in females; $p < 0.015$; 1.3-fold increase). No significant differences were observed between males and females in other measured cytokines and chemokine. This data suggests that CCL2 and CXCL10 chemokines may be influenced by gender.

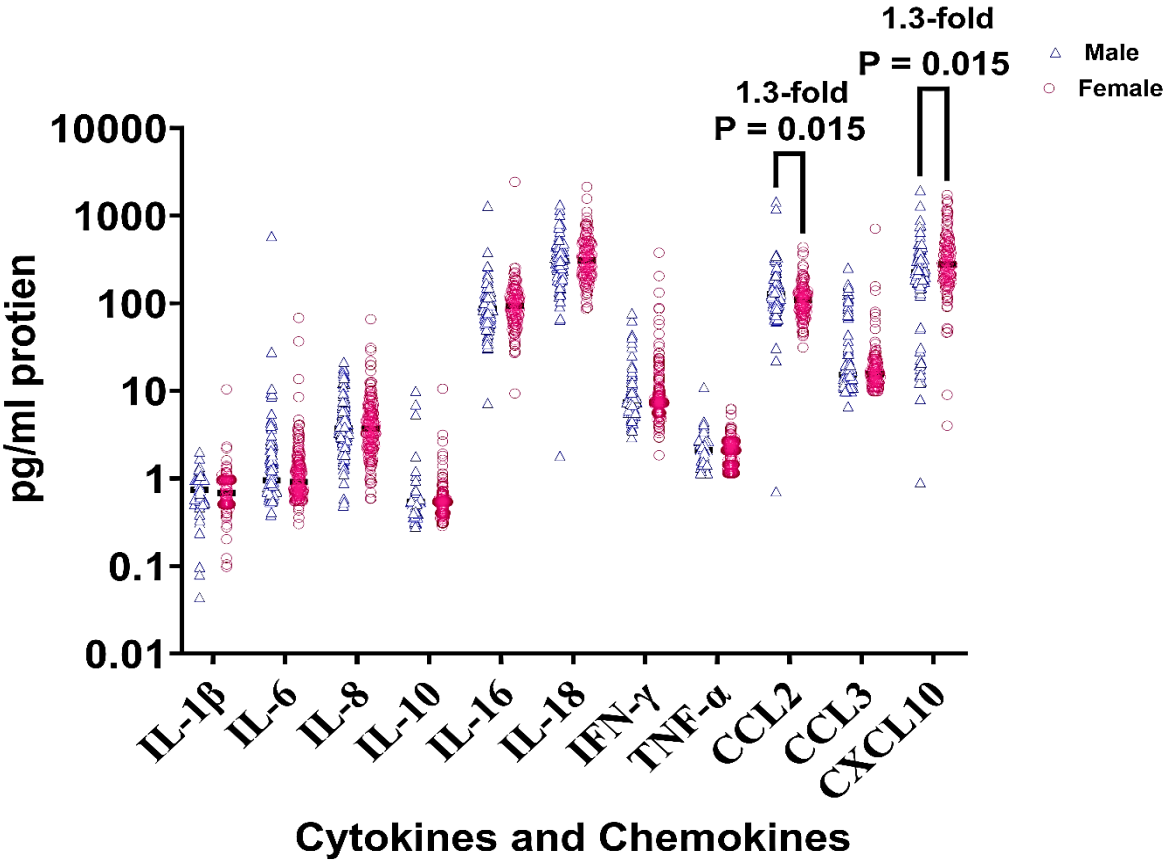


**Figure 35. Effect of gender on plasma cytokines and chemokines level**

The plasma concentrations of IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-16, IL-17, IL-18, IL-23, CCL2, CCL3, CXCL10, INF-γ, and TNF-α were assessed in male (n=128) and female (n=192) subjects. The results are displayed as scatter dot plots. Statistical significance was determined using multiple Mann-Whitney tests. Median levels for each cytokine are indicated by a horizontal line. IL = interleukin, CCL = CC chemokine ligand, CXCL= chemokine (C-X-C motif) ligand, TNF-α= tumour necrosis factor alpha; IFN-γ = interferon gamma. Fold differences indicated are differences between the medians.

### 4.4.3 Plasma cytokines and chemokines Levels in male vs female patients with different gastric conditions

To investigate the impact of gender on cytokine and chemokine responses in patients with various gastric conditions (NAG, AG, IM, GC), the levels of cytokines and chemokines (including IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-16, IL-17, IL-18, IL-23, CCL2, CCL3, CXCL10, INF-γ, and TNF-α) were in plasma

samples from both male and female patients across these conditions. As illustrated in **(Figure 36A)**, male patients with NAG exhibited significantly higher levels of CCL2 (median of 164.2 pg/ml in males versus 126.2 pg/ml in females; $p = 0.006$; 1.6-fold increase), while showing a notable decrease in CXCL10 levels compared to females (median of 75. 7 pg/ml in males versus 298.6 pg/ml in females; $p < 0.001$; 1.8-fold increase). The data indicated that the levels of CCL2 and CXCL10 chemokines are affected by gender in patients with NAG, but other cytokines and chemokines measured across different gastric conditions were not influenced by gender **(Figure 36A, B, C, and D).**

