Host cell responses to *Helicobacter pylori* secreted factors

Raquel Garcia Lobato Tavares

Academic dissertation for the Degree of Doctor of Philosophy in Molecular Bioscience at Stockholm University to be publicly defended on Thursday 14 December 2017 at 10.00 in Vivi Täckholmssalen (Q-salen) NPQ-huset, Svante Arrhenius väg 20.

Abstract
The infection of the human gastric mucosa by the bacterium *Helicobacter pylori* can lead to the development of gastritis, gastroduodenal ulcers, and cancer. The factors that determine disease development in a small percentage of infected individuals are still not fully understood.

In this thesis, we aimed to identify and functionally characterize novel virulence factors of *H. pylori* and to understand their effect on host cell responses.

In Paper I, we found that JHP0290, an uncharacterized secreted protein of *H. pylori*, induced macrophage apoptosis concomitant to the release of pro-inflammatory cytokine TNF via the regulation of the Src family of kinases and ERK MAPK pathways. In paper II, we demonstrated that JHP0290 exhibits both proliferative and anti-apoptotic activity, together with a faster progression of the cell cycle in gastric epithelial cells. During these responses, ERK MAPK and NF-κB pathways were activated. Paper III revealed a pro-apoptotic effect of another *H. pylori*-secreted protein HP1286 in macrophages via the TNF-independent and ERK-dependent pathways. No apoptosis was observed in HP1286-treated T cells or HL60 neutrophil-like cells, suggesting cell-type specific effect of HP1286. In Paper IV, we observed the pro-inflammatory activity of *H. pylori* secreted protein HP1173 in macrophages. The protein was found to induce TNF, IL-1β, and IL-8 in macrophages through MAPKs, NF-κB, and AP-1 signaling pathways. Furthermore, differential expression and release of JHP0290, HP1286, and HP1173 homologues was observed among *H. pylori* strains (papers II, III, IV).

Due to their ability to regulate multiple host cell responses, proteins JHP0290, HP1286, and HP1173 could play an important role in bacterial pathogenesis.

Keywords: *Helicobacter pylori*, Secreted proteins, Host cell responses, Macrophages, Apoptosis, Pro-inflammatory cytokines, MAPKs.

Stockholm 2017
http://urn.kb.se/resolve?urn=urn:nbn:se:diva-148427

ISBN 978-91-7797-047-7
ISBN 978-91-7797-048-4

Department of Molecular Biosciences, The Wenner-Gren Institute
Stockholm University, 106 91 Stockholm
HOST CELL RESPONSES TO *HEICOBACTER PYLORI* SECRETED FACTORS

Raquel Garcia Lobato Tavares
Host cell responses to *Helicobacter pylori* secreted factors

Raquel Garcia Lobato Tavares
“Tudo vale a pena, se a alma não é pequena.”

— Fernando Pessoa (Mar Português)
SUMMARY

The infection of the human gastric mucosa by the bacterium *Helicobacter pylori* can lead to the development of gastritis, gastroduodenal ulcers, and cancer. The factors that determine disease development in a small percentage of infected individuals are still not fully understood.

In this thesis, we aimed to identify and functionally characterize novel virulence factors of *H. pylori* and to understand their effect on host cell responses.

In **Paper I**, we found that JHP0290, an uncharacterized secreted protein of *H. pylori*, induced macrophage apoptosis concomitant to the release of pro-inflammatory cytokine TNF via the regulation of the Src family of kinases and ERK MAPK pathways. In **paper II**, we demonstrated that JHP0290 exhibits both proliferative and anti-apoptotic activity, together with a faster progression of the cell cycle in gastric epithelial cells. During these responses, ERK MAPK and NF-κB pathways were activated. **Paper III** revealed a pro-apoptotic effect of another *H. pylori*-secreted protein HP1286 in macrophages via the TNF-independent and ERK-dependent pathways. No apoptosis was observed in HP1286-treated T cells or HL60 neutrophil-like cells, suggesting cell-type specific effect of HP1286. In **Paper IV**, we observed the pro-inflammatory activity of *H. pylori* secreted protein HP1173 in macrophages. The protein was found to induce TNF, IL-1β, and IL-8 in macrophages through MAPKs, NF-κB, and AP-1 signaling pathways. Furthermore, differential expression and release of JHP0290, HP1286, and HP1173 homologues was observed among *H. pylori* strains (**papers II, III, IV**).

Due to their ability to regulate multiple host cell responses, proteins JHP0290, HP1286, and HP1173 could play an important role in bacterial pathogenesis.
POPULÄRVETENSKAPLIG
SAMMANFATTNING


**I studie I** visar vi att det utsöndrade proteinet JHP0290 från *H. pylori*, vars funktion hittills varit okänd, sätter igång produktion av den inflammatoriska signalmolekylen tumörnekrosfaktor (TNF) samt apoptos (programmerad cellldöd) hos makrofager. Makrofager är immunceller vars uppgift är att bekämpa bakterieinfektioner i ett tidigt skede. Induktionen av TNF är också beroende av att makrofager aktiverar två olika signaleringsvägar kallade Src och ERK MAPK. **I studie II** undersöker vi effekterna av proteinet JHP0290 på magepitelceller. När dessa celler exponeras för JHP0290 ser vi en minskad apoptos samt ökad celldelning genom att cellcykeln ”snabbas på”. Dessutom aktiveras även signaleringsvägarna ERK MAPK och NF-κB, vilka har en central roll i aktiveringen av immunförsvar. **I studie III** fokuserar vi på ett annat protein kallat HP1286. Vi undersöker dess förmåga att aktivera apoptos i olika typer av immunceller såsom makrofager, T celler och neutrofiler. Våra resultat visar att HP1286 endast aktiverar apoptos i makrofager men inte i de andra immuncellerna, vilket betyder att detta protein har cell-specifika effekter.

**I studie IV** studerar vi ett tredje protein kallat HP1173 som visar sig vara mycket inflammatoriskt. När makrofager exponeras för HP1173 tillverkas och utsöndras flera olika inflammatoriska signalmolekyler såsom TNF, interleukin-1β och interleukin-8 vars funktioner i kroppen är att aktivera och ledsaga övriga delar av immunförsvar under den pågående infektionen.

Sammanfattningsvis har vi i denna avhandling visat att proteinerna JHP0290, HP1286 och HP1173 från *H. pylori* modifierar beteendet och funktionen av olika typer av värdceller och att dessa proteiner sannolikt spelar en viktig roll i bakteriens förmåga att orsaka infektion.
Helicobacter pylori é uma bactéria que habita preferencialmente no estômago do ser humano há mais de 50 000 anos. Estima-se que mais de cinquenta por cento da população mundial seja infectada por H. pylori. No entanto, apenas uma pequena percentagem dos indivíduos infectados demonstra sintomas de doenças gastroduodenais como gastrites, úlceras e cancro. Os fatores que efetivamente determinam o desenvolvimento de disfunções gástricas causadas por H. pylori ainda não são inteiramente compreendidos. O estudo de eventos que resultam das interações entre bactéria e hospedeiro, contribui para o conhecimento mais aprofundado do modo como H. pylori infecta, atua e prevalece no seu habitat natural que é o estômago humano. Os trabalhos apresentados nesta tese tiveram como objetivo identificar e caracterizar a função de novos fatores da H. pylori com potencialidade em causar doença, e compreender o seu efeito no funcionamento normal das células do hospedeiro.

No estudo I, descobrimos que JHP0290, uma proteína libertada por H. pylori e anteriormente não caracterizada, induz morte celular (apoptose) em macrófagos, células do sistema imunitário que desempenham um papel importante no combate a infeções. Para além disso, verificámos que o efeito anterior é acompanhado da liberação da citocina pró-inflamatória TNF, uma das moléculas responsáveis por dirigir a orquestra de respostas imunitárias do hospedeiro através da emissão de sinais entre células. Porém, no estudo II observámos que JHP0290 exibe efeitos de proliferação (divisão de células descontrolada) e de anti apoptose celular juntamente com a rápida progressão do ciclo celular, em células do epitélio gástrico. Os resultados do estudo III revelaram que HP1286, outra proteína segregada por H. pylori, exerceu um efeito de apoptose em macrófagos independente da liberação de TNF. No mesmo estudo verificámos que HP1286 não causou o efeito de apoptose na presença de linfócitos T e neutrófilos, outros tipos de células do sistema imunitário importantes para o desenvolvimento de respostas imunitárias no hospedeiro. Esta observação sugere que HP1286 atua de forma específica consoante o tipo de célula com que contacta. Por fim no estudo IV, verificámos que outra proteína libertada por H. pylori designada HP1173, apresenta atividade pro-inflamatória em macrófagos uma vez que promove a indução de citocinas de perfil pro-inflamatório como TNF, IL-1β e IL-8.

Os resultados destes estudos são indicadores de que as proteínas JHP0290, HP1286 e HP1173 podem representar um papel importante no desenvolvimento de doenças causadas por H. pylori devido ao facto de terem a capacidade de regular processos celulares no hospedeiro.
CONTENTS

SUMMARY ...................................................................................................... ii
POPULÄRVETENSKAPLIG SAMMANFATTNING ....................................... iii
RESUMO DE DIVULGAÇÃO CIENTÍFICA......................................................... iv
LIST OF PUBLICATIONS ................................................................................. vii
ABBREVIATIONS ......................................................................................... viii
INTRODUCTION ............................................................................................. 1

Chapter 1 Helicobacter pylori........................................................................ 2
  1.1 Classification ............................................................................................ 2
  1.1.1 Helicobacter ......................................................................................... 2
  1.1.2 Helicobacter pylori ................................................................................ 2
  1.2 Epidemiology, disease, and treatment ...................................................... 3
  1.2.1 Epidemiology and transmission ............................................................ 3
  1.2.2 Diagnosis and eradication .................................................................... 4
  1.2.3 Antibiotic resistance ............................................................................ 5

Chapter 2 Pathogenesis.............................................................................. 6
  2.1 Helicobacter pylori genetic diversity ......................................................... 6
  2.1 Major virulence factors .......................................................................... 7
  2.1.1 Urease and Flagella ............................................................................. 7
  2.1.2 Outer membrane proteins ..................................................................... 7
  2.1.3 CagA ..................................................................................................... 8
  2.2 Helicobacter pylori Secretome ................................................................. 9
  2.2.1 Toxins .................................................................................................. 9
  2.2.1.1 VacA .............................................................................................. 9
  2.2.1.2 Other toxins .................................................................................. 10
  2.2.2 Binding and transport proteins ............................................................ 11
  2.2.3 Enzymes .............................................................................................. 12
  2.2.4 Uncharacterized secreted proteins ...................................................... 13
  2.2.5 Mechanisms of protein secretion ....................................................... 13
  2.3 Other virulence-associated factors ......................................................... 14
  2.4 Impact of host factors in Helicobacter pylori pathogenesis .................... 15
  2.5 Influence of environmental factors ......................................................... 16

Chapter 3 Host immune responses to Helicobacter pylori ........................................... 17
  3.1 Human stomach ...................................................................................... 17
### Table of Contents

3.2 Gastric mucosa ..................................................................................................... 17
3.3 Innate Immunity ..................................................................................................... 18
  3.3.1 *H. pylori* recognition by PRRs ....................................................................... 19
  3.3.2 Other host cell receptors of *H. pylori* ............................................................. 20
3.4 Macrophages ........................................................................................................ 20
  3.4.1 Macrophages responses to *H. pylori* ............................................................. 21
3.5 Interaction of *H. pylori* with other host cells .................................................. 24
  3.5.1 Gastric epithelial cell responses to *H. pylori* ................................................ 24
  3.5.2 Dendritic cells ............................................................................................... 25
  3.5.3 T-cells ............................................................................................................ 25
  3.5.4 Neutrophils ..................................................................................................... 26
  3.5.5 B-cells ............................................................................................................. 26

### PRESENT INVESTIGATION ........................................................................ 27
Aims .......................................................................................................................... 27
Results and discussion .............................................................................................. 28
  Paper I ................................................................................................................... 28
  Paper II .................................................................................................................. 29
  Paper III ............................................................................................................... 30
  Paper IV ................................................................................................................. 31
Future Perspectives .................................................................................................. 33

### ACKNOWLEDGEMENTS ........................................................................ 35

### REFERENCES .............................................................................................. 38
This thesis is based on the following original papers, which will be referred to by their Roman numerals in the text.