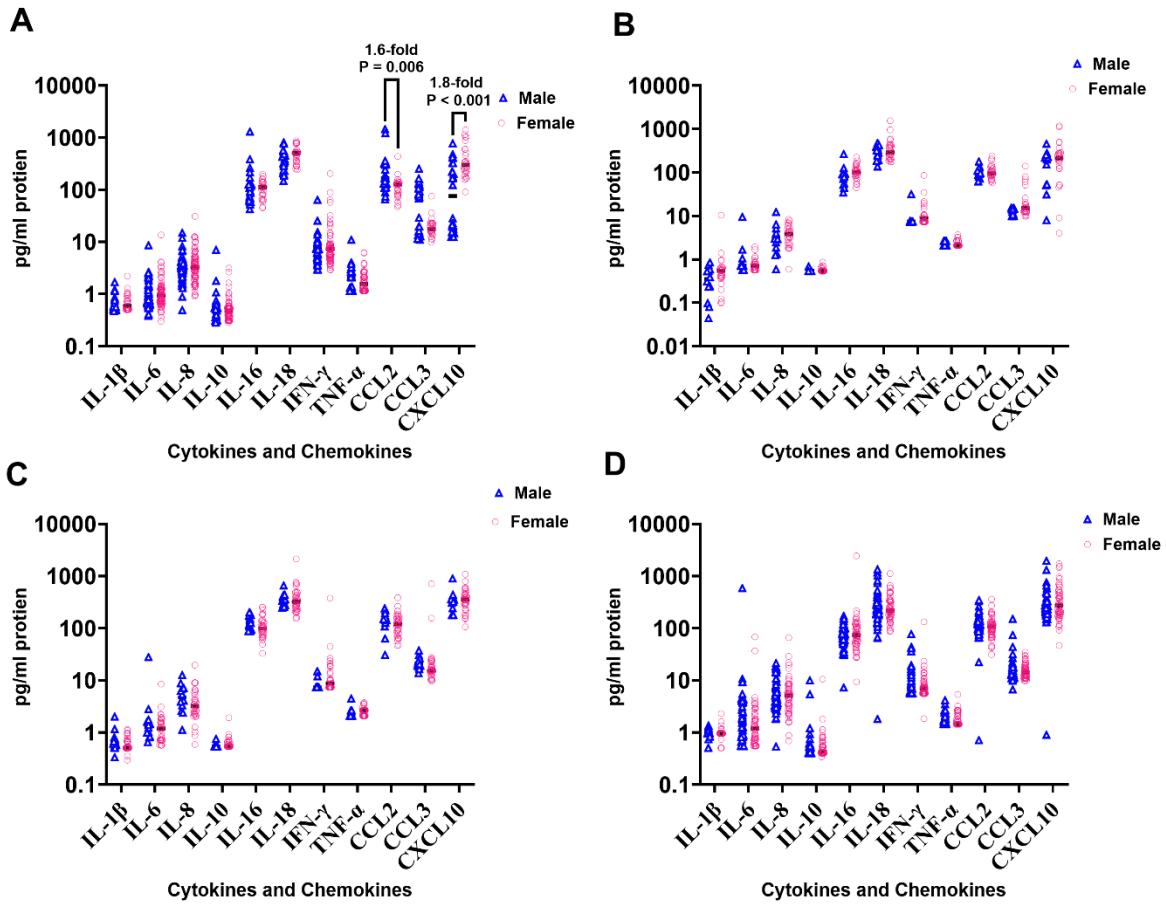


**Figure 36. Sex-specific differences in systemic immune responses in patients with different gastric conditions.**

The plasma concentrations of IL-1β, IL-4, IL-6, IL-8, IL-10, IL-13, IL-16, IL-17, IL-18, IL-23, CCL2, CCL3, CXCL10, INF-γ, and TNF-α were assessed in male (n=128) and female (n=192) patients with various gastric conditions, including **(A)** non-

atrophic gastritis (NAG)(n=133)(male=56 and female=77), **(B)** atrophic gastritis (AG)(n= 39) (male=12 and female=51)**(C)** intestinal metaplasia (IM) (n=47)( male=12 and female=35), and **(D)** gastric cancer (GC) (n=101) (male=48 and female=53). The results are displayed as scatter dot plots. Statistical significance was determined using multiple Mann-Whitney tests. IL = interleukin, CCL = CC chemokine ligand, CXCL= chemokine (C-X-C motif) ligand, TNF-α= tumour necrosis factor alpha; IFN-γ = interferon gamma.

### 4.4.4 Plasma cytokine and chemokine levels in patients with various gastric conditions.

The concentrations of IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-16, IL-17, IL-18, IL-23, CCL2, CCL3, CXCL10, INF-γ, and TNF-α were measured in plasma samples collected from patients with different gastric conditions including NAG, AG, IM, and GC. In the entire study population, plasma levels of IL-6 and IL-8 were significantly higher in patients with GC (n=101) (Median= 1.27 pg/ml and 4.8 pg/ml, respectively) compared to those with NAG (n=133) (Median=0.851 pg/ml and 3.4 pg/ml, respectively) and AG (n=39) (Median= 0.71 pg/ml and 3.3 pg/ml, respectively) as shown in **(Figure 37B and C)**. Conversely, plasma levels of IL-18, IL-16, and CCL3 in patients with NAG (n=53) (Median= 450 pg/ml, 111 pg/ml, and 16.6 pg/ml, respectively) and intestinal metaplasia (IM) (n=47) (Median= 333 pg/ml, 109 pg/ml, and 17 pg/ml, respectively) were significantly higher than those in patients with GC (n=101) (Median=222 pg/ml, 75 pg/ml, and 14 pg/ml, respectively) as illustrated in **(Figure 37E, J, and K)**. The plasma levels of other chemokines, CCL2 and CXCL10, did not show significant changes across the stages of gastric cancer.

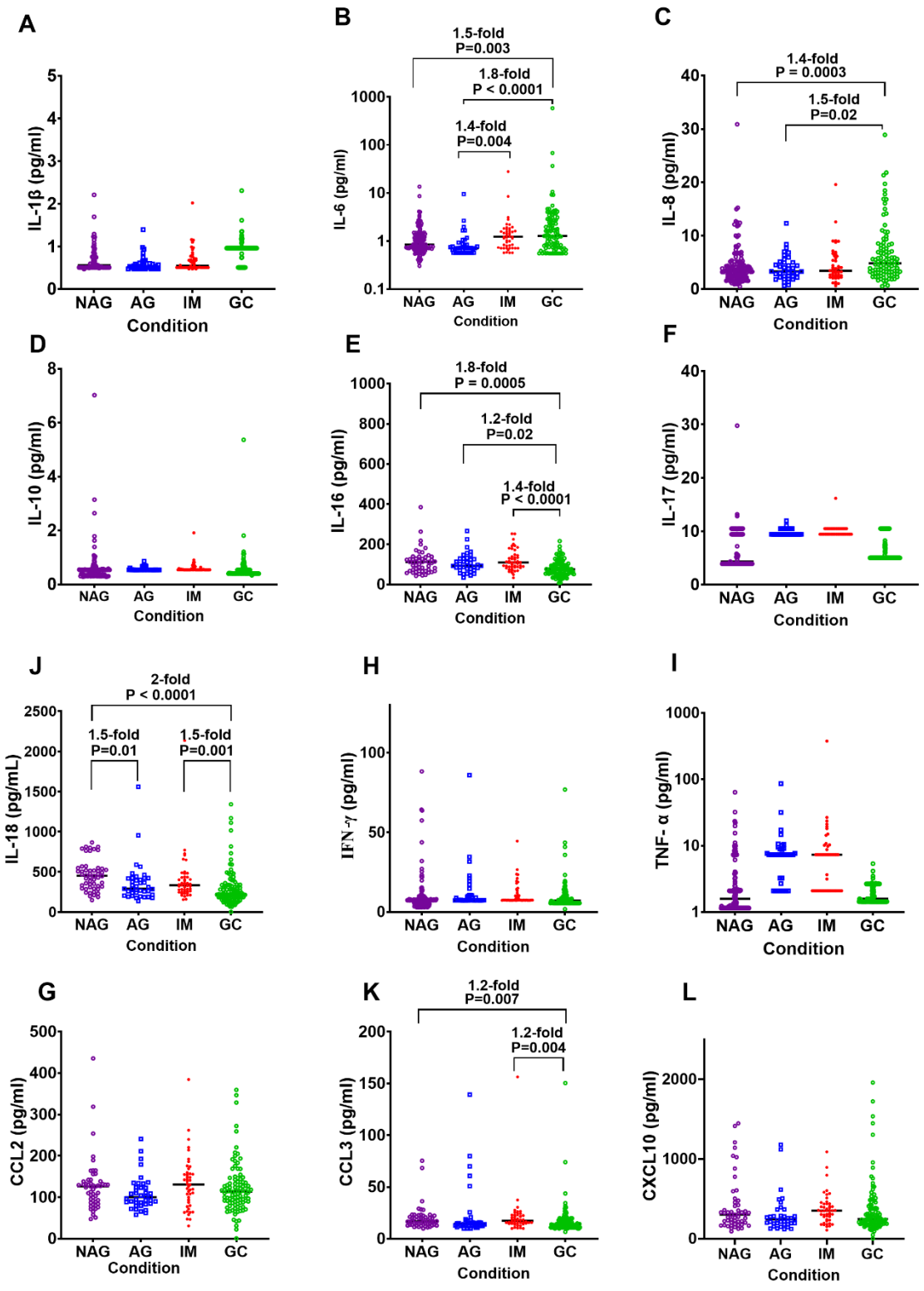
A
IL-1β (pg/ml)
NAG AG IM GC
Condition
B
1.5-fold P=0.003
1.8-fold P < 0.0001
1.4-fold P=0.004
IL-6 (pg/ml)
C
1.4-fold P = 0.0003
1.5-fold P=0.02
IL-8 (pg/ml)
D
IL-10 (pg/ml)
E
1.8-fold P = 0.0005
1.2-fold P=0.02
1.4-fold P < 0.0001
IL-16 (pg/ml)
F
IL-17 (pg/ml)
J
2-fold P < 0.0001
1.5-fold P=0.01
1.5-fold P=0.001
IL-18 (pg/mL)
H
IFN-γ (pg/ml)
I
TNF- α (pg/ml)
G
CCL2 (pg/ml)
K
1.2-fold P=0.007
1.2-fold P=0.004
CCL3 (pg/ml)
L
CXCL10 (pg/ml)

**Figure 37. Plasma cytokine and chemokine levels in patients with different gastric conditions.**

The plasma concentrations of IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-16, IL-17, IL-18, IL-23, CCL2, CCL3, CXCL10, INF-γ, and TNF-α were evaluated in patients with various gastric conditions, including non-atrophic gastritis (NAG), atrophic gastritis (AG), intestinal metaplasia (IM), and gastric cancer (GC). The results are presented as scatter dot plots, with statistical significance determined using nonparametric or mixed One-Way ANOVA. Median levels for each cytokine are indicated by a horizontal line. Fold differences indicated are differences between the medians. The numbers of samples in the groups were GC=101, NAG=133, AG=39, and IM=47. IL = interleukin, CCL = CC chemokine ligand, CXCL= chemokine (C-X-C motif) ligand, TNF-α= tumor necrosis factor alpha; IFN-γ = interferon gamma.

### 4.4.5 Plasma cytokine and chemokine levels in *H. pylori-infected* patients with various gastric conditions.

The data indicated that individuals infected with *H. pylori* who had AG, IM, and GC exhibited significantly elevated plasma levels of IL-6 (median IL-6 levels: 1.2 pg/ml, 1.3 pg/ml, and 1.1 pg/ml, respectively; representing 1.5-fold, 1.8-fold, and 1.5-fold increases, respectively) compared to *H. pylori*-positive patients with NAG (median level = 0.7 pg/ml)**(Figure 38B)**. Conversely, a significant reduction in IL-18 plasma concentrations was observed in *H. pylori*-positive patients with GC (median = 185 pg/ml) compared to those with NAG, AG, and IM (median levels: 485 pg/ml, 311 pg/ml, and 338 pg/ml, respectively) **(Figure 38G)**. Similarly, *H. pylori*-positive patients with GC showed a significant decrease in IL-16 plasma levels (median = 31.5 pg/ml) compared to those with IM (59.3 pg/ml) **(Figure 38E)**. No significant differences were reported in plasma levels of IL-8, CCL2, CCL3, and CXCL10 **(Figure 38C, I, K, and L).** Other cytokines (IL-1β, IL-10, IL-17, IFN-γ, and TNF-α) were excluded from the analysis as the plasma concentrations were below the detection thresholds for most patients **(Figure 38A, D, F, H, and I)**. Additionally, IL-4, IL-12p70, and IL-

13 were analyzed but excluded from the study as their levels fell below the Fit Curve Range for the measured cytokines.

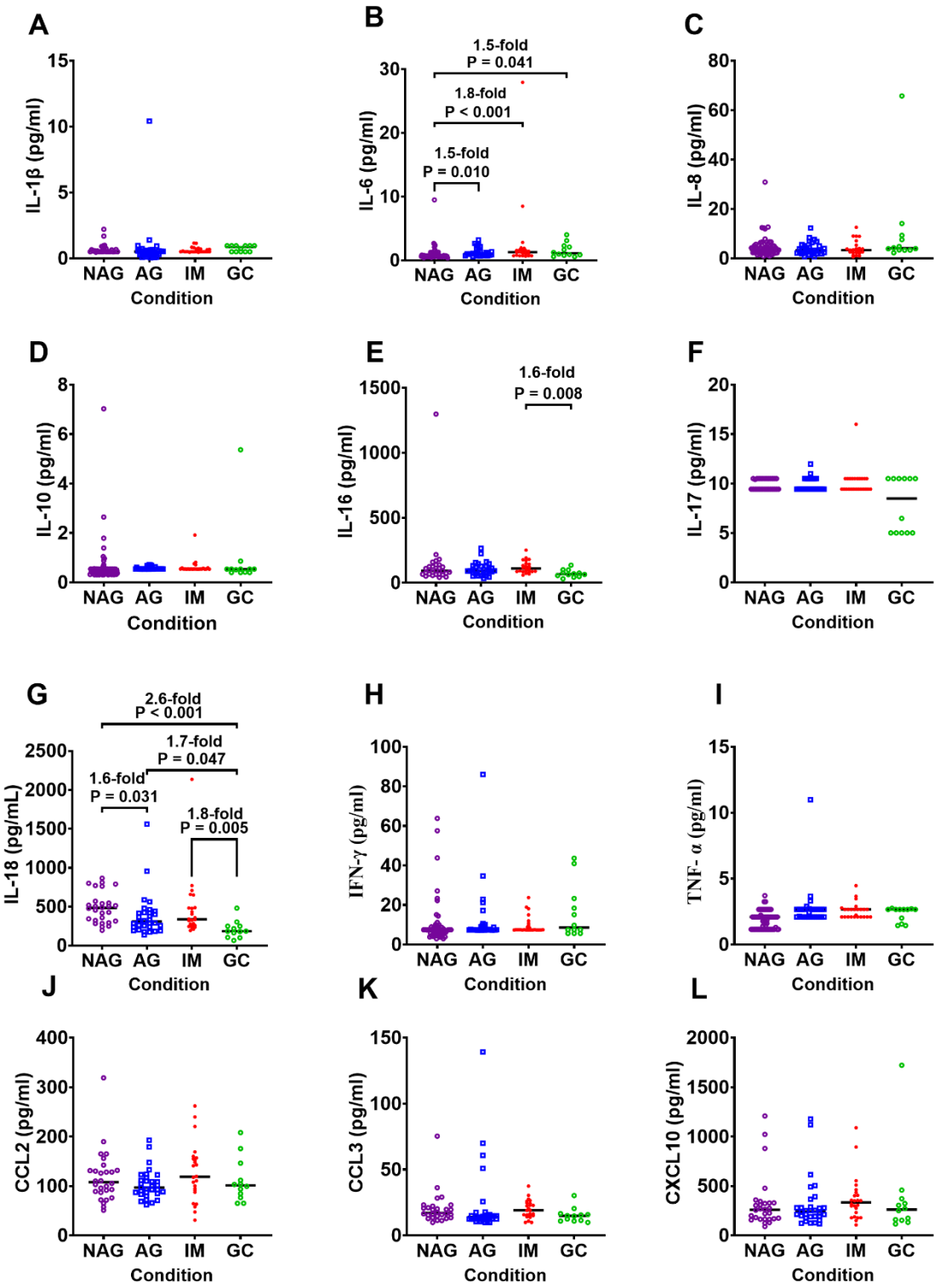
A
IL-1β (pg/ml)
15
10
5
0
NAG AG IM GC
Condition
B
IL-6 (pg/ml)
30
20
10
0
1.5-fold
P = 0.041
1.8-fold
P < 0.001
1.5-fold
P = 0.010
NAG AG IM GC
Condition
C
IL-8 (pg/ml)
80
60
40
20
0
NAG AG IM GC
Condition
D
IL-10 (pg/ml)
8
6
4
2
0
NAG AG IM GC
Condition
E
IL-16 (pg/ml)
1500
1000
500
0
1.6-fold
P = 0.008
NAG AG IM GC
Condition
F
IL-17 (pg/ml)
20
15
10
5
0
NAG AG IM GC
Condition
G
IL-18 (pg/mL)
2500
2000
1500
1000
500
0
2.6-fold
P < 0.001
1.7-fold
P = 0.047
1.6-fold
P = 0.031
1.8-fold
P = 0.005
NAG AG IM GC
Condition
H
IFN-γ (pg/ml)
100
80
60
40
20
0
NAG AG IM GC
Condition
I
TNF- α (pg/ml)
15
10
5
0
NAG AG IM GC
Condition
J
CCL2 (pg/ml)
400
300
200
100
0
NAG AG IM GC
Condition
K
CCL3 (pg/ml)
150
100
50
0
NAG AG IM GC
Condition
L
CXCL10 (pg/ml)
2000
1500
1000
500
0
NAG AG IM GC
Condition