IV. Tavares R and Pathak SK. Induction of TNF, IL-8 and IL-1β in macrophages by *Helicobacter pylori* secreted protein HP1173 occurs via MAP-kinases, NF-κB and AP-1 signaling pathways. *Manuscript, submitted*

*Papers not included in this thesis:

Saroj SD, Maudsdotter L, Tavares R and Jonsson A-B. Lactobacilli interfere with *Streptococcus pyogenes* haemolytic activity and adherence to host epithelial cells. *Frontiers in Microbiology*, 2016 Jul 29; 7:1176


*MG and LM contributed equally*
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BabA</td>
<td>Blood group antigen binding adhesion</td>
</tr>
<tr>
<td>CagA</td>
<td>Cytotoxic-associated gene A</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptors</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Dendritic cell-specific ICAM3-grabbing non-integrin</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EPIYA</td>
<td>Glutamic Acid-Proline-Isoleucine-Tyrosine-Alanine</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FaaA</td>
<td>Flagella-associated autotransporter</td>
</tr>
<tr>
<td>GECs</td>
<td>Gastric epithelial cells</td>
</tr>
<tr>
<td>GGT</td>
<td>γ-glutamyl transpeptidase</td>
</tr>
<tr>
<td>Hop</td>
<td>Helicobacter outer porins</td>
</tr>
<tr>
<td>HP-NAP</td>
<td><em>H. pylori</em> neutrophil-activating protein</td>
</tr>
<tr>
<td>HspB</td>
<td><em>H. pylori</em> Heat-shock protein B</td>
</tr>
<tr>
<td>HtrA</td>
<td>High temperature requirement protein A</td>
</tr>
<tr>
<td>ImaA</td>
<td>Immunomodulatory-associated autotransporter</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase enzyme</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinases</td>
</tr>
<tr>
<td>Mc1-1</td>
<td>Induced myeloid leukaemia cell differentiation protein</td>
</tr>
<tr>
<td>NLRs</td>
<td>NOD-like receptors</td>
</tr>
<tr>
<td>OipA</td>
<td>Outer inflammatory protein A</td>
</tr>
<tr>
<td>OMPs</td>
<td>Outer membrane proteins</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated patterns</td>
</tr>
<tr>
<td>PPI</td>
<td>Proton pump inhibitor</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pathogen recognition receptors</td>
</tr>
<tr>
<td>RLRs</td>
<td>Retinoic acid-inducible gene (RIG)-I-like receptors</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SabA</td>
<td>Sialic acid-binding adhesin</td>
</tr>
<tr>
<td>SFKs</td>
<td>Src family of tyrosine kinases</td>
</tr>
<tr>
<td>SHP-2</td>
<td>Tyrosine phosphatase</td>
</tr>
<tr>
<td>SMO</td>
<td>Spermine oxidase enzyme</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Tipg</td>
<td>Tumor necrosis factor-α inducing protein</td>
</tr>
<tr>
<td>T4SS</td>
<td>Type IV secreted system</td>
</tr>
<tr>
<td>VacA</td>
<td>Vacuolating cytotoxin A</td>
</tr>
</tbody>
</table>
INTRODUCTION

The existence of microorganisms that today we called bacteria was no more than a matter of conjecture until they were first observed by Anton van Leeuwenhoek in the mid-17th century. Over the course of time, the previously considered “invisible living creatures” were characterized in a wide variety of species and identified in almost all habitats on earth. For many bacteria, the optimal or only living environment is within another species. The human body is no exception and is known to harbor a large and diverse community of bacteria [1]. Most bacterial species colonize the human host asymptomatically, but a small percentage is classified as pathogenic due to their ability to cause damage or disease in the host. Throughout the years, the terms pathogen and pathogenicity have been redefined. In 1890, Robert Koch presented guidelines, known as Koch’s postulates, on how to classify a microbe as pathogenic [2]. The latter was soon revised since it considered the ability of the microorganism to cause disease as an invariant trait, excluding microbes that did not cause disease in every host or microbes that were not classified as pathogenic but did cause disease in certain hosts [3]. Further scientific and technological advances contributed to a new view of microbial pathogenicity that identified the host as an equally important factor for disease development. Understanding the concept of host immunodeficiency led to the recognition of microbes that can cause disease only in some hosts. Furthermore, the ability to isolate known-pathogenic microbes from asymptomatic individuals, challenged the scientific community to reflect on the host-pathogen relationship and to consider notions such as carrier and commensal [3].

Long-term colonizing bacteria are known to establish a close relationship with their hosts. Mutual adaptation has generated a balance between the commensal and pathogenic role of certain bacteria in humans. However, either party can disrupt this equilibrium. The development of complex strategic mechanisms aiming at survival has become a hallmark of human pathogens. On the other side, the human host focuses on the pathogen clearance to avoid colonization, further persistence, and disease development.
Chapter 1 *Helicobacter pylori*

1.1 Classification

1.1.1 Helicobacter

The genus *Helicobacter* belongs to the phylum *Proteobacteria*, family *Campylobacteriaceae*, and consists of non-spore-forming Gram-negative bacteria. *Helicobacters* are classified as microaerophilic organisms since they require a concentration of oxygen for its growth that is less than that in the air. To date, the *Helicobacter* genus comprises 37 validated species [4] of pathogenic and non-pathogenic bacteria, which can be classified according to the site of bacterial colonization. Enterohepatic *Helicobacter* species colonize the intestinal tract or the liver of humans, other mammals, and birds [5]. Gastric *Helicobacter* species represent a group of bacteria that are identified in the stomach niche of a wide range of hosts, including humans, non-human primates, domesticated animals, and other mammals [6].

1.1.2 Helicobacter pylori

*Helicobacter pylori* were first isolated in 1982 by Barry Marshall and Robin Warren from the stomach of patients suffering from gastritis [7]. Due to its resemblance to *Campylobacter* species, the bacterium was initially classified as *Campylobacter pylori*. However, further investigation revealed that *C. pylori* differed from other *Campylobacter* species in features such as flagella morphology, fatty acid composition, and enzymatic activity [8-10]. Sequence analysis of the 16S ribosomal RNA gene confirmed the separation between *C. pylori* and the rest of the *Campylobacter* species [11]. Consequently, *C. pylori* were placed into a new genus, *Helicobacter*, and later renamed *H. pylori* [8]. The cultivation of *H. pylori* represented a turning point in the perception of the relationship between bacterial colonization and disease development. The spiral shape and the presence of multiple unipolar sheathed flagella confer motility to the organism, allowing rapid movements within the viscous layer of the gastric mucosa [12]. Its microaerophilic profile requires the production of cytochrome c oxidases to use oxygen, even at low concentrations, as a terminal electron acceptor. Catalase protects the bacteria against possible damage by hydrogen peroxide excreted from phagocytes [13]. Urease is an essential enzyme for bacterial
survival in the acidic niche of the stomach since it hydrolyzes urea into carbon dioxide and ammonia, thus creating a pH-neutral environment [14].

1.2 Epidemiology, disease, and treatment

*H. pylori* have been a colonizer of the human gastric mucosa for over 50,000 years. Bacterium coevolution with humans is a relationship that preceded the anatomically modern human migrations from Africa. Phylogeographic and genetic patterns have revealed that, similarly to humans, the genetic diversity of *H. pylori* declines with the geographic distance from East Africa, the origin of modern humans [15, 16]. Decades of intimate association has selected *H. pylori* as one of the most prevalent human bacterial pathogens.

1.2.1 Epidemiology and transmission

More than half of the world’s population is colonized by *H. pylori*. However, the prevalence of bacterial infection varies geographically, with 80% of infected individuals in developing countries compared to 30–50% in developed countries [17]. Additionally, variation in infection prevalence also occurs within the same population, according to the individual’s age, gender, ethnic background, and socioeconomic status [17, 18]. The risk of acquiring *H. pylori* infection is higher during early childhood and is consistently associated with poor hygienic and crowded living conditions. Bacterial mechanisms of transmission are yet to be fully understood, but multiple routes have been proposed, including oral-oral, gastric-oral, and fecal-oral pathways [19-21]. Regardless of *H. pylori* not being considered a waterborne pathogen, its presence in water streams, rivers, and pipes represents a spring of exposure and enable its rapid spread, especially in developing countries [19, 22-24]. Although most of the infections are asymptomatic, long-term colonization of the organism throughout the life of the host can lead to chronic inflammation and development of site-specific diseases. Approximately 10–20% of infected individuals reveal clinical symptoms of gastrointestinal disorders. Peptic ulceration is diagnosed in about 10% of *H. pylori* carriers, gastric adenocarcinoma in 1–3%, and gastric B-cell lymphoma, known as mucosa-associated lymphoid tissue (MALT) lymphoma, in less than 0.1% [7, 17, 25]. Gastric adenocarcinoma is the fifth most common cancer in the world and occupies the 19th position in the world’s cause of death list. In 1994, the World Health Organization International Agency for Research on Cancer recognized *H. pylori* as a type I carcinogen. Since then, chronic infection with *H. pylori* has been considered as the strongest risk factor for stomach cancer. The last global cancer statistics reported an estimated number of 951,600 new stomach cancer cases and the occurrence of 723,100 deaths during the year of 2012 [26]. On the other hand, over the past decades, bacterial infection and prevalence have diminished in industrialized countries among children and young adults. This drop is directly associated with the improvement of
population hygienic and sanitary conditions, dietary habits, and socioeconomic status [27]. Interestingly, some studies have also established associations between \textit{H. pylori} infection and extragastric diseases with high impact on society’s healthcare, such as neurological disorders (Parkinson’s and Alzheimer’s diseases) or cerebrovascular and cardiovascular disorders [28-32].

The 2011 World Gastroenterology Organization Global Guideline report classified Sweden as having a low \textit{H. pylori} infection incidence (11% of bacteria prevalence in adults between 25 and 50 years-of-age) [33]. In order to evaluate the current situation in the Swedish population, a recent study analyzing the prevalence of \textit{H. pylori} over a period of 23 years in adults revealed that bacteria incidence was significantly decreased in all age groups over that period, with special focus on the elderly group where the vast majority of people did not have gastric atrophies [34]. Nevertheless, it is substantial to continue exploring not only the mechanisms of bacterial colonization and prevalence but also the physiological role in the human host.

\textbf{1.2.2 Diagnosis and eradication}

Invasive and non-invasive methods are available for the diagnosis of \textit{H. pylori} infection. The most reliable method consists of culturing bacteria from a biopsy collected from the upper gastrointestinal mucosa of a likely infected individual [35-37]. The urea breath test is less invasive and is usually selected for a first diagnosis. This test accesses the urease activity of a potential \textit{H. pylori}-infected patient.

According to the 2016 Toronto Consensus for the Treatment of \textit{H. pylori} Infection in Adults report, the recommended first-line treatments for \textit{H. pylori} infection are the following: Bismuth quadruple (PBMT) regimen including proton pump inhibitor (PPI), bismuth, metronidazole, and tetracycline, and the concomitant nonbismuth quadruple therapy (PAMC) that replaces bismuth with amoxicillin and tetracycline with clarithromycin for a period of 7, 10 or 14 days [38]. Although, the action mechanisms of some of these chemicals, such as bismuth, are yet well understood, the combination of antibiotics used should target specific components of the bacterial cell (in an attempt to avoid resistance) and contribute to the bacterial eradication. Even though high cure rates are associated with this therapy, the complexity of administration protocols represents an obstacle to its acceptability for general use [39]. A recent Swedish study aimed to correlate the efficacy of antibiotic treatment against \textit{H. pylori} with the bacteria eradication levels within the Swedish population over a 10-year period. The authors had accessed data from the Prescribed Drug Register [40] and analyzed the prescriptions for the antibiotic combination package of the standard PPI triple therapy administrated for 7 days, which represent the dominant recommended regimen in Northern Europe [41]. The authors concluded that around 140,000 individuals were treated with standard PPI
triple therapy in the past decade and that PPI can be effective in eradicating *H. pylori* [41].

To date, no one has succeeded in developing a safe and efficacious vaccine against *H. pylori* infection. There is an apparent need to develop novel therapies since the current ones are expensive, result in a high frequency of side effects and contribute to the increase of antibiotic resistance. Promising solutions for *H. pylori* eradication are the bioavailable potassium-competitive acid blocker vonoprazan, developed and approved in Japan for the treatment of acid-related diseases [42-44], and the use of probiotics as adjuvant therapies contributing not only to the increase of eradication rates but also to the reduction of therapy-related side effects [45-48].

Recently, the question whether *H. pylori* eradication is beneficial for the human physiology has also been addressed. Several epidemiologic and experimental studies have suggested an inverse correlation between *H. pylori* infection and the occurrence of esophageal diseases, inflammatory bowel diseases, obesity, multiple sclerosis (MS), food allergy, and asthma [49-52]. However, contradictory results and limited evidence of the studies have diverted the consensus of opinion among the scientific community regarding the role of *H. pylori* in the development of those diseases, calling for further and better conducted experiments in the matter [51, 53, 54].

1.2.3 Antibiotic resistance

The emergence of antibiotic-resistant strains has led to a drop in the treatment efficacy rates of *H. pylori* eradication, especially in countries where the antibiotic prescription is overstated. The 2016 Toronto Consensus for the Treatment of *H. pylori* Infection in Adults report suggested the restriction of PPI triple therapy to areas with known low clarithromycin resistance (<15%) or high eradication cases (>85%) [38]. Because of the increased incidence of clarithromycin-resistance *H. pylori* strains, the latter was considered as a high priority in the 2017 WHO Priority Pathogens List for research and development of new antibiotics. Furthermore, increasing rates of metronidazole and fluoroquinolone (e.g., levofloxacin) resistance have also been observed [55-57].
Chapter 2 Pathogenesis

2.1 *H. pylori* genetic diversity

The *H. pylori* genome, successfully sequenced in 1997, was found to be in the lower range of sizes among pathogenic bacteria, with a mean of 1670 kb [58, 59]. The bacterial microevolution is driven by selective pressures that result in diversification. These pressures can result from human host constraints via immune responses and developmental changes in the gastric epithelium, acidity or nutrient availability, but also from signals within large bacteria populations [60]. Consequently, the bacteria reveal high rates of mutation and recombination frequencies [61]. Therefore, *H. pylori* populations are considered to behave like certain viral quasispecies that have a high mutation rate; the offspring is also expected to contain one or more mutations compared to the parent. Accordingly, the human host is not colonized by a single bacterial clone but instead by a group of related genotypes [62]. Endogenous mutation is a mechanism that leads to the appearance of adaptive mutations. In the case of *H. pylori*, most strains are thought to have a mutated phenotype that allows the development of variants considerably more adapted to the required living conditions. Diversity within *H. pylori* strains was highlighted in a study where the genomes of two different strains (26695 and J99) were compared. Furthermore, it was observed that about 6% of the genome consisted of strain-specific genes, mainly located in a part of the genome called the plasticity region [63]. A good example of the current increased strain diversity is the rapid emergence of bacterial populations with high-level resistance to antibiotics used to eradicate *H. pylori*, such as clarithromycin [64]. The high natural competent profile of *H. pylori* allows it to increase in diversity through intergenomic recombination. Evidence that allelic diversity in *H. pylori* populations is created by recombination within strains was shown by multi-locus sequence typing (MLST) analysis of housekeeping gene fragments [65]. The uptake of foreign DNA from other strains might happen during a persistent or transient mixed infection, in a chronic colonization scenario [65-67]. Consequently, the bacteria can maximize the diversity of sequences targeted by selected pressure while, at the same time, maintaining those alleles essential for survival. These strategies allow a heterogeneous bacterial population to colonize parts of the stomach that vary in features like acidity, expression of host cell products, or level of inflammation of gastric mucosa, therefore promoting bacterial adaptation to a constant changing host [61].
2.1 Major virulence factors

*H. pylori* have evolved specific mechanisms and features essential for a successful colonization and persistence in the gastric mucosa of the human host. Several bacterial components contribute to gastric inflammation, either by interfering with host-signaling pathways, which are important for keeping gastric epithelium homeostasis, or by interacting with host immune cells, manipulating them for its own benefit (Fig. 1). Thus, these components are believed to be major determinants of bacterial virulence.

### 2.1.1 Urease and Flagella

The first step of the *H. pylori* colonization and pathogenesis process is to survive the harsh conditions of the human stomach. One of the key mechanisms developed by the bacterium consists in the adjustment of the periplasmic pH through the regulation of urease activity [68]. Urease is mainly found in the cytoplasm, where its activity is regulated by UreaI, a proton-gated channel that allows the entry of urea in acidic conditions, thus preventing, the alkalinization of the cytoplasm [68]. Due to the lysis of some organisms, the enzyme urease can also be found on the surface of *H. pylori*. This enzyme is responsible for the conversion of urea into carbon dioxide and ammonia, which in turn produces ammonia hydroxide when combined with water [68]. Consequently, the acidic pH of the *H. pylori* microenvironment is neutralized, leading to a reduction of the mucus layer viscoelasticity, thereby allowing a safe movement of the bacterium through the respective layer [69, 70].

The helical shape of the bacteria facilitates the movement within the thick layer of the gastric mucosa. However, it is the sheathed flagella that mediate bacteria motility, conferring screw-like movements that enable the organism to penetrate the mucosa layer [71]. The flagellar sheath is continuous with the outer membrane and contains lipopolysaccharide (LPS) and proteins [72]. A correlation between bacterial motility and disease has been proposed. *H. pylori* strains presenting a lower motility capacity showed a reduced ability to colonize and survive in the host when compared to fully motile strains [12]. Production of interleukin-8 (IL-8) was shown to be higher in strains with high motility than in low-motility strains, which can be related to the better colonization efficacy of high motile strains [73].

### 2.1.2 Outer membrane proteins

About 4% of the *H. pylori* genome is predicted to encode outer membrane proteins (OMPs). The semipermeable phospholipid bilayer, which makes up the outer membrane, is composed of several groups of proteins. The *Helicobacter* outer porins (Hop) represent the largest family of OMPs. The Hop proteins are primarily involved in adhesion of the bacteria to the gastric epithelium, but also perform transport functions, essential for the metabolism and selective permeability of the outer membrane [35, 74].
Outer inflammatory protein (OipA) is a member of the Hop protein group and plays a role in the process of \textit{H. pylori} attachment to gastric epithelial cells (GECs). This protein is present in all \textit{H. pylori} strains, but the \textit{oipA} gene status is regulated by phase variation. Strains containing the functional \textit{oipA} gene have been associated with the presence of peptic ulcers and gastric cancer [75]. OipA has also been linked to increased levels of mucosal IL-8 and $\beta$-catenin signaling, important for cell-to-cell junctions and proliferation [75, 76]. In some countries, \textit{H. pylori} strains with an active \textit{oipA} gene, as well as the high production of \textit{H. pylori} associated-virulence factors, cytotoxic-associated gene A (CagA), and vacuolating cytotoxin A (VacA), are classified as highly pathogenic strains [77].

The blood group antigen-binding adhesin (BabA) is an outer membrane protein that binds to ABO-histo-blood group antigens and Lewis b antigens (Leb), on the surface of GECs [78]. The expression of BabA allows the bacteria to rapidly adapt to the changes in host mucosal glycosylation that occur upon infection [79]. Sialic acid-binding adhesin (SabA) is another \textit{H. pylori} adhesin that binds to the carbohydrate structure sialyl Lewis$^+$ antigen [75]. SabA expression is highly induced during chronic gastric inflammation [80]. SabA has been related to an increased risk of gastric cancer but low risk of peptic ulcer development [81].

2.1.3 CagA

The \textit{cagA} gene is located in the 40 kb DNA insertion element region called \textit{cag} Pathogenicity Island (\textit{cag} PAI), which contains about 31 genes, many of which are responsible for encoding the type IV secreted system (T4SS) [35]. T4SS is a syringe-like structure that injects CagA protein into host GECs, as well as B lymphoid cells and dendritic cells [82-84]. Once translocated into GECs, CagA localizes in the inner surface of the plasma membrane, where it might be phosphorylated at the Glu-Pro-Ile-Tyr-Ala (EPIYA) motif by host Src and Abl kinases [85]. The carboxy-terminal polymorphic region of CagA, possess four different EPIYA motifs, EPIYA-A to EPIYA-D that vary in amino-acid sequence. Phosphorylated CagA binds and subsequently activates eukaryotic tyrosine phosphatase (SHP-2), which leads to the activation of ERK 1/2, Crk adaptor or C-terminal Src kinase, which are involved in the regulation of cell processes, including meiosis, mitosis, cell growth, differentiation, migration or immune response [86]. The number of EPIYA motif sites varies among strains and countries and represents an important risk indicator of developing gastric cancer [87]. Morphological changes in the human gastric adenocarcinoma cell line (AGS), characterized by cell elongation (which is associated with cell-scattering and increased cell motility), is a reported consequence of CagA translocation and subsequent phosphorylation [88]. These alterations indicate the interference of CagA with signaling pathways involved in cellular processes essential for the maintenance of cell health. Thus, bacterial strains containing CagA with a greater number of EPIYA motifs and inducing morphological alterations in
GECs have been linked to an increased risk of gastric cancer [89, 90]. A correlation between *H. pylori* strains possessing the cag PAI locus and the risk of developing a peptic ulcer and gastric cancer has been shown in Western populations [91]. The frequency of *H. pylori* CagA+ strains is geographically distributed with a representation of 60% in developing countries and almost 100% in East Asian countries [92]. Due to the high incidence of CagA+ strains in patients with *H. pylori*-associated disease, CagA has become the best-studied bacterial factor and an important determinant of *H. pylori* virulence.

### 2.2 *H. pylori* Secretome

In an *H. pylori*-infected individual, the majority (70–80%) of bacteria are found in the mucous layer rather than in contact with the underlying epithelium [93]. Due to the general non-invasive nature of *H. pylori*, it is believed that bacterium-released products, such as secreted proteins, play an important role in the disease development. Furthermore, the bacterium uses secreted proteins of different structure, biochemical composition, and functions, with the purpose of adapting to the mucosal environment [74, 94]. In the literature, the secretome is considered an ambiguous term since there is no consensus about which protein types to include. The definition of secreted proteins refers to polypeptides that are transported outside the outer membrane through a secretion mechanism [74]. However, in this section of the thesis, I will include those proteins that have a secretion signal and have been shown to be secreted by the bacterium, either to the periplasmic or external spaces. To date, there are five major studies that have attempted to identify the composition of the *H. pylori* secretome [95-99]. Some authors have suggested that the *H. pylori* secretome encodes at least 160 proteins. The number of proteins reported to make up the *H. pylori* secretome likely varies because of the ambiguity of the secretome definition, as well as the nature of the experiments performed. Proteins released by the bacterium can be divided into several groups according to their function. Only a portion of the *H. pylori* secretome is discussed below, leaving out groups such as outer membrane proteins, flagella components, and cytotoxin-associated gene pathogenicity island (*cag*-PAI).