**Figure 38. Plasma cytokine and chemokine levels in *H. pylori* positive patients with various gastric conditions.**

The plasma concentrations of IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-16, IL-17, IL-18, IL-23, CCL2, CCL3, CXCL10, INF-γ, and TNF-α were assessed in *H. pylori* positive patients with different gastric diseases. The results are displayed as scatter dot plots. Statistical significance was determined using multiple Mann-Whitney tests. Median levels for each cytokine is indicated by a horizontal line. Fold differences indicated are differences between the medians. The number of tested *H. pylori*-positive plasma samples were NAG = 28, AG = 31, IM = 24, GC = 12 for IL-1β, IL-16, IL-18, CCL2, CCL3, and CXCL10. For IL-4, IL-6, IL-8, IL10, IL-12p70, IL-13, IL-17, IFN-γ, and TNF-α, the sample sizes were NAG = 62, AG = 31, IM = 24, GC = 12.

### 4.4.6 Plasma cytokine and chemokine levels in *H. pylori* uninfected patients with various gastric conditions.

The data indicated that *H. pylori* uninfected patients with GC showed significantly elevated plasma levels of IL-6 (median IL-6 levels: 1.2 pg/ml representing 1.6-fold increases) compared to *H. pylori*-negative patients with NAG (median level = 0.8 pg/ml **( Figure 39B)**. Similarly, a significant increase in IL-8 plasma concentrations (median = 5 pg/ml) was observed in *H. pylori*-negative patients with GC compared to those with NAG (median levels: 3.4 pg/ml) **(Figure 39C).** Surprisingly, *H. pylori*-negative patients with GC showed a significant decrease in IL-16 plasma levels (median = 75.6 pg/ml) compared to those with NAG and IM (112 pg/ml and 109 pg/ml respectively) **(Figure 39 E).** Likewise, *H. pylori*-negative patients with GC showed a significant decrease in IL-18 plasma levels (median = 231.5 pg/ml) compared to those with NAG (433 pg/ml) **(Figure 39G).** No significant differences were reported in plasma levels of CCL2, CCL3, and CXCL10 **( Figure 39J, K, and L)**. Other cytokines (IL-1β, IL-10, IL-17, IFN-γ, and TNF-α) were excluded from the analysis as their plasma concentrations were below the detection thresholds for most patients **(Figure 39A, D, F, H, and I).** Additionally, IL-4, IL-12p70, and IL-13 were analyzed but

excluded from the study as their levels fell below the Fit Curve Range for the measured cytokines.

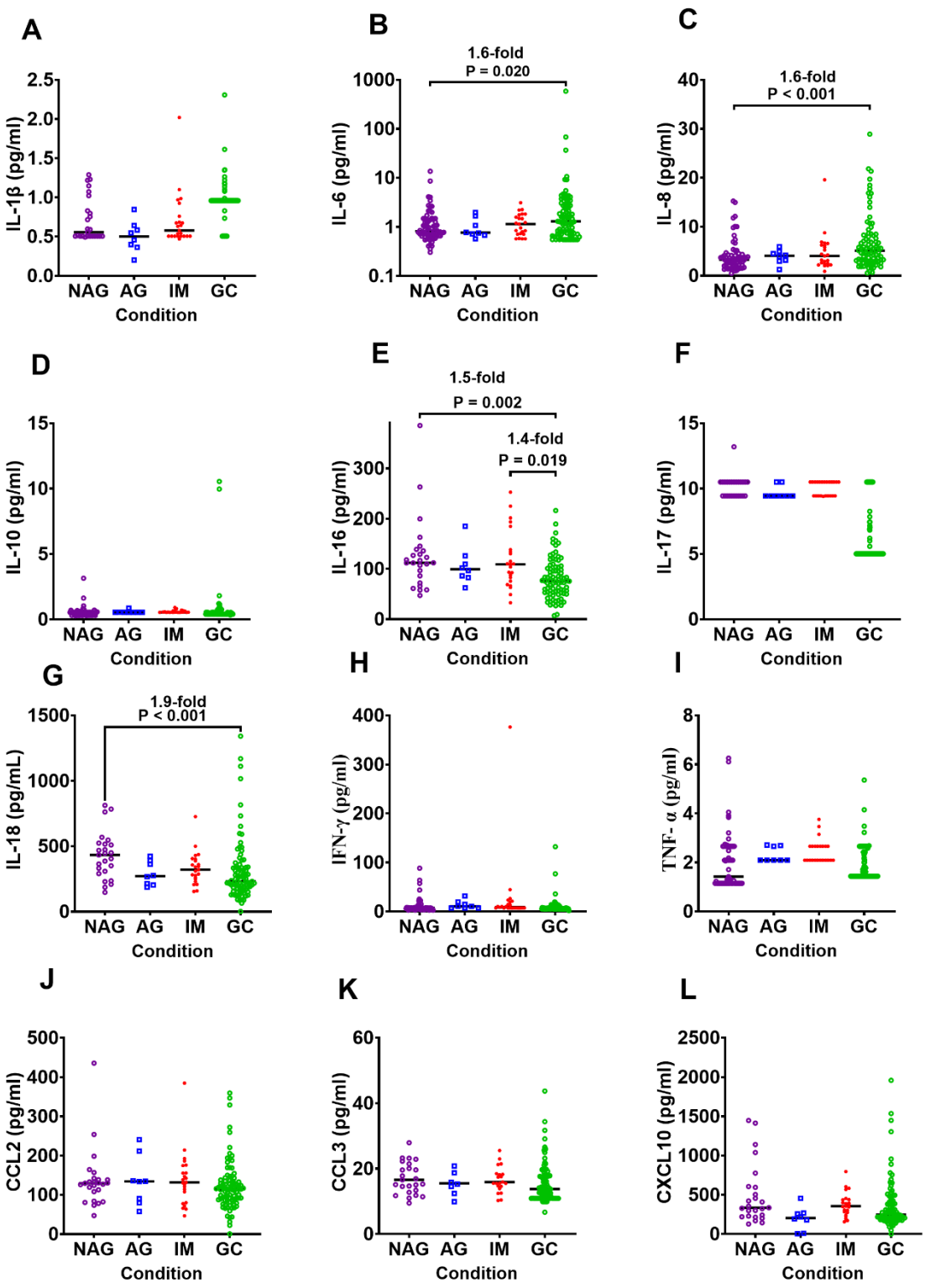
A
IL-1β (pg/ml)
2.5
2.0
1.5
1.0
0.5
0.0
NAG AG IM GC
Condition
B
1.6-fold
P = 0.020
IL-6 (pg/ml)
1000
100
10
1
0.1
NAG AG IM GC
Condition
C
1.6-fold
P < 0.001
IL-8 (pg/ml)
40
30
20
10
0
NAG AG IM GC
Condition
D
IL-10 (pg/ml)
15
10
5
0
NAG AG IM GC
Condition
E
1.5-fold
P = 0.002
1.4-fold
P = 0.019
IL-16 (pg/ml)
300
200
100
0
NAG AG IM GC
Condition
F
IL-17 (pg/ml)
15
10
5
0
NAG AG IM GC
Condition
G
1.9-fold
P < 0.001
IL-18 (pg/mL)
1500
1000
500
0
NAG AG IM GC
Condition
H
IFN-γ (pg/ml)
400
300
200
100
0
NAG AG IM GC
Condition
I
TNF- α (pg/ml)
8
6
4
2
0
NAG AG IM GC
Condition
J
CCL2 (pg/ml)
500
400
300
200
100
0
NAG AG IM GC
Condition
K
CCL3 (pg/ml)
60
40
20
0
NAG AG IM GC
Condition
L
CXCL10 (pg/ml)
2500
2000
1500
1000
500
0
NAG AG IM GC
Condition