#### 2.2.1 Toxins

##### 2.2.1.1 VacA

The *vacA* gene is expressed by the majority of *H. pylori* strains and encodes the pore-forming exotoxin vacuolating cytotoxin, VacA, a major secreted virulence factor of *H. pylori*. This toxin consists of two domains, a passenger, and an autotransporter domain. The mature virulent form of the
The toxin is 88-kDa in size and is released as a soluble protein in the host cell upon cleavage of the passenger domain by the autotransporter domain [100]. The toxin is composed of p33 (N-terminal) and p55 (C-terminal) subunits that assemble into an oligomeric complex and further disaggregates upon exposure to pH differences, thereby allowing the insertion of VacA into lipid bilayers. Consequently, anion-selective, voltage-dependent channels are formed and cells are vacuolated as a result of the channel, which targets to late endosomes and early lysosomes [101]. VacA is actively transported into cells via endocytosis and is accumulated inside different cell compartments. Once inside the cells, the toxin can induce mitochondrial damage and cytochrome c release, which may lead to cell apoptosis [101]. The disturbance of mitochondrial morphological dynamics is one of the mechanisms suggested for VacA-induced apoptosis. On the other hand, it was also proposed that induction of gastric epithelial cell death by VacA could be related to the release of pro-inflammatory protein high-mobility group box 1 (HMGB1), which is associated with programmed cell necrosis rather than apoptosis [102]. By *H. pylori* genetic diversity studies, the *vacA* gene has been shown to contain three regions of great diversity: s, signal sequence region; m, mid-region; and i, intermediate region. Every region presents two subdivisions with possible variations that reflect different virulent profiles of the bacteria. For instance, the s1m1 strains have been shown to be the most cytotoxic, followed by s1m2 strains, whereas the s2m1 and s2m2 strains have no cytotoxic activity [35]. The increased risk of gastric cancer development has been mainly associated with the *H. pylori* genotypes *vacA* and *s1m1* [103]. Interestingly, for certain populations, the phenotype *vacA* *il* was shown to be a better determinant of gastric adenocarcinoma development [104].

### 2.2.1.2 Other toxins

Studies have identified two proteins (ImaA and FaaA) that are exported by the type V autotransporter system and localized on the bacterial cell surface. Since they share characteristics with the VacA protein, including copies of a VacA-conserved motif (pfam03077) and the N- and C-terminal external motifs for the type V export pathway, they were classified as VacA-like proteins [74]. These proteins were observed to be upregulated *in vivo*, and respective mutants showed a deficiency in colonization and persistence compared to wild-type phenotype with FaaA contributing to flagella stability and functionality [105]. The presence of FaaA antibodies in the serum of *H. pylori*-infected patients was strongly associated with gastric cancer risk [106]. Based on these results, the authors proposed FaaA as a new noninvasive biomarker for early detecting of gastric cancer risk [106]. The ImaA protein is upregulated under acidic conditions and exhibited pro-inflammatory activity in contact with gastric epithelial cell line AGS through the stimulation of TNF and chemokine IL-8 [107].
Tumor-necrosis-factor α inducing protein (Tipα) is a secreted protein localized to the periplasm and extracellular space but also attached to the inner membrane. Similar to the major virulence-associated factor CagA, Tipα can also penetrate GECs but in a T4SS independent manner [108]. In mouse stomach cancer cells and gastric epithelial cell lines, Tipα has been reported to induce some pro-inflammatory cytokines via the NF-κB pathways and chemokines. As the name suggests, the protein stimulates TNF but also IL-1β expression, as well as the chemokines Cc12, Cc17, Cc120, Cxc11, Cxc15, and IL-8 [109, 110]. Since all of these signaling molecules are associated with the activation of inflammatory responses and, ultimately, with cancer progression, the authors have decided to classify Tipα as a carcinogenic factor.

2.2.2 Binding and transport proteins

HP1286 is the most relevant example of H. pylori secreted protein under the group of binding or transport proteins. This protein has been suggested to play an important role in bacterial colonization and persistence in the stomach, as it was observed to be overexpressed under acidic conditions together with other virulence factors. Besides the presence of a secretion signal at the N-terminal, several independent studies have found the protein in culture supernatants [95, 99, 111, 112]. Crystal structure analysis led to the placement of HP1286 in the protein binding family of lipocalins due to the presence of a cavity formed by an eight-stranded β-barrel [113]. The cavity acts as a transporter of the bacterial protein ligand. Lipocalins belong to the YceI-like family of proteins, which all contain an internal cavity with the function of binding and transport of amphiphilic molecules. However, the 3D structure of HP1286 suggests a divergence from other members of the family, principally because of a unique binding specificity and the presence of a signal peptide [113]. According to structure features, HP1286 is thought to have a role in sequestering fatty acids or amides from the environment. The protein could either function to supply the bacterium with fatty acids essential for its metabolism or to protect H. pylori from the detergent-like antimicrobial activity of fatty acids present in the gastric mucosa [113]. One study observed a strong upregulation of HP1286 expression in a UreA negative H. pylori strain, a mutant unable to transport urea inside the cell [114]. This observation could further indicate a possible link between HP1286 expression and survival/virulence of H. pylori. Also, recombinant HP1286 was shown to induce apoptosis in a gastric epithelial AGS cell line, contributing to the pathological outcome of the infection by disrupting the balance between the rate of new cell production and cell loss [111].

The CeuE1 and CeuE2 proteins are annotated as “Iron (III) ABC transported periplasmic iron-binding proteins” [74]. CeuE1 is not secreted to the extracellular space remaining localized to the periplasm where it transports the metal iron to the ABS transporter, FecD. FecD is formed by the pair of proteins HP0888/HP0889, where HP0888 is extracellularly
secreted and shows a highly immunogenic profile [115]. Another secreted protein involved in metal homeostasis in *H. pylori* is CnzB (HP0970), part of a cluster that forms a cadmium, zinc and nickel (cznABC) metal export pump. CznABC is not only required for bacterial resistance to cadmium, zinc, and nickel, but has also been reported to play a role in urease modulation and gastric colonization [116].

### 2.2.3 Enzymes

Among the *H. pylori* secreted proteins with enzymatic activity, some are involved in antioxidant systems (e.g., the catalase, KatA/HP0785). This protein contributes to ROS detoxification by catalyzing the conversion of hydrogen peroxide to molecular oxygen and water, thus, protecting the bacteria against oxidation species [13]. Furthermore, a recent study has observed a strong correlation between serum KatA and gastric cancer risk, suggesting the use of KatA as a novel biomarker for gastric cancer screening [117]. The high-temperature requirement A (HtrA) protein is upregulated under acidic conditions and, because of its role as a protein quality control chaperone and a trypsin-like serine protease, is grouped with proteins with proteolytic activity [74]. The effector mechanism of HtrA contributes to the disruption of the cell adhesion junctions that make up the gastric epithelium, through the cleavage of the cell adhesion protein E-cadherin, and seems to be conserved among Gram-negative bacteria. Furthermore, HtrA has been proposed as a candidate for novel therapeutic approaches since the silencing of the *htrA* gene results in the reduction of E-cadherin proteolysis and further *H. pylori* intercellular penetration [118]. The γ-glutamyl transpeptidase protein (GGT/HP1118) has an enzymatic function, converting glutamine into glutamate and ammonia, as well as catalyzing the breakdown of glutathione into glutamate and cysteinylglycine through hydrolysis [119]. These observations suggest that the physiological role of GGT is to provide the bacterial cells the possibility to use extracellular glutamine and glutathione as sources of glutamate [120]. Also, GGT-produced ammonia can be used by *H. pylori* as a source of nitrogen for the cells and contribute to pH buffering to resist the acidic environment. The uptake systems of glutamine, glutamate or nitrogen are activated at neutral (rather than acidic) environments, indicating the proximity to the gastric epithelium during the occurrence of these processes [121]. Extracellular availability of glutamine, glutathione, and ammonia, and subsequent consumption of these compounds can change the redox balance of the host cells and, thereby increasing their susceptibility to ROS [122]. Studies have reported damage effects induced by GGT in GECs, including cell-cycle arrest, apoptosis, and necrosis [122-126]. Additionally, evidence points to GGT as a possible modulator of T-cell immunity since it was shown to block T-cell proliferation through cell cycle arrest in the G1 phase by disruption of the Ras signaling pathway [127].
2.2.4 Uncharacterized secreted proteins

This group of secreted proteins is considered one of the largest, with 28 genes encoding proteins of no characterized function. Presumably, about 30–40% of those proteins are designated as hypothetical proteins since their function remains unknown [74]. For most of these proteins, their function cannot be predicted because of a lack of orthologues in other bacterial species. In some cases, the protein’s function is predicted by bioinformatics, without supporting experimental evidence.

The need for studying new virulence-associated factors resulted from the contradicting evidences between known H. pylori virulent factors and differences in the disease outcome [128, 129]. This indicates the presence of additional bacterial-derived components involved in pathogenesis. HP0305 is an uncharacterized H. pylori hypothetical secreted protein. HP0305 is highly conserved among H. pylori strains and has been reported to be overexpressed under acidic conditions, which are the conditions encountered by the bacteria in the human stomach [96]. HP0305 is strongly recognized by the sera of infected individuals, suggesting a highly antigenic profile and it has been identified as a potential biomarker for gastric cancer risk in a Chinese population [130, 131]. HP0305 homologs have different annotations among H. pylori strains. For instance, the HP0305 homolog in H. pylori strain J99 is designated as JHP0290. Additionally, the presence of this protein in outer membrane vesicles suggests that it could be transported from the bacteria into target cells through these vesicles [132]. Another protein with the identified secreted signal peptide is HP1173, previously reported to be released into the extracellular medium without homologs of clear function [95]. Two studies have reported HP1173 in their experiments, confirming this protein’s localization to the outer membrane surface of H. pylori, as well as its immunogenic profile (based on its strong recognition with murine and human sera from H. pylori-infected individuals) [133, 134].

2.2.5 Mechanisms of protein secretion

To deliver proteins to the extracellular milieu, H. pylori use several mechanistic pathways. Similar to other Gram-negative bacteria, H. pylori has to transport its polypeptides across three barriers: the inner membrane (IM), the periplasmic space (PS), and the outer membrane (OM). The so-called general secretion comprises the most common mechanisms of unfolding protein translocation across the IM [135, 136]. Proteins containing an amino-terminal signal sequence, which is further cleaved by a signal peptidase upon release to the PS, are translocated via the Sec system. According to genomic studies, H. pylori is predicted to synthesize proteins that can cross the IM in a Sec-dependent manner [63]. Once in the PS, the proteins can be exported across the OM to the extracellular space by multiple mechanisms. The major H. pylori toxin VacA is delivered through an autotransporter composed of three domains: an amino-terminal sequence for sec-dependent transport through IM; the domain to be secreted, called the
passenger; and a carboxyl-terminal that allows the OM crossing of the passenger (β domain) [137]. One of the well-established mechanisms for protein release in *H. pylori* is the type IV secretion system. The latter is characterized by the direct translocation of proteins over the IM and OM, straight into the extracellular space or inside the recipient eukaryotic cells, similar to the delivery of the highly immunogenic virulence factor, CagA [138]. The alternative pathway of altruistic autolysis is used by some Gram-negative bacteria and is based on the principle that autolysis of some bacteria can benefit the remaining viable population. Some studies have identified this mechanism as a possible method for delivery of *H. pylori* proteins, such as urease, heat shock proteins, and HP-NAP [139, 140]. Additionally, outer membrane vesicles (OMVs) have been suggested to play a role in protein transportation. OMVs are bleb-like shapes shed by Gram-negative bacteria resulting of the budding from the OM surface and holding mainly OM and periplasmic contents [141]. In *H. pylori*, these vesicles are suggested to have a crucial role in bacterial pathogenesis due to their composition. The presence of virulence-associated factors such as VacA, LPS or HP-NAP in *H. pylori* OMVs, indicate a possible function of these vesicles as vehicles for the transport of antigens from non-adherent bacteria to the gastric mucosa [142-145].

2.3 Other virulence-associated factors

*H. pylori* neutrophil-activating protein (HP-NAP) is a highly conserved protein known to induce high production of oxygen radicals in neutrophils and participate in the adhesion to endothelial cells [146, 147]. On the other hand, NAP has also been shown to contribute to *H. pylori* survival by binding to bacterial DNA and protecting it from damage by free radicals [148]. Besides the dual role in oxidative stress, NAP induces TNF, IL-6, and IL-8 from monocytes, as well as IL-8 from neutrophils [146, 149]. In this way, NAP is suggested to function as a trigger of inflammation and gastric mucosa damage stimulating bacterial growth through nutrient uptake released from damaged tissue. *H. pylori* heat shock protein 60 (Hsp60) is expressed under low pH conditions and induces IL-8 from monocytes through NF-κB via the TLR2 pathways [150]. Furthermore, the presence of antibodies against Hsp60 in *H. pylori*-infected individuals associated this protein with gastric disease development [151]. Duodenal ulcer promoting gene (dupA) is a virulence factor that has been shown to be associated with peptic ulcer and gastric cancer development [152, 153]. Bacterial peptidoglycan, delivered into host cells through a cag-secreted system, is sensed by host intracellular pattern recognition molecules, leading to the activation of pro-inflammatory responses and proliferative signaling pathways [154, 155]. Contrary to other Gram-negative bacteria, the *H. pylori* LPS show very low endotoxin activity, allowing the bacteria to persist and establish a chronic colonization rather than trigger a systemic inflammatory
response leading to septic shock [156]. Nevertheless, the action of this molecule should not be underestimated since LPS exhibits both pro- and anti-apoptotic activity in GECs and *H. pylori* has been reported to activate monocytes through a LPS-dependent manner [156-158]. As a result of bacterial phagocytosis, LPS is degraded and releases components such as lipid A, which can contain some immunological activity and, thus, induce gastric damage in case of long-term infection [159, 160].

![Diagram](image)

**Figure 1.** Schematic summary of *H. pylori* major virulence factors and some of its functions on host gastric mucosa and epithelium.

2.4 Impact of host factors in *H. pylori* pathogenesis

For some time, the contribution of host genotypes to the development of *H. pylori*-associated diseases was ignored. Host responses, including gastric inflammation and reduction in acid secretion, are determinants of the final disease outcome [161]. Studies have reported the importance of polymorphisms in the expression of proteins within *H. pylori*-infected individuals. For instance, within *H. pylori*-infected individuals, high-expression of pro-inflammatory cytokine IL-1β polymorphisms are associated with a significantly increased risk of hypochlorhydria, gastric atrophy, and gastric adenocarcinoma, compared to individuals that limit IL-1β expression [162]. Polymorphisms promoting the increased expression of
the acid-suppressive pro-inflammatory cytokine TNF have also been reported to be linked with an increased risk of gastric disorders [163, 164]. The risk of developing distal gastric cancer have been associated with low-expression polymorphisms of the anti-inflammatory cytokine IL-10, which led the authors to conclude that a higher number of genetic polymorphisms of pro-inflammatory proteins is related to higher risk of developing cancer [165]. Assessing the genotypic information of infected individuals could constitute an essential tool for patient-targeted treatment in order to prevent the development of gastric malignancies.

2.5 Influence of environmental factors

The development of *H. pylori*-associated diseases is influenced by bacteria strain-specific features, the host genotype, and the environment. High dietary salt intake has received major attention in respect to increased risk of gastric cancer development. Case-control studies of Japanese and South Korea populations revealed that *H. pylori*-infected subjects following a high-salt diet showed an increased risk of gastric cancer compared to infected individuals who consumed lower levels of salt [166, 167]. Other studies using *H. pylori*-infected Mongolian gerbils and mice have also reported a positive correlation between high dietary salt intake and increased risk of gastric malignancies development [168-170]. However, the mechanisms involved in the increased risk of gastric cancer by high salt intake are not yet understood. Possible explanations highlight the direct effects of the salt on the gastric epithelium, possibly allowing the entry of carcinogens into the gastric tissue or lowering the threshold for malignant transformation [75]. Recent observations suggest a possible modulation of the expression of the *H. pylori* cagA gene by high concentrations of salt [171]. Other external factors have also been correlated with *H. pylori* disease development. A few studies have reported a possible beneficial effect of helminth infection on the reduction of *H. pylori*-induced gastritis [172, 173]. A population control based study in Sweden suggested a link between high intake of dietary vitamin C and β-carotene and a lower risk of developing gastric cancer in *H. pylori*-infected subjects [174]. Nevertheless, the protective effect of food antioxidants against *H. pylori* threat of disease development among infected individuals has yet to be established. Additionally, a possible association between *H. pylori* infection and cigarette smoking has been reported for certain populations [175].
Chapter 3 Host immune responses to *H. pylori*

During thousands of years of interaction with its host, *H. pylori* has evolved strategies to modulate the human immune system for its benefit and survival in the gastric mucosa.

3.1 Human stomach

The human stomach operates as a transitional storage space, mechanically and chemically digesting food before it passes to the intestine. Furthermore, the stomach constitutes an important physical barrier against orally ingested microorganisms. Gastric juice, a fluid characterized by the combination of gastric hydrochloric acid (HCl) and proteolytic enzymes (pepsins), is the main contributor to this barrier function [176]. Gastric acidity is preserved among vertebrates and individuals with gastric acid secretion impairment (hypochlorhydria [pH 4–7] or achlorhydria [pH 7]) show an increased susceptibility towards infection [176, 177]. The human stomach can be divided into three histologically distinct parts (Fig. 2A): cardia, the cardiac opening connecting the stomach and the esophagus; the fundus/corpus, the largest and central part (about 80%); and the antrum, the lower funnel-shaped part of the stomach. The antrum region is often referred to as the pylorus or pyloric antrum since it comprises the pyloric sphincter muscle, a narrowing that joins the stomach to the duodenum. As the name suggests, *H. pylori* are mainly found in the antrum/pylorus region of the stomach.

3.2 Gastric mucosa

The gastric mucosa is the outer tissue lining the stomach lumen, which is formed by a single layer of columnar epithelial cells. Different regions of the stomach are characterized by specific types of epithelial cells. For instance, the fundus/corpus area is composed of acid-secreting parietal cells, mucous neck cells, and pepsinogen-secreting zymogenic cells, while the antrum contains gastrin-secreting cells and gland cells [178]. These cell lineages are derived from precursors of multipotent gastric stem cells, and their function depends on the respective secretory product and site of migration within the mucosal epithelium. The latter is composed of different cell types organized by the structure into gland-like invaginations called oxyntic glands in the region of the fundus/corpus or pyloric glands in the antrum or respective
gastric pits [179]. The surface of these pits contains mucus-producing cells that cover the gastric mucosa with a viscous mucus layer. The thick mucus is divided into an outer and inner layer, where the former is looser and easily removed, and the latter is tightly connected to the underlying epithelium [180].

A pH gradient is formed along the mucosa, ranging from 1–2 in the lumen to pH 7 in the gastric epithelium. Together, the epithelial cell and mucus layers constitute a physical barrier that separates and protects the underlying epithelium from the low pH of the gastric lumen and the microbiological content of the lamina propria.

Figure 2. Illustration of the human stomach (A) and histology of a pyloric gland from the antrum region of the stomach (B).

3.3 Innate Immunity

The constant exposure of the human body to microorganisms led to the development of a specialized defense system. Many microbes are successful in breaking through these physical (skin, gastric mucosa, and epithelial cell layer) and chemical barriers (pH and antimicrobial compounds) of the body. Consequently, the immune system will be alerted of foreign presence and generate a response to effectively eliminate the invading microbes. In mammals, the immune system is characterized by the innate and adaptive responses. The innate immunity comprises cellular and biochemical
mechanisms that are positioned to respond quickly, even before an infection takes place [181, 182]. Activation of innate responses occurs upon recognition of microbes through pattern recognition receptors (PRRs) or detection of products derived from injured cells [183]. Besides the physical and chemical barriers, innate immunity involves multiple cell types, including granulocytes (e.g., neutrophils) and antigen-presenting cells (macrophages and dendritic cells), as well as several proteins that function to mediate the inflammation (e.g., blood proteins) or to regulate the activity of the innate immune cells (e.g., the cytokines).

3.3.1 H. pylori recognition by PRRs

The PRRs recognize conserved pathogen-associated patterns (PAMPs), which are specific structures common to groups of related microorganisms, such as the LPS, peptidoglycan or foreign DNA [184, 185]. These receptors are expressed by innate immune cells and make up a response that acts in a generic and broad-range manner. Upon PAMPs recognition, PRRs induce extracellular and intracellular signaling pathway cascades, including the activation of transcription factors such NF-κB and activator protein 1 (AP-1), further triggering inflammatory responses with the production of pro-inflammatory cytokines and chemokines such TNF, IL-1β or IL-8 [186, 187].

According to protein domain homology, the majority of PRRs can be categorized in one of the following five families of receptors: Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), or by cytoplasmic PRRs, NOD-like receptors (NLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and AIM2-like receptors (ALRs) [188]. H. pylori can be recognized by membrane-bound PRRs, TLRs, and CLRs, or by cytoplasmic PRRs, NLRs, and RLRs [186]. During infection, the H. pylori PAMPs, LPS and unmethylated CpG motifs, are identified by TLR4/TLR2 and TLR9 respectively. Through the modulation of its surface molecules, the bacterium can avoid detection by TLRs. For instance, H. pylori express variable O-antigens, which are units of the LPS that are recognized as “self” due to their carbohydrate composition, which is biochemically related to the human blood group antigens [189]. Furthermore, modifications of the lipid A portion of the LPS leads to alterations in the net charge of the microbial surface, thereby blocking antimicrobial molecules from binding to this structure (which is usually negatively charged) [190]. The intracellular receptors NOD1 and NOD2 are the best studied NLRs during H. pylori infection, and they recognize peptidoglycan-derived peptides such as γ-D-glutamyl-mesodiaminopimelic acid and muramyl dipeptide, respectively [186]. Polymorphisms within these receptors have been associated with an increased rate of gastric cancer incidence. The RLRs and CLRs subfamilies are reported to sense RNA from H. pylori [191] and to bind carbohydrates (mannose, fucose, and glucan) present on the bacterial surface, respectively. DC-specific ICAM3-grabbing non-integrin (DC-SIGN) is a CLR expressed
in dendritic cells (DCs) and macrophages. *H. pylori* contain fucosylated DC-SIGN ligands that have been shown to break the signaling complex downstream of DC-SIGN, resulting in a reduction of pro-inflammatory cytokine production [192]. The evasion from TLRs recognition and the inhibition of DC-SIGN-mediated signaling are adaptations developed by *H. pylori* that contribute to its survival and help to establish persistent infection.

### 3.3.2 Other host cell receptors of *H. pylori*

In addition to PRRs, *H. pylori* interact with host cells through many other cell surface protein receptors. Bacterial factors can bind to different receptors depending on the cell type or even within the same cell. For instance, the bacterium exotoxin VacA targets several receptors on the surface of epithelial cells, such as receptor protein tyrosine phosphatases RPTP-β, RPTP-α, epidermal growth factor (EGF) or fibronectin [193-196]. On the other hand, VacA has been reported to enter into activated cells by binding to the surface CD18 receptor, which is also referred to as integrin β2 [197]. For a successful translocation of the virulence-associated factor CagA into the host cells, several proteins of the T4SS complex interact with integrin α5β1 at the surface of GECs [198, 199]. Furthermore, the secreted protein TipA binds to and further translocated into GECs through a surface nucleolin; TieA secreted protein has been reported to exert pro-inflammatory and pro-apoptotic effects on various cell types via TNF receptor 1 (TNFR1), and HP-NAP is thought to interact with neutrophils by a G protein-coupled receptor (GPCR) [147, 200-202]. According to present literature, there are about 25 host protein receptors interacting with 17 known *H. pylori* bacterial factors and a list of at least 19 other receptors with unknown correspondent bacterial ligands [198]. These facts contribute to the insight that the crosstalk between host and pathogen reflects a complex network of cellular and signaling processes that play an essential role in bacterial pathogenesis and ultimately in disease development.