**Figure 39. Plasma cytokine and chemokine levels in *H. pylori* uninfected patients with various gastric conditions.**

The plasma concentrations of IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-16, IL-17, IL-18, IL-23, CCL2, CCL3, CXCL10, INF-γ, and TNF-α were assessed in *H. pylori* negative patients with different gastric diseases. The results are displayed as scatter dot plots. Statistical significance was determined using multiple Mann-Whitney tests. Median levels for each cytokine are indicated by a horizontal line. Fold differences indicated are differences between the medians. The data showed elevated plasma IL-6 and IL-8 concentrations in *H. pylori*. The number of tested *H. pylori*-negative plasma samples were NAG = 25, AG = 8, IM = 23, GC = 89 for IL-1β, IL-16, IL-18, CCL2, CCL3, and CXCL10. For IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17, IFN-γ, and TNF-α, the sample sizes were NAG = 72, AG = 31, IM = 24, GC = 12.

## 4.5 Discussion

The precise cause of GC remains unclear, though chronic gastritis and *H. pylori* infection are significant risk factors (Wroblewski et al., 2010a). Surgery and chemotherapy are the primary curative treatments. Advances in early detection, chemotherapy, molecularly targeted therapies, and multidisciplinary approaches have led to a decline in mortality rates over recent decades. Despite these advancements, the global 5-year survival rate for GC remains low at around 20% (Eom et al., 2022). Consequently, new strategies focus on early and advanced-stage detection and personalized treatment optimization.

Given the crucial role of cytokines as signaling and effector molecules in the TME of GC (Oya et al., 2020, Chung and Lim, 2014), and the relatively limited research in this area, understanding the correlations between cytokines and GC prognosis could provide valuable insights into the underlying mechanisms of the disease (Yang et al., 2021a). Discrepancies between tumor cytokine levels and those found in circulation may explain some of the contradictory data in the literature. However, conducting these studies in humans is particularly

challenging because repeated biopsies throughout disease progression are not standard clinical practice. Therefore, analyzing peripheral cytokines is necessary to investigate their role at various stages of the disease (Yang et al., 2021a). This study explored the hypothesis that circulating levels of inflammatory/proinflammatory cytokines and chemokines could serve as indirect markers of tissue damage. Measuring these levels might provide a valuable biomarker for the early detection of GC, potentially leading to improved long-term outcomes.

Because 50% of the participants in our study tested positive for *H. pylori*, it was crucial to determine whether *H. pylori* infection amplifies the impact of elevated circulating cytokine levels on the risk of GC. The data showed that no significant difference was observed in plasma levels of IL-1β, IL-6, IL-8, IL-10, IL-16, IL-17, IL-18, IFN-γ, TNF-α, CCL2, CCL3, and CXCL10 in *H. pylori-positive* patients versus *H. pylori-negative* patients. There were increases in plasma levels of IL-18 in *H. pylori-infected* patients compared to uninfected individuals, but this did not reach statistical significance ($p=0.05$). These findings are similar to a previous study where no changes in serum levels of IL-6, IL-8, IL-10, IFN-γ CCL2, and CCL3, were found between *H. pylori* positive and negative patients (Khaiboullina et al., 2016). Another study also found no significant correlation between *H. pylori* status and IL-6, IL-10, IL-12, and IL-18 serum levels (Thong-Ngam et al., 2006b).

Due to the variation in cytokine and chemokine secretion by innate immune cells between sexes (Klein and Flanagan, 2016), this study also investigated the impact of age and gender on cytokine and chemokine concentrations. The findings revealed that female subjects had significantly higher CXCL10 plasma levels compared to male subjects. In contrast, female participants exhibited significantly lower plasma levels of CCL2 compared to their male subjects. Additionally, when classifying male and female patients based on their gastric conditions, it was found that female patients with NAG

conditions had a 1.8-fold increase in plasma CXCL10 levels compared to males with the same condition, while male subjects exhibited a 1.6-fold increase in plasma CCL2 levels compared to females with NAG conditions. These results align with a recent study indicating that females have a significantly greater induction of CXCL10/IP-10 in response to Rhinoviruses A16 (RV-A16) compared to males (Regis et al., 2021). No correlation was found between the patient's age and the expression of the measured cytokines and chemokines (data is not shown).

The analysis also showed that elevated plasma concentration of IL-6 in patients with gastric cancer compared to those with NAG in all examined groups regardless of their *H. pylori* status. Additionally, in *H. pylori-positive* patients, significant evaluation of IL-6 was observed across all gastric conditions compared to NAG while in *H. pylori-negative* patients, it was only observed in GC patients compared to patients with non-atrophic gastritis. This finding complements the established knowledge that IL-6 increases in serum concentrations in gastric cancer patients compared to healthy controls (Ashizawa et al., 2005). Moreover, several research have examined the relationship between clinical characteristics in gastric cancer patients and IL-6, all reporting a significant association. For instance, elevated serum IL-6 concentrations were linked to the depth of tumor invasion (Ikeguchi et al., 2009), tumor size, tumor depth, lymph node metastases, and stage (Kim et al., 2009). IL-6 serum levels were higher in advanced gastrointestinal cancer patients and linked to both overall survival (OS) and time to disease progression (TTP) (De Vita et al., 2001). Additionally, previous reports have documented elevated gastric mucosal levels of IL-6 in *H. pylori*-positive patients with gastritis, which decrease following successful eradication (Sugimoto et al., 2010, Mejías-Luque et al., 2008). These findings are consistent with our observations of increased IL-6 cytokine levels across all gastric conditions in *H. pylori*-infected patients. In contrast, among *H. pylori*-negative patients, elevated

IL-6 levels were only noted in those with GC. This underscores the role of *H. pylori* in triggering a robust inflammatory response mediated by proinflammatory cytokines, including IL-6.