### 3.4 Macrophages

Mononuclear phagocytes consist of a group of cells that originate from a common precursor in the bone marrow. These cells have specialized phagocytic functions and play pivotal roles in innate and adaptive responses against pathogens. Once that mononuclear phagocytes go in the peripheral blood, they are completely undifferentiated and are called monocytes, which in turn mature into macrophages when they enter tissues [203]. Besides the ingestion and elimination of microbes, through proteolytic digestion or production of reactive oxygen and nitrogen species, macrophages also detect and engulf apoptotic and dead host cells. In this way, after infection, the tissue will be clean of dead cell components and leftover residues that otherwise could trigger inflammatory responses. Macrophages can also act
as antigen-presenting cells (APCs), that activate T-lymphocytes as a result of antigen display [181]. Furthermore, activated macrophages modulate the immune responses through the release of cytokines that regulate the manner in which other cell types act towards targeted stimuli.

3.4.1 Macrophages responses to H. pylori

Macrophages are an important line of defense against H. pylori infection. Their action can be induced by either bacterium-derived products or signals from GECs arising from the direct contact of the bacterium with the gastric mucosal surface. Macrophages coordinate the immune response against H. pylori infection, possibly by activating adaptive immunity through the production of IL-12 [204]. The production of IL-12 in the gastric mucosa has been associated with the presence of peptic ulcers in individuals infected with H. pylori CagA+ strains [205]. The inflammatory response activated upon bacterial infection can be amplified by the production of macrophage cytokines, such as IL-1, TNF and IL-6 [206]. The effector functions of macrophages also play a role in H. pylori infection clearance. One such mechanism is through the generation of nitric oxide (NO) from the enzyme inducible NO synthase (iNOS) in macrophages. In vitro studies revealed that iNOS expression is upregulated in the presence of H. pylori [207].

Furthermore, bacteria can be eliminated by macrophages via the antimicrobial effect of NO. However, the efficacy of this pathway is insufficient to eradicate the bacteria. H. pylori use the enzyme arginase to compete with macrophages for the iNOS substrate, L-arginine, leading to a reduction of NO production and further enhancement of bacteria survival [208]. Arginase II is upregulated in macrophages upon contact with bacterial components resulting in the production of L-ornithine, in addition to urea [209]. Despite the depletion of substrate availability for iNOS, the arginase II enzyme has been reported to have a role in inducing macrophage apoptosis via the metabolism of L-ornithine into polyamines [209]. One of the polyamines produced is spermidine that, upon its back-conversion from spermine to spermidine by the enzyme spermine oxidase (SMO), releases hydrogen peroxide (H₂O₂) [210]. SMO upregulation by H. pylori leads to the activation of mitochondrial membrane depolarization and the caspase-3 pathway, which is associated with the intrinsic apoptosis pathways in macrophages [210].
*H. pylori* use several mechanisms to induce apoptosis in macrophages. The bacterium activates the ERK1/2 signaling pathway followed by the formation and activation of the AP-1 complex, which in turn induces the expression and further nuclear translocation of a c-Myc gene that leads to increased expression of ornithine decarboxylase (ODC) and further macrophage apoptosis [211, 212]. *H. pylori*-derived products have also been reported to play a role in macrophage apoptosis. Bacterial protein TNFR-1 interacting endonuclease A (TieA/HP986) induces apoptosis in human macrophages through both TNF- receptor 1 (TNFR1) and Fas-dependent pathways, concurrent with the induction of TNF and IL-8 via NF-κB activation [202]. Later, another study confirmed the pro-apoptotic and pro-inflammatory profile of TieA in murine macrophages [213]. The importance of TNF signaling in the long-term survival of macrophages has been previously highlighted [214, 215]. *H. pylori* contain proteins, such as CtkA/JHP0940, with the ability to induce macrophage apoptosis concurrent with the release of TNF and other pro-inflammatory cytokines and chemokines, such as IL-1β and IL-8 [216, 217]. Furthermore, the VacA protein also induces monocytes apoptosis through the activation of NF-κB followed by the production of pro-inflammatory cytokines such as TNF and IL-1β, and further induction of ROS and NO, ultimately leading to apoptosis [218].
Besides demonstrating the action of _H. pylori_ antigens on macrophages, these studies also present the critical role of pro-inflammatory signaling in the regulation of events during immune responses.

Also, _H. pylori_ can escape being phagocytosed by macrophages. The engulfed bacteria can prevent the fusion of macrophage phagosomes with lysosomes, essential for bacteria killing, via the production of a functional VacA protein [219]. Another study has demonstrated the ability of _H. pylori_ to delay actin polymerization, preventing phagosome formation. Consequently, the constant clustering and fusion of phagosomes resulted in the formation of “megasomes” containing several bacteria, causing resistance to killing by macrophages [220].

**Figure 4.** The cell on the left represents a summary of the pro-apoptotic pathways induced by _H. pylori_ secreted factors in macrophages. On the right is depicted the mechanisms of _H. pylori_ evasion from macrophage phagocytosis.
3.5 Interaction of *H. pylori* with other host cells

### 3.5.1 Gastric epithelial cell responses to *H. pylori*

The normal function of the gastrointestinal mucosa relies on the maintenance of gastric epithelial cell homeostasis. This implies that rates of cell proliferation should be accompanied and compensated by similar rates of cell loss. *H. pylori* infection is associated with the disruption of this cellular balance that may further lead to disease development [221]. Several studies have shown the induction of gastric epithelial cell apoptosis by *H. pylori* [222-225]. However, the level of cell apoptosis may vary among infected individuals due to *H. pylori* strain-specific variations. On the contrary, *H. pylori* are also known to exert an anti-apoptotic effect on cells from the gastric epithelia [226]. This effect can be performed through activation of anti-apoptotic pathways or the expression of anti-apoptotic genes, such as induced myeloid leukemia cell differentiation protein (Mcl-1) and cellular inhibitor of apoptosis protein 2 (cIAP-2) in GECs [226-228]. *H. pylori* cag+ strains have been shown to stimulate the pro-apoptotic MAPK ERK1/2 signaling pathway and expression of Mcl-1 anti-apoptotic protein within gastric pits [227]. Moreover, certain bacterium-virulence associated factors are known to regulate gastric epithelial cell proliferation responses. For example, co-expression of CagA and *H. pylori* heat shock protein B (HspB) induce gastric epithelial cell proliferation independently of bacterial infection [229]. *H. pylori* LPS and SlyD protein have also been shown to induce proliferation and anti-apoptotic signaling pathways in gastric epithelial cell lines [156, 230]. Furthermore, *H. pylori* infection alters cell cycle progression in GECs. The arrest of the cell cycle at G1 phase has been reported during *H. pylori* infection [229]. However, some authors suggest that this effect appears to be *H. pylori* strain specific and dependent on the type of cell and bacterial multiplicity of infection (MOI) [231, 232]. For instance, at lower MOIs the cell cycle was not arrested and instead progressed into S phase, indicating a complexity in cell cycle regulation [231]. Other studies have also observed a faster progression into cell cycle from G1 into G2-M in GECs induced by *H. pylori* factors such as co-expression of CagA and HspB proteins [229]. During infection, the bacteria activate several signaling pathways involved in cell responses regulation. MAPK, Akt, and Wnt signaling pathways are known to be activated and further participate in the regulation of *H. pylori*-induced gastric epithelial cell proliferation [154]. The transcription factor NF-κB is a key factor in the immune response against *H. pylori* and is known to be involved in the regulation of pro-inflammatory cytokines and anti-apoptotic signaling pathways during bacterial infection [233]. A good example is the interaction of TieA *H. pylori* secreted protein with GECs, which results in the stimulation of pro-inflammatory chemokine IL-8 secretion through a NF-κB-mediated mechanism [201].
3.5.2 Dendritic cells

Dendritic cells (DCs) constitute a bridge between innate and adaptive immunity. To escape the acidic environment of the gastric lumen, *H. pylori* disrupt the tight junctions of the gastric mucosal barrier. This disruption can also contribute to the increased interaction between DCs and *H. pylori* from luminal and sub-epithelial layers [234]. DCs interact with *H. pylori* by binding of glycol-conjugate carbohydrate structures to the DC-SIGN receptor [75]. *Ex vivo* studies have reported the production of IL-12 and IL-10 by DCs as a result of *H. pylori* stimulation [235]. Interestingly, IL-12 responses are reduced with the inhibition of bacterial colonization, suggesting that phagocytosis of *H. pylori* by DCs activates intracellular receptors [236].

DCs function as APCs and, after being activated, can activate T cells. The latter activation can be done through induction of Th1 or regulatory T-cell (Treg) responses [237]. Evidence suggests that the DC response against *H. pylori* might contribute to bacterial evasion from the immune system. This was supported by the activation of DCs by *H. pylori*, which resulted in an alteration of the Th17/Treg balance towards Tregs, followed by a compensation of the immune system that includes the depletion of Tregs and induction of Th17 response [238]. This imbalance and compensation can lead to chronic inflammation and cancer risk development.

3.5.3 T-cells

T cell-mediated immunity is important for the generation of a protective immune response against bacteria. The T cells of *H. pylori*-infected patients that develop malignancies have been much studied. Gastric lymphocytes from *H. pylori*-infected patients showed increased levels of IFN-γ-producing T cells, compatible with a Th1 cytokine response [239]. Furthermore, *H. pylori*-specific T-cell clones from gastric mucosa exhibit a Th1 profile in patients with peptic ulcer disease [240]. The interferon-γ (IFN-γ) produced by T cells activates macrophages in an attempt to destroy bacteria through phagocytosis processes. On the other hand, macrophages and DCs stimulated by the bacteria already present at the site of infection produce IL-12, which is important for the activation of the Th1 response [204, 241]. CD4+ helper T cells are also activated upon bacterial infection. These cells produce cytokines, such as IL-17, responsible for inducing local inflammation and increase the effector functions of neutrophils and macrophages. A defective IL-17 response in *H. pylori*-infected gastric tissues has been associated with the contribution of the chronic persistence of the bacterium [242]. Some studies have indicated a possible influence of *H. pylori*-specific Tregs in bacterial pathogenesis through the suppression of T-cell memory responses, leading to the persistence of infection [243]. Interestingly, other findings have attributed a stabilizer role to Tregs, thereby contributing to the balance between host and bacterium, by allowing the survival of *H. pylori* but also preventing the risk of strong inflammation in the host [244]. *H. pylori* strains
containing a functional VacA protein have been associated with a reduction of lymphocyte T-cell proliferation through the downregulation of the transcription factor nuclear factor of activated T cells (NFAT) or by activating MAPK signaling, which results in an actin rearrangement and further disruption of the cellular cytoskeleton [245].

3.5.4 Neutrophils

Neutrophil migration through the gastric mucosa is triggered upon *H. pylori* infection. These cells perform phagocytosis by engulfing the microbe and induce killing through oxygen-dependent and independent mechanisms [196]. The bacteria can avoid opsonization by neutrophils through low pH and mucins, that prevent antibody binding to the bacterial surface, but also by the production of catalase and superoxide dismutase that detoxify ROS [246-248]. Down-regulation of CXCR1 and CXCR2 chemokines on the surface of neutrophils by the bacterium leads to a decrease in neutrophil migration to the site of inflammation and, thus, bacterial killing, due to the reduction of receptors for the neutrophil-recruiting chemokine, IL-8 [249].

The unique outer membrane lipid composition of *H. pylori* influences the uptake of the organism by phagocytes. For instance, glucosylation of cholesterol in the outer membrane increases the ability of the bacterium to escape phagocytosis [250]. Additionally, neutrophil production of ROS can be disrupted by *H. pylori* through the suppression of the NADPH oxidase system. The large amounts of ROS produced by neutrophils containing bacteria are accumulated outside the phagosome, resulting in an inefficient assembly of NADPH oxidase [220]. Consequently, the NADPH oxidase system assembles on the cell surface of the neutrophil, releasing ROS into the extracellular space and further maximizing the inflammation [220].

3.5.5 B-cells

There are several studies indicating a possible contribution of B-cells to *H. pylori* pathogenesis. Much research has focused on the development of gastric MALT lymphoma and non-Hodgkin lymphoma of the stomach, which are malignancies composed by transformed B-cells [75, 196]. It has been reported that naïve mouse splenocytes exposed to bacteria are protected from spontaneous apoptosis and experience proliferation in response to low, but not high, multiplicity of infection and that the responding cells are derived from a B-cell population [251]. B-cells have also been shown to produce autoreactive antibodies that might be pathogenic [252].
PRESENT INVESTIGATION

Aims
The studies included in this thesis were conducted with the overall purpose of contributing to the current knowledge of host-pathogen interactions during *H. pylori* pathogenesis, with focus on host cell responses to novel *H. pylori* secreted virulence-associated factors.

Specifically, the aims of this thesis were:

- To investigate the role of *H. pylori* secreted protein JHP0290 on macrophage responses (**Paper I**)
- To characterize the human gastric epithelial cell responses against JHP0290 (**Paper II**)
- To understand the effect of *H. pylori* secreted protein HP1286 on host cell responses, with major focus on macrophages (**Paper III**)
- To study the responses of macrophages to the secreted *H. pylori* protein HP1173 (**Paper IV**)
Results and discussion

**Paper I**

*H. pylori* infection generates strong innate and adaptive immune responses that often fail to eradicate the bacterium. Activated macrophages of the sub-mucosal space play an important role in generating innate immune responses during *H. pylori* infection. However, the bacteria have developed several strategies to evade the action of these innate immune cells.

Primarily, we observed that recombinant purified JHP0290 (rJHP0290) was able to bind to multiple cell types, including macrophages, human GECs, human monocyte-derived dendritic cells (MoDC), and human neutrophils. These observations were confirmed by flow cytometry, Western blot, and immunofluorescence analysis. Considering the ability of rJHP0290 to interact with macrophages, we further investigated the type of effect exerted on these innate immune cells. Exposure to rJHP0290 induced macrophage apoptosis in a time- and dose-dependent manner. However, the same effect was not observed in GECs under similar conditions, indicating a cell-type specificity of rJHP0290. Also, rJHP0290-induced macrophage apoptosis was concomitant with the release of pro-inflammatory cytokine TNF. The latter is an acid-suppressive cytokine that is upregulated during *H. pylori* infection. The effect of JHP0290 on macrophages was further confirmed by the generation of a mutant strain lacking the *jhp0290* gene (*Δjhp0290*). The mutant strain was significantly impaired in its ability to induce macrophage apoptosis and TNF release.

We were further interested in identifying the signaling pathways involved in the regulation of rJHP0290-induced effects on macrophages. Macrophages were pre-treated with a set of chemical inhibitors followed by incubation with rJHP0290. Only the PP2 and U0126 inhibitors, which block Src family of tyrosine kinases and ERK MAPK signaling pathways respectively, produced a decrease in macrophage apoptosis and TNF release. Immunoblot analysis confirmed the activation of the ERK MAPK pathway by JHP0290 through the assessment of ERK1/2 phosphorylation levels.

*H. pylori* is known to induce the activation of NF-κB and AP-1 transcription factors that, in turn, initiate a cascade of events characteristic of the host immune response, including the regulation of several chemokines and cytokines. Using the inhibitors Wedelolactone and SR11302, that specifically block NF-κB nucleus translocation and AP-1 complex activation respectively, we observed a partial reduction of TNF release in Wedelolactone pre-treated macrophages and no effect upon inhibition of AP-1 factor. These results indicate that JHP0290-induced TNF from macrophages was a process mediated by NF-κB, together with one or more transcription molecule(s). Furthermore, we observed that rJHP0290-induced macrophage apoptosis was only partially blocked by neutralizing antibodies.
against TNF, suggesting the involvement of TNF-independent pathways, possibly via the activation of the AP-1 complex (since SR11302 significantly impaired rJHP0290-induced apoptosis).

In conclusion, this study provides evidence of the mechanistic role of *H. pylori* secreted protein JHP0290 during interaction with macrophages. By induction of macrophage apoptosis, JHP0290 might help in survival and persistence of the bacterium in the gastric mucosa.

**Paper II**

In paper I, we report that the *H. pylori* secreted protein JHP0290 binds to multiple cell types, including GECs. However, the effect of JHP0290 on this cell type was not known. GEC homeostasis relies on the maintenance of cell growth and cell loss rates. A disruption in the equilibrium between cell gain and loss is associated with disease development in the gastric mucosa.

In this project, we explored the action of JHP0290 in the modulation of gastric epithelial cell responses.

Sequence homology analysis indicated that JHP0290 is highly conserved among *H. pylori* strains. We first determined the expression of JHP0290 homologs in the whole cell lysates of various strains isolated from patients with different geographic background and infection-associated disorders. The level of expression and protein release varied among strains. Interestingly, we noted that the level of JHP0290 expression in certain strain does not correlate with the amount of protein released to the culture supernatant of the respective strain. These differences in expression can be associated with strain-specific properties or occur because of distinct environmental conditions experienced by each *H. pylori* strain at their particular site of isolation.

rJHP0290 showed both monomeric and dimeric forms, with the latter exhibiting a better binding ability to GECs. This feature was confirmed by creating a protein mutated in a cysteine at position 162 (rJHP0290 C162A) and verified that the mutant protein existed only in monomeric form. In paper I, we reported that JHP0290 was not capable of inducing apoptosis in GECs. As *H. pylori* and/or bacterium-derived molecules are known to promote proliferation of GECs, we investigated whether JHP0290 could exhibit a similar effect. MTT and BrdU proliferation assays showed that rJHP0290 induces GEC proliferation in a dose- and time-dependent manner. Furthermore, a faster progression into the cell cycle was observed in rJHP0290-treated GECs. Simultaneous treatment of GECs with rJHP0290 and an apoptosis inducer called camptothecin (CPT) revealed the anti-apoptotic activity of the protein. This effect was regulated via the inhibition of caspase 3 enzymatic activity, which is an important marker for cell death. Pre-treatment of GECs with rJHP0290 resulted in the reduction of CPT-induced caspase 3 activity.
Additionally, we were keen to investigate the signaling pathways activated and involved in the regulation of these cell responses by rJHP0290. Because MAPK pathways are important regulators of cell cycle processes during *H. pylori* infection, we analyzed the activation of ERK MAPK in rJHP0290-treated GECs and observed an activation of the signaling pathway in a time-dependent manner. GECs transfected with an NF-κB SEAP reporter and subject to treatment with various concentrations of rJHP0290 showed activation of NF-κB, suggesting a role of this transcription factor in mediating rJHP0290-induced GECs responses. Altogether, these data indicate that JHP0290 might contribute to the development of *H. pylori* infection-associated diseases through the regulation of proliferative and anti-apoptotic pathways in GECs.

**Paper III**

Previous literature has suggested a possible role for *H. pylori* secreted protein HP1286 in bacterial colonization and persistence in the stomach. This is largely because of its structural features, the fact that it is expressed under acidic conditions, and its pro-apoptotic activity in GECs [111, 113, 114]. However, the role of HP1286 during interactions with host immune cells remains unknown. Therefore, in paper III, we explored the effect of HP1286 on various cell types, with focus on its interaction with macrophages.

The HP1286 sequence is highly conserved among the tested *H. pylori* strains. We observed differences in the expression levels and the amount of protein released into the culture supernatant. The high genetic diversity of *H. pylori* is known to drive the appearance of strain-specific features that are considered to play an important part in disease development. In this case, the different environmental pressures encountered by each strain at their isolation site could be the possible cause of differential protein expression.

Flow cytometry analysis revealed the binding ability of recombinant purified HP1286 (rHP1286) to distinct cell types, including macrophages, monocytes, neutrophils, and T cells. From these observations, we presumed that the protein was recognized by receptor(s) that is/are present in various cell types. We detected differences in the rHP1286-binding efficacy to cells, suggesting a distinct level of receptor expression according to the cell type. rHP1286 induced apoptosis in macrophages in a dose- and time-dependent manner. Under similar conditions, rHP1286 failed to induce apoptosis in primary monocytes, T cells, or neutrophil-like HL 60 cells. These results indicate that rHP1286 might exert a cell type-specific effect, similar to JHP0290 protein in paper I and paper II. One explanation for this could be the involvement of different signaling pathways in the regulation of pro- and anti-apoptotic activities of these proteins.
To confirm the role of endogenous HP1286 on macrophage apoptosis, we constructed an isogenic mutant strain disrupted in the hp1286 gene (26695Δhp1286). Absence of hp1286 gene product had no impact on bacterial growth or viability when comparing 26695Δhp1286 to the wild-type strain. However, apoptosis was reduced in macrophages infected with 26695Δhp1286, confirming the involvement of endogenous HP1286. Furthermore, we also observed a decrease in apoptosis of macrophages when treated with culture supernatant (Condition medium, CM) from 26695Δhp1286.

H. pylori infection induces TNF in macrophages and TNF has been reported to induce macrophage apoptosis [202, 216]. However, rHP1286-induced macrophage apoptosis was not affected in the presence of TNF neutralizing antibodies and rHP1286 failed to induce TNF under similar conditions, which indicated that the response was mediated via TNF-independent pathways. Inhibitor studies revealed the involvement of ERK MAPK pathways in the regulation of macrophage responses to rHP1286, since apoptosis was impaired in cells treated with U0126 prior to incubation with rHP1286. The assessment of ERK phosphorylation levels in rHP1286-challenged macrophages, including detection in the cytoplasmic and nuclear fractions of the cells, further confirmed the relevance of the ERK MAPK pathway in the regulation of rHP1286-induced macrophage apoptosis. Finally, we observed that rHP1286 failed to activate ERK, p38 and JNK MAPK pathways in T cells, supporting our hypothesis that multiple mechanisms regulate these cell processes according to the cell type and activating stimuli.

Our results identified HP1286 as a new regulator of macrophage apoptosis during H. pylori infection. Through the modulation of macrophage responses, H. pylori can evade the action of these innate immune cells and persist in the gastric mucosa, increasing the risk of disrupting the host-pathogen homeostasis and, ultimately affecting the development of infection-associated diseases.

**Paper IV**

One of the earliest responses generated by the host immune system against H. pylori infection is the induction of cytokines and chemokines, such as TNF, IL-1β, and IL-8 from both GECs and immune cells [253]. These signaling molecules play an important role in mediating events during the interaction between H. pylori (or bacteria-derived factors) and host cells.