IL-8 is a pro-inflammatory chemokine produced by variety of cells, including neutrophils, macrophages, endothelial cells, and cancer cells. It induces the chemotaxis of neutrophils to sites of inflammation (Baggiolini, 2015). When secreted by tumor cells, IL-8 is involved in the chemotactic recruitment of granulocytes (Alfaro et al., 2016). Additionally, IL-8 has direct pro-tumorigenic effects, such as promoting angiogenesis, invasion, or metastasis (David et al., 2016). Elevated levels of IL-8 activate malignant biological behaviors in cancer cells, resulting in a poor clinical prognosis (Chen et al., 2015). Moreover, elevated serum IL-8 levels are linked to tumor load and resistance to immune checkpoint inhibitors (ICIs). Inhibiting the IL-8/IL-8R receptor axis can enhance the effectiveness of ICIs (Schalper et al., 2020). The findings of this study enhance the existing understanding of IL-8. The result showed significant upregulation in IL-8 systemic concentrations in patients with gastric cancer compared to those with gastritis. A similar finding was reported in *H. pylori-negative* patients. The result does not show significant increases in IL-8 in *H. pylori-positive* perhaps due to a small sample size (12 *H. pylori-positive* patients with GC vs 62 *H. pylori-positive* with non-atrophic gastritis). These findings are like previous reports where higher mean levels of serum IL-8 were found in GC cases than in healthy controls (Macri et al., 2006, Konturek et al., 2002). Additionally, a recent meta-analysis revealed that elevated IL-8 expression is significantly associated with poor clinical outcomes in GC patients and serves as an independent risk factor influencing the prognosis of GC (Wang et al., 2021).

IL-18 is a cytokine also known as IFN-γ-inducing factor, and generated by Kupffer cells, activated macrophages, keratinocytes, intestinal epithelial cells, and osteoblasts (Thong-Ngam et al., 2006a), DCs, some tumor cells (Tas et al.,

2015). IL-18 is synthesized as an inactive precursor and cleaved by caspase-1 enzyme into the active form of IL-18 (Fantuzzi and Dinarello, 1999). Various investigations have documented the significance of IL-18 as a Th1 cytokine, mainly in cooperation with IL-12, in anti-tumor activity. Its antitumor effects include enhanced NK cell activity, decrease of tumorigenesis, initiation of apoptosis, and inhibition of angiogenesis in tumor cells to suppress tumor neovascularization (Tanaka et al., 2000, Coughlin et al., 1998, Cao et al., 1999).The antitumor effect of IL-18 is mainly facilitated through Fas–FasL interactions, whereas IL-12 antitumor activity is mediated by the perforin pathway. Other research indicates that IL-18 helps maintain a balance between inflammation and repair in the lamina propria beneath the epithelium (Dupaul-Chicoine et al., 2010). For instance, in osteosarcoma, elevated IL-18 expression is linked to increased tumor resistance due to myeloid-derived suppressor cell infiltration (Guan et al., 2017). Conversely, in melanoma, IL-18 has been observed to boost the antitumor response by inducing tumor-infiltrating $CD8^+$ T lymphocytes (Kunert et al., 2018). These findings underscore the significant role IL-18 plays in regulating tumor cells. Therefore, in this study, IL-18 was included in the list of cytokines to investigate changes in systemic levels of cytokines and chemokines to identify potential biomarkers for gastric cancer. The results of this study showed that IL-18 plasma levels are significantly reduced in patients with GC compared to those with intestinal metaplasia and non-atrophic gastritis. Similar findings were reported in *H. pylori*-positive patients, while in *H. pylori*-negative patients, significant downregulation was observed in GC patients compared to those with NAG only. Reduced IL-18 levels could serve as a mechanism for tumor cells to evade the immune response, given that IL-18 is essential for activating $CD4^+$ T cells, $CD8^+$ T cells, and NK cells (Kaplanski, 2018). Furthermore, IL-18 works in tandem with IL-12 to stabilize INF-$\gamma$ production, enhancing T cells' cytotoxic activity. Additionally, IL-18-deficient NK cells exhibit decreased activity and immune function, and studies have shown that IL-18-deficient mice develop heightened inflammation

and cancerous lesions in the intestinal epithelium (Salcedo et al., 2010). These results are in agreement with a previous study that noted that *IL18* mRNA, protein, and IL-1β-converting enzyme (ICE) mRNA levels are reduced or absent in colonic cancers compared to normal mucosa. This suggests a lack of mature IL-18 in tumors and indicates a compromised IL-18-dependent immune response (Pages et al., 2000). This was further supported by the absence of IFN-γ and FasL transcripts in half of the tumors analyzed. In the same study, the clinical outcome analysis demonstrated that the absence of IL-18-induced molecules, specifically IFN-γ and FasL, was linked to lymph node and/or liver metastases (Pages et al., 2000). Additionally, a recent study revealed the risk of pre-neoplastic lesions progressing to cancer was 2.08 times higher in women with lower *IL18* expression, and a notable decrease in *IL18* expression when comparing high-grade squamous intraepithelial lesions (HSIL) to cancer. This decreased *IL18* mRNA expression may elevate the risk of cervical cancer development (Matamoros et al., 2019). Furthermore, In uterine cervix cancer, certain *IL18* polymorphisms have been associated with cancer progression (Tavares et al., 2016), and it has been observed that lower plasma levels of IL-18 heighten the risk of developing cervical cancer (Gening et al., 2014). Although the IL-18 result of the present study is in line with previous studies, it contradicted previously published findings in the GC field. Thong-Ngam *et al*. (2006) reported that serum IL-18 levels were elevated in GC patients compared to those with gastric ulcers, suggesting IL-18 as a potential diagnostic marker, though not a prognostic one for overall survival (Thong-Ngam et al., 2006b). However, the Thong-Ngam study included all participants without applying exclusion criteria for health conditions such as autoimmune diseases, chronic diseases, and previous or current treatments. Elevated systemic IL-18 levels have been associated with various inflammatory and autoimmune diseases, including type 1 diabetes (Dong et al., 2007), Inflammatory bowel diseases (Naftali et al., 2007), rheumatoid arthritis (Gualberto Cardoso et al., 2021), multiple sclerosis (Jahanbani-Ardakani et al., 2019), Myasthenia gravis (Jander

and Stoll, 2002), Psoriasis (Ohta et al., 2001). Elevated IL-18 levels have also been documented in several cancers, such as lung cancer (Naumnik et al., 2004), breast cancer (Inoue et al., 2019), leukaemia (Takubo et al., 2000), and hepatocellular carcinoma (Tangkijvanich et al., 2007). In the current study, patients with diabetes, autoimmune diseases, cancer, or those undergoing previous/current cancer treatment were excluded to enhance result accuracy. Unlike Thong-Ngam's study, which focused on advanced-stage GC (stages III and IV) with poor 5-year survival rates, the current study included all cancer stages (I, II, III, IV). These differences could explain the discrepancies between the two studies findings. A Japanese study published in 2001 also found elevated serum IL-18 levels in gastric cancer patients. Interestingly, they observed that patients with stage I or IV gastric carcinoma had serum IL-18 levels comparable to healthy controls, while those with stage II or III had elevated levels. This suggests that increased IL-18 production may reflect the impact of gastric tumors on systemic immune responses, with minimal effects in stage I tumors. They interpreted the variation in IL-18 levels across cancer stages as a possible mechanism for tumor cells to evade immune surveillance, explaining why stage IV patients had IL-18 levels similar to healthy individuals, which were significantly lower than those in stage II patients (Kawabata et al., 2001).

Like IL-18, IL-16 plasma concentrations were significantly lower in GC patients compared to those with other gastric conditions. This finding is consistent with previous results discussed in Chapter 2. In the earlier study, IL-16 levels were measured in plasma samples from patients undergoing routine upper-intestinal endoscopy at the Queens Medical Centre, and the data showed a significant reduction in IL-16 plasma levels in GC patients compared to those with gastritis and peptic ulcer disease (Chapter 2).