In paper IV, we aimed to characterize the role of HP1173 secreted protein during H. pylori interaction with macrophages. Previous information on HP1173 is limited, with just a few studies reporting the detection of the protein in culture medium. One study has described the strong recognition of HP1173 by murine and human sera from H. pylori-infected individuals, suggesting an antigenic profile for HP1173 [95, 133]. To start, using various H. pylori strains, we checked the expression level of HP1173 in whole cell
lysates, as well as the amount released into the culture supernatant. HP1173 was expressed by all strains tested but with differences in the amount of protein released. In the cell lysate samples, HP1173 expression was similar among the tested strains. Similar to for JHP0290 and HP1286 (paper II and III, respectively), we detected no correlation between the protein expression and amount of protein released in some bacterial strain. Recombinant purified HP1173 (rHP1173) was able to interact with differentiated THP-1 cells in a dose-dependent manner. Next, we investigated the outcome of this interaction by exposing THP-1 to various concentrations of rHP1173 and assessing the effect on cellular processes, including apoptosis and pro-inflammatory cytokine induction. THP-1 apoptosis was not detected after treatment with rHP1173. On the other hand, the protein induced the production of TNF, IL-1β, and IL-8 in THP-1 cells in a dose- and time-dependent manner. mRNA levels of TNF, IL-1β, and IL-8 were also upregulated in rHP1173-treated THP1 cells. Furthermore, an isogenic mutant disrupted in the \( hp1173 \) (26695\( \Delta hp1173 \)) gene was constructed, and growth/viability studies showed no difference between \( \Delta hp1173 \) and Wt ruling out the possibility of HP1173 playing a role in bacterial growth. Furthermore, less induction of TNF, IL-8 and IL-1β was observed in macrophages treated with CM from 26695\( \Delta hp1173 \), confirming the involvement of endogenous HP1173.

The identification of regulatory mechanisms behind the processes triggered by the bacteria during inflammation is pivotal for a better understanding of the bacterial strategies of survival in the stomach. We detected a decrease in TNF, IL-8, and IL-1β induction upon blocking of ERK and JNK MAPKs signaling pathways by U0126 and SP600125 respectively. In addition to ERK and JNK MAPK, induction of IL-1β was also partly reduced by the SB203580 inhibitor, suggesting the involvement of p38 MAPK in cytokine regulation. NF-κB activation was observed in rHP1173-challenged macrophages and pre-treatment of cells with Wedelolactone, an NF-κB inhibitor, significantly inhibited rHP1173-induced TNF, IL-8, and IL-1β release. Furthermore, blocking AP-1 complex with an SR11302 inhibitor impaired IL-8 and IL-1β induction.

In an attempt to identify the possible receptor implicated in recognition of HP1173 in macrophages, we explored the role of TLRs by pre-treating differentiated THP-1 cells with MyD88 adaptor molecule inhibitor, followed by rHP1173 incubation, and observed no difference in pro-inflammatory cytokine expression. This data suggests that TLRs requiring a MyD88 adaptor might not contribute to HP1173 detection. However, this observation does not rule out the involvement of TLRs since some TLRs can signal via other adaptors such as TRIF, TIRAP and TRAM. These findings demonstrate the pro-inflammatory activity of HP1173 in macrophages.
Future Perspectives

A common principle within the scientific community is the recognition of a never-ending project, since new questions will always arise whilst tackling an initial hypothesis. This thesis is no exception, as our studies generated new findings but also many questions, which are described in this section of the thesis.

The identification of receptor(s) involved in the recognition of JHP0290, HP1286, and HP1173 remains a major question that would be interesting to investigate further. Our papers demonstrate the interaction of these proteins with different cell types, which suggests the interaction with a general receptor present in several cells, including macrophages, monocytes, dendritic cells, and neutrophils. In papers I and IV, we accessed the role of TLRs dependent on MyD88 adaptor molecule, in the recognition of JHP0290 and HP1173. The absence of an observed effect led us to the assumption that these proteins might not be detected by TLRs that require MyD88 adaptor molecule signaling. However, the current observation dose not rule out the involvement of TLRs since other TLR adaptors such as TRIF, TRAM and TIRAP could be required. There are other known receptors such as nucleolin, and TNFR1, which can also recognize bacterial factors [200, 202]. Standardized co-immunoprecipitation and pull-down studies are possible approaches that we could follow to identify physical protein-protein interactions. Furthermore, determining the protein structures of JHP0290 and HP1173 would be an important contribution to the better understanding of their functions, as well as the possibility to place them under a certain group of proteins.

In our studies, we observed that JHP0290, HP1286, and HP1173 were conserved among the tested *H. pylori* strains, with differences in expression in whole cell lysates or/and distinct amounts of protein release into the cell medium. To explore the reason for this observation, we could proceed by studying the expression of these proteins in an increased number of *H. pylori* strains, including strains isolated from individuals with different gastric-associated diseases or geographic backgrounds, which could help to build a pattern of expression. Also, the screening for JHP0290, HP1286, and HP1173 expression and release in a larger and more diverse group of *H. pylori* strains might reveal a correlation between the expression of these proteins and the virulence nature of the strain. Studying the mechanisms that regulate expression and release of these proteins would be an important step in further understanding their possible role in bacterial pathogenesis.

As mentioned above, the proteins tested showed the ability to bind to different cell types. An overall conclusion that we can take from our four studies is that these proteins exert a cell type-specific effect. Therefore, as a continuation of papers I and II, it would be interesting to investigate the responses of T cells, dendritic cells, and neutrophils to JHP0290, as well as to identify the pathways involved. In paper I, we detected JHP0290 binding to macrophages, but further experiments in GECs (unpublished data)
suggested translocation of JHP0290 inside the cells. To further investigate this observation, we could follow the protein translocation overtime using immunofluorescence techniques, thereby identifying the protein’s localization inside the cell. The identification of the mechanistic signaling activated during the process of translocation, would provide essential knowledge on JHP0290 intracellular functions. Preliminary data also indicated a possible effect of JHP0290 on GECs migration.

In paper III, we showed that HP1286 was able to interact with multiple cell types. Also, we showed that HP1286 did not induce apoptosis in T cells and neutrophils, even though it was capable of binding to these cells. In another study, HP1286 has also been reported to induce apoptosis in GECs. Thus, it would be interesting to investigate the effect of HP1286 on other cell types, as well as the signaling pathways mediating these responses.

In paper IV, a microarray analysis could provide a better cytokine/chemokine profile in rHP1173-challenged macrophages. Furthermore, binding and responses of other possible targets cells such as GECs, dendritic cells, T-cells, neutrophils could be explored. Our preliminary data has indicated that rHP1173 binds to GECs and induces IL-8 (unpublished observations).

In vivo experiments are undoubtedly crucial to gain a better insight into the role of JHP0290, HP1286, and HP1173 during H. pylori-infection-associated disease development. The virulence-associated effects of JHP0290, HP1286, and HP1173 could be validated in an animal model using the mutant strains.
ACKNOWLEDGEMENTS

This work would have never been possible without the help, patience and support of others, whom I am truly thankful. In particular, I would like to express my gratitude:

To my supervisor Sushil Pathak, for giving me the opportunity to do a PhD and trusting me as your first student. I am thankful for your hard-working, scientific guidance and perseverance during these years. The knowledge I gained from working with you, literally side by side, in the lab is invaluable and for that I am sincerely grateful.

To my co-supervisor, Ann-Beth Jonsson, for the advice, support and consideration when sharing new opportunities. Thank you for the encouragement to continue on a scientific path.

To all former members of Ann-Beth Jonsson’s group, Hong Sjölinder’s group and Helena Aro’s group many thanks for valuable discussions, support and guidance but mostly for the great moments shared during “labbing”, teaching, “lunching” and “after-working”. I am honoured and very glad to have had the opportunity to meet and work with you.

To all group leaders and members of the F5 corridor for always being helpful and friendly even on Monday mornings. I very much appreciated all your scientific insights, discussions and guidance but also your good company during lunch time, fika or just “kitchen break”. Thank you for contributing to a great and enjoyable working environment.

To the present members of Ann-Beth Jonsson’s group, whom I not only share the lab and office facilities but also good friendship. Sara S, for being an awesome company, foodie, TV-series addict and talented scientist. We may not come from the same neighbourhood but we definitely make an awesome teaching team even under high levels of “stressing mode”!! Gabby, for being the free-spirit of the lab and always spread good vibes (and chocolate, and sweets, and cheese!!). Thank you for contagious me with your enthusiasm for outdoor running! Hanna, for all the valuable discussions and for sharing the torments of working with H. pylori, AGS cells, macrophages and dealing with the nitrogen gas. Thank you for your friendship and for introducing me to the amazing Ethiopian food and culture! Fanglei and Sara Y thank you for all the great discussions and the good environment in the office! Past members of the associated groups have left an important mark in my PhD journey. Sunil, thank you for sharing your
knowledge, scientific and life insights. You are a true mentor and very much missed around here. Nele, your perseverance and hard-working are inspiring. Thank you for all good talks and moments! Linda, three years have passed since your defence and I very much miss those countless great/crazy office moments!! I am so happy that our friendship still growth strong outside academia world!! Pilar, your kindness, good company and delicious desserts are very much missed in F561 lab! Xiao, you are a strong, talented woman whom I very much respect. Thank you for a good friendship!

To all former and present awesome members of the pub-team and PhD-board. Mattias, the person who started with me in the same office back in 2011 and ended up becoming a dear and close friend. Thank you for the countless adventures of recording dissertation movies, pub nights, lost keys, getting stuck in elevators, beer tastings! The “chitchat group” would never be complete without Alice, thank you for being a good friend and for introducing me to your lovely family, and Fredrik, for all the great talks about science, movies, music, TV-series, travels, beer! But most importantly, thank you for telling me the truth about my height. Since then I live in a much bigger world! Andreas, for always speaking your mind even if it means being politically incorrect. Thank you for all the talks and moments spent together my friend! Mikkis, for being an awesome “MOSS-partner” and making me realize that “people will start look at you differently”! Thank you for all the great pub nights! Steffie, for being a caring friend, a natural bartender, a fantastic cook and baker! Carlota and Jutta, your strong personalities and passion for science are damn inspiring! Thank you for all the good talks and moments but mostly to all the support given when I most needed! Lexi and Albin for being great “MOSSers”, colleagues and “pubbers”!! Einar, for great scientific discussions and intense life insights. Wearing black trousers was never the same! António Martins and Ana Rita, for the great Portuguese companionship at MBW.

To the administration section at MBW: Bea, for being caring, competent, helpful and hard-working. You are the best technical professional that an academic department could have. Jerker, for being competent, a good company and creative bartender! Gelana and Eva Norman, for always being kind and helpful with administrative issues.

To my Lappis/Stockholm family, Alvaro, Hanna, Gonçalo, Victoria, Francesca, Shahul, Davide, Justyna, for all the memorable conversations, AW’s, brunches, dinners, fikas, celebrations, holidays. I am thankful for your friendship, all your love and care throughout these years.

My dear and most amazing friends in Portugal. Not even a distance of 3000 km is sufficient to break us. Your support, love, laughs, fights, weirdness and greatness no matter what, were and are extremely valuable. Obrigada, Ana, Bi, Piro, Luís, Tê, Pipo, JC, Carol, JB, Diogo, Paulo, André, Zé.
To my Swedish family Anette, Jesus, Tommy, Sara, Mona, for welcoming with wide open arms and for always being caring and loving! I am very grateful to you all.

Manuel, my partner in crime in this world of fools. There are not enough words to describe how incredibly thankful I am for everything you have done for me. Du är “Fabuloso”!

Finally, to my dear and beloved family. Mãe, Paulo e Avô, for the unconditional love, support and wisdom. For accepting my choices of “exploring” the world and finding my dreams while leaving my adored country behind. Mami, obrigada por estares sempre presente independentemente de para nós isso significar telefonar todos os dias. Obrigada por sempre me apoiare e acreditares nas minhas capacidades. Obrigada pelos valores e exemplos de integridade, dedicação, perseverança e força. “Colhemos muitas pedras ao longo do caminho...mas no fim construiremos um castelo...”. Eles estarão sempre comigo. Assim como tu. Obrigada.
REFERENCES

47. Tongtawee, T., et al., Improved Helicobacter pylori Eradication Rate of Tailored Triple Therapy by Adding Lactobacillus delbrueckii and Streptococcus thermophilus in Northeast Region of Thailand: A


203. Kenneth Murphy, P.T., Mark Walport, Janeway's Immunobiology. 7th ed. 2008: Garland Science, Taylor and Francis Group, LLC.


213. Ansari, S.A., et al., Helicobacter pylori protein HP0986 (TieA) interacts with mouse TNFRI and triggers proinflammatory and