Mice studies has shown that reduction in IL-16 levels is linked to impaired B cells development (Szabo et al., 1998). On the other hand, most of the human studies demonstrated an increase in IL-16 levels rather than a decrease, particularly in conditions such as rheumatoid arthritis (Lard et al., 2004), lupus erythematous, and multiple sclerosis (Skundric et al., 2006). However, IL-16 slows down human immunodeficiency virus replication in cells from infected subjects, and serum IL-16 amounts decline with disease progression (Amiel et al., 1999). Notably, Indinavir, a medication used in HIV patients, is known to increase circulating IL-16 levels. Since IL-16 functions as a chemoattractant for various CD4-expressing immune cells to the site of inflammation, low levels of IL-16 might signal inadequate immune surveillance and an impaired ability of the immune system to effectively respond to infections or tumors, potentially resulting in a poorer prognosis for cancer patients. Therefore, IL-16 reduction in GC patients suggests a potentially inhibitory role of IL-16 in GC progression, although the precise mechanisms remain to be fully elucidated.

Unfortunately, other cytokines such as IL-1β, IL-10, IL-17, IFN-γ, and TNF-α were excluded from the analysis because their plasma concentrations were below the detection thresholds for most of the patients. Similarly, IL-4, IL-12p70, and IL-13 were examined but excluded from the study since their levels were below detection limits in all patients.

In conclusion, this data reinforces the evidence of elevated IL-6 and IL-8 levels in GC patients. Additionally, it is the first to demonstrate that plasma levels of IL-18 and IL-16 are reduced in these patients, providing new insights into the potential roles of IL-16 and IL-18 GC. There is also significant interest in exploring the correlation between IL-18 and IL-16 in the context of GC.

This study has several advantages, including a large number of samples encompassing various precancerous and cancer stages, which allows the identification of differential cytokine expression at each stage, rather than merely comparing healthy controls to GC cases. Another advantage is the use

of the MSD assay, which enables the simultaneous measurement of multiple cytokines in a single sample of less than 50 microlitres. By assessing a broader range of cytokines and chemokines, the study aimed to uncover their relationships with GC development, but disappointingly, some cytokines could not be included as their concentrations were below the detection levels. However, one limitation is the absence of healthy controls.

# Chapter 5. General Discussion

## 5.1 Association of IL-16 with *H. pylori* and its related diseases.

Initially, this thesis aimed to investigate the expression of the cytokine IL-16 during *H. pylori* infection and its correlation with *H. pylori*-associated diseases, especially GC. The interest stemmed from IL-16's unique properties of chemoattraction and inactivation of $CD4^+$ T-cells, its strong link to various inflammatory diseases, and different types of cancers. Limited research has focused on IL-16 in *H. pylori*, primarily through *in vitro* studies involving GECs. A previous study has shown elevated IL-16 serum levels in patients with GC (Yang et al., 2017), and another study has reported the ability of *H. pylori* to induce IL-16 production by GECs (Alzahrani, 2014). our study is the first to investigate IL-16 expression levels in human samples (plasma and gastric biopsies). In addition to *H. pylori* status, the study also considered various factors such as *cagA* status, age, gender, smoking status, related diseases, oesophageal diseases, and histopathological scores, and conducted correlation analyses between IL-16 and other cytokines (IL-6, IL-10, IL-17).

Interestingly, IL-16 was detected in all plasma samples, showing significant individual variation (ranging from 135.68 to 2788.456 pg/ml), which can be due to genetic polymorphisms or microbiota differences. No association was found between IL-16 levels and *H. pylori* status, *cagA* status, age, gender, smoking status, related diseases, oesophageal diseases, or histopathological scores. Furthermore, an eradication study measuring IL-16 concentrations before and after *H. pylori* elimination over 24 months showed that plasma IL-16 levels remained unchanged post-eradication, suggesting *H. pylori* might not significantly impact systemic IL-16 levels. There was no correlation between systemic and gastric IL-16 protein levels. Interestingly, GC patients showed a significant reduction in IL-16 plasma levels compared to patients with gastritis, PUD, and healthy controls. This contradicts a previous study that reported elevated IL-16 serum concentrations in GC patients compared to healthy controls, possibly due to different patient recruitment criteria.

Further examination of IL-16 in a Mexican population with well-characterized precancerous lesions and conditions (NAG, AG, IM, and GG) showed similar results. No significant differences in IL-16 plasma levels between *H. pylori*-infected and uninfected patients. GC patients again showed a significant reduction in IL-16 plasma levels compared to patients with NAG, AG, and IM. Then, the role of *H. pylori* on IL-16 secretion by DCs and monocytes was also investigated. *H. pylori* was co-culture with THP-1 cells, KG-1 cells, CD14+ cells, or moDCs. Unfortunately, neither *H. pylori* nor its virulence factors (*cagA, cagE, vacA*) altered IL-16 secretion by monocytes or DCs, indicating that *H. pylori* may not impact IL-16 secretion by monocytes and DCs. The impact of bile metabolites on the IL-16 cytokine responses of monocytes was also investigated. Unfortunately, no change in IL-16 levels was observed.

This comprehensive study utilized different sample types (plasma, gastric tissue, cell lines, and peripheral blood monocytes) and techniques (ELISA, MSD, RT-qPCR, and Flow Cytometry). The study included healthy controls, *H. pylori*-infected and uninfected patients, patients with different gastric conditions, and participants from diverse ethnic backgrounds. It also considered multiple parameters that could influence plasma IL-16 levels, such as age, gender, smoking status, *cagA* status, and oesophageal disorders. In conclusion, we determined that *H. pylori* does not affect IL-16 cytokine levels.

## 5.2 Plasma cytokines and GC biomarkers

This study builds upon the initial focus in the previous IL-16 study on IL-16 as a promising factor for understanding the mechanisms underlying GC development in *H. pylori* patients. we aimed to expand our knowledge of the differential expression of cytokines and chemokines in *H. pylori*-positive and negative individuals across various stages of GC. This study also aimed to identify which cytokines or chemokines are elevated or reduced during the precancerous stages (AG, NAG, and IM), rather than concentrating solely on one cytokine at the stage of gastric cancer. Sixteen cytokines, including IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-16, IL-17, IL-18, IL-23, INF-γ, TNF-α, CCL2, CCL3, and CXCL10, were analyzed using the MSD assay. This assay was selected for its ability to measure multiple cytokines in a small sample volume, and with low detection limits. Disappointingly, only seven out of sixteen cytokines were included in the analysis because most were below the detection threshold for all or most patients. Despite this, some intriguing results were observed. Notably, this study is the first to show decreased plasma levels of IL-18 and IL-16 in GC patients, The observed reduction of IL-16 in GC patients suggests a potentially inhibitory role of IL-16 in the progression of GC, although the precise mechanisms remain to be fully elucidated, and reduced IL-18 could lead to a diminished anti-tumor immune response, allowing for cancer progression.

## 5.3 *H. pylori* virulence factors and immune response

*H. pylori* virulence factors are associated with gastric pathogenic processes. *H. pylori* exhibits significant genetic variability, and certain strains are more closely linked to gastric cancer. Notably, strains expressing the virulence factors *vacA* and *cag*-PAI are the most prominent (Molina-Castro et al., 2018). Thus, this thesis also examined the impact of *H. pylori* virulence factors, specifically *cagA, cagE, and vacA*, on the production of cytokines IL-10

and IL-6, which are involved in the development of GC. The gastric microbiota is also a potential source of factors that play a role in gastric cancer progression. the impact of bile metabolites on the cytokine responses of monocytes was also considered. In the study on IL-16, the influence of the *cagA* status of the strain on IL-16 responses was evaluated. To determine whether the *cagA* status was associated with increased IL-16 production in *H. pylori*-infected patients, plasma IL-16 levels were measured using ELISA and compared based on the presence or absence of the plasma anti-CagA IgG antibody. The results indicated no significant difference in IL-16 levels between patients infected with a *cagA*+ strain and those infected with a *cagA*- strain. Additionally, the effect of these virulence factors on cytokine production by monocytes and DCs was investigated. Monocytic and dendritic cell lines, as well as peripheral blood monocytes, were utilized, along with different bacterial strains, including wild-type Tx30a, wild-type 60190, and the isogenic *H. pylori* strains *cagA, cagE,* or *vacA*. Cytokine levels were measured using ELISA and flow cytometry. However, the virulence factors of *H. pylori* did not affect cytokine production by DCs and monocytes.

## 5.4 Alternative approaches

### 5.4.1 Combining the analyses

This thesis focuses on plasma cytokines and chemokines as cancer biomarkers, though they have certain limitations. Cytokines, despite their value, are not specific to cancer and can be elevated in various conditions such as infections, autoimmune diseases, and inflammatory states. Genetic differences, physical activity (Windsor et al., 2018), treatments, and stress lead to the alteration of individual cytokine levels (Kim and Maes, 2003). Moreover, inconsistencies in pre-analytical sample handling and storage can influence cytokine measurements (Vincent et al., 2019). Hence, complementary approaches are essential when using cytokines as cancer biomarkers. Combining circulatory

cytokine profiling with other diagnostic methods, such as measuring soluble cytokine receptors, cancer-related proteins in the circulation, and quantification of gene expression could offer a cost-effective and accessible solution for cancer diagnosis, prognosis, and treatment outcomes. For instance, Li *et al* (2018) enhanced the screening efficacy of carcinoembryonic antigen (CEA) and cancer antigen (CA)-724 by incorporating the measurements of IL-6, IL-8, and TNF (Li et al., 2018).

Long-term follow-up of patients with atrophic gastritis and intestinal metaplasia, analyzing gastric and plasma samples at different intervals, could also be valuable. However, such studies are time-consuming; a previous study yielded promising results after a 66-month follow-up (Chapelle et al., 2020). Additionally, given the lower incidence of gastric cancer in the UK, this type of analysis would be more viable in Eastern Asia, where the incidence rate is significantly higher.

### 5.4.2 Animal model

Many researchers prefer to use mouse models due to their availability, which includes inbred strains and genetically modified variants, their short breeding cycles, and the ease of access to experimental reagents (Zhang and Moss, 2012). We did not consider using a mouse model to study the cytokine immune response during the development stage of gastric cancer. The limitations associated with wild-type mouse models restrict their use for experimental *H. pylori* infections.

*H. pylori* is well adapted for colonizing the human stomach, but it does not easily infect the gastric mucosa of animals. This is because of the complex interaction of *H. pylori* with the human gastric epithelium, which takes decades to develop into gastric cancer. It is difficult to determine the pathogenesis of *H. pylori* infection and the immune response generated by this pathogen (Ansari and Yamaoka, 2022).

A mouse model infected with the *H. pylori* Sydney strain (HpSS1) exhibited chronic gastritis and gastric atrophy. However, wild-type mouse models such as C57BL/6, BALB/c, and C3H typically develop only mild gastritis or slowly progressing diseases when infected with *H. pylori*, offering limited insights into *H. pylori* pathogenicity. Infections of mouse models with *H. pylori* and *H. felis* have resulted in lymphocytic gastritis but do not progress to severe conditions like peptic ulcers or gastric cancer. Additionally, the anatomical structure of the murine stomach differs from that of the human stomach and lacks the necessary components for developing severe gastric pathologies. Moreover, the presence of other bacteria in the murine stomach may influence the outcomes of *H. pylori* infection (Ansari and Yamaoka, 2022). A vast array of chemokines and chemokine receptors orchestrates the movement of immune cells within and through tissues. Unsurprisingly, there are notable differences between the murine and human systems. For instance, CXCR1 is found in humans but not in mice. Chemokines such as IL-8, neutrophil-activating peptide-2 (CXCL7), IFN-inducible T cell-chemoattractant (CXCL11), monocyte chemoattractant protein-4 (CCL13), HCC-1 (CCL14), hemofiltrate CC chemokines-2 (CCL15), pulmonary and activation-regulated chemokine (CCL18), myeloid progenitor inhibitory factor-1 (CCL23), and eotaxin-2/3 (CCL24/CCL26) have been identified in humans but not in mice. Conversely, CCL6, CCL9, lungkine (CXCL15), and MCP-5 (CCL12) are found in mice but not in humans (Mestas and Hughes, 2004). These disadvantages limit the usefulness of wild-type mouse models for studying experimental *H. pylori* infections.

### 5.4.3 Human gastroid monolayer models of *H. pylori* infection

This thesis investigated the impact of *H. pylori* on cytokine production by DCs and monocytes by directly co-culturing them with the bacterium. However, this setup does not accurately represent a real infection scenario, where immune cells are separated from *H. pylori* by the epithelial barrier. *H. pylori* secretes the serine protease HtrA, which disrupts cell-to-cell junctions by cleaving epithelial

junctional proteins such as occludin, claudin-8, and E-cadherin, thereby enabling *H. pylori* to access the epithelial barrier and interact with immune cells (Tegtmeyer et al., 2017).

A new *ex-vivo* gastroid monolayer model of *H. pylori* infection uses healthy, untransformed human epithelial cells infected in a two-dimensional model system to facilitate the study of the interaction between the host cell and the pathogen in a manner that closely mimics actual infection. These gastric organoids, or gastroids, are derived from human gastric biopsies and encompass the full characteristics of epithelial cell types found in a normal stomach. They are notable for their stability, ability to be cultured semi-permanently, and capacity to be revived after being stored in a freezer. Unlike cell lines developed from tumor-derived cells, this multicellular model offers several advantages, as it contains polarized, differentiated normal human cells without malignant alterations. Two-dimensional cultures provide a physiological environment where *H. pylori* infection is both straightforward and reliable (Uotani et al., 2019). Previous studies have indicated that two-dimensional polarized monolayers derived from human stomach tissue are an appropriate ex vivo model for studying the interactions between the gastric epithelium, DCs, and luminal *H. pylori* bacteria (Uotani et al., 2019, Sebrell et al., 2019)

## 5.5 Conclusion

In conclusion, this research has initiated the process of addressing crucial gaps in understanding how *H. pylori* sustains infection and leads to GC from a cytokine perspective. Although no link was found between IL-16 cytokines and *H. pylori* infection, this result contributes novel information to the field. The thesis also demonstrated that even with advanced techniques measuring multiple cytokines and chemokines across numerous samples, a single approach is insufficient for definitive cancer biomarker identification. Instead,

combining methods, such as proteomic and transcriptomic analyses alongside cytokine analysis, could provide a more reliable and comprehensive understanding of useful gastric cancer biomarkers. Moreover, long-term monitoring of patients with gastric precancerous lesions and periodic assessment of their cytokine levels could offer clearer insights into cytokine biomarkers.

The suggested correlation between IL-16 and IL-18 cytokines in the context of gastric cancer provides a novel avenue for research. Both interleukins could be part of a larger network of immune regulation that, when disrupted, leads to a failure in controlling tumor growth. Further studies can investigate how these interleukins interact and influence each other, which can provide deeper insights into their combined roles in cancer immunity. Overall, this information expands the understanding of how IL-16 and IL-18 might contribute to immune response modulation and the pathophysiology of GC, potentially opening new therapeutic pathways.

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