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Estudio de los factores angiogénicos ANGPT1, ANGPT2, TNF-α, VEGFA y miR-203a en la carcinogénesis gástrica inducida por la infección de *Helicobacter pylori in vitro* e *in vivo*

Study of proangiogenic factors ANGPT1, ANGPT2, TNF-α, VEGFA and miR-203a in gastric carcinogenesis induced by *Helicobacter pylori in vitro* and *in vivo*

Tesis sometida a la consideración de la Comisión del Programa de Doctorado en Ciencias para optar al grado y título de Doctorado Académico en Ciencias

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RESUMEN

El adenocarcinoma gástrico es el quinto tipo de cáncer más común y el cuarto en mortalidad a nivel mundial. El factor de riesgo más importante es la infección crónica con la bacteria *Helicobacter pylori*, que coloniza el estómago e induce una respuesta inflamatoria que puede desembocar en varias patologías gástricas incluido el cáncer. La infección puede alterar la vasculatura gástrica mediante la desregulación de factores angiogénicos y microARNs. Sin embargo, los mecanismos por los que la bacteria induce una neovascularización temprana en la mucosa gástrica y cómo influye esto sobre los eventos precancerosos que preceden el cáncer, no está completamente comprendido.

En esta tesis se presentan tres distintos estudios que fueron realizados para investigar la expresión de varios factores proangiogénicos y proinflamatorios como VEGFA, TNF-A, ANGPT1, ANGPT2, TEK, y microRNAs miR-203a, miR-200a y miR-135. Para eso, se usaron líneas celulares de cáncer gástrico (AGS, MKN28 y MKN45) co-cultivadas con *H. pylori*, un modelo murino y se realizó el genotificado de SNPs de genes proinflamatorios (*TNF-A, IL-6, IL-8* e *IFNGR1*) en ADN de muestras de sangre humana.

Los resultados sugieren que *H. pylori* podría contribuir al proceso de carcinogénesis por la desregulación de miR-203a, que a su vez promueve la angiogénesis en la mucosa gástrica al incrementar la expresión de ANGPT2 en el modelo in vitro. En el epitelio gástrico murino, la bacteria indujo la expresión de Angpt2, *TNF-A* y Vegf-A. Además se encontró una asociación entre el polimorfismo TNF-A-857*T y el riesgo de padecer cáncer gástrico en Costa Rica. Este polimorfismo produce una mayor expresión del gen TNF-A, cuya proteína TNF-α, es un activador del endotelio y encargado de la secreción de ANGPT2.

ABSTRACT

Gastric adenocarcinoma is the fifth most common type of cancer worldwide, and the fourth cause of death by cancer in both sexes. The most important risk factor worldwide is chronic infection with *Helicobacter pylori*, which colonizes the stomach and induces an inflammatory response that can lead to several gastric pathologies, including cancer. The infection can alter the gastric vasculature by the deregulation of angiogenic factors and microRNAs. However, the molecules and mechanisms by which the bacterium induces early neovascularization in gastric mucosa, and how this influences the precancerous series of events that precede GC is not completely understood.

Three distinct studies were performed in order investigate the expression several pro-angiogenic and inflammatory factors as VEGFA, *TNF-A*, ANGPT1, ANGPT2, TEK, and microRNAs miR-203a, miR-200a, and miR-135, using gastric cancer cell lines (AGS, MKN28, MKN45) co-cultured with *H. pylori*, a mouse model and the genotyping of SNP in proinflammatory genes (*TNF-A, IL-6, IL-8* and *IFNGR1*) in DNA from human blood samples.

The results suggest that *H. pylori* could contribute to the process of carcinogenesis by downregulating miR-203a, which further promotes angiogenesis in gastric mucosa by increasing ANGPT2 expression in the *in vitro* model. In the murine gastric epithelium, the bacterium induced the expression of Angpt2, *Tnf-A* and Vegf-A. An association was found between *TNF*-A-857*T SNP and the risk developing of gastric cancer in Costa Rica. That polymorphism produces a higher expression of TNF-A gene, which protein TNF-α, is an activator of the endothelium and related with the secretion of ANGPT2.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

ANGPT1/Angpt1	Angiopoietin 1 (human/mouse)
ANGPT2/Angpt1	Angiopoietin 2 (human/mouse)
BabA	Blood group antigen-binding adhesin A
c-Abl (ABL1)	Cellular-Abelson murine leukemia virus oncogene
c-Src	SRC proto-oncogene, non-receptor tyrosine kinase
CagA	Cytotoxin-associated gene A
CTNN1B	Catenin beta 1
CAM	Chick Chorioallantoic Membrane
CDH1	E-cadherin gene
COX-2	Cyclo-oxygenase 2
DupA	Duodenal ulcer promoting gene A
EBV	Epstein Barr Virus
ECs	Endothelial cells
EMT	Epithelial-mesenchymal transition
FGFs	Fibroblast growth factors
FOXO1	Forkhead box protein O1
GC	Gastric adenocarcinoma
HDGC	Hereditary diffuse gastric cancer
HIF	Hypoxia Inducible Factor
IL-1B	Interleukin
IL-1RN	Interleukin 1 Receptor Antagonist gene
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
INEC	Instituto Nacional de Estadística y Censos de Costa Rica
IARC	International Agency for Research on Cancer
MALT	Mucosa-associated lymphoid tissue
MMPs	Matrix metalloproteinases
NF-κB	Nuclear factor kappa B

OipA	Outer inflammatory protein A
PECAM-1 (CD31)	Platelet endothelial cell adhesion molecule-1
PG	Pepsinogen
PDGF	Platelet-derived growth factor
SHP-2	Src homology region 2-containing protein tyrosine phosphatase 2
SabA	Sialic acid-binding adhesin A
SNP	Single nucleotide polymorphism
TCGA	The Cancer Genome Atlas Consortium
Tie1 and Tie2	Tyrosine Kinase with Ig and EGF Homology Domains 1 and 2
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TLRs	Toll-like receptors
TSGs	Tumor suppressor genes
T4SS	Type 4 secretion system
uPA	Plasminogen activator urokinase
vacA	Vacuolating cytotoxin A gene
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
WHO	World Health Organization

INTRODUCTION

1. Epidemiology of gastric adenocarcinoma

Gastric adenocarcinoma (GC), is the most common (~95%) of all malignancies originating in the stomach. The other 5% uncommon variants of gastric cancer include mesenchymal, neuroendocrine tumors and mucosa-associated lymphoid tissue lymphoma (MALT) (1). GC is the fifth most common type of cancer worldwide, preceded by breast, lung, colorectal and prostate cancers, and the fourth cause of death by cancer in both sexes. In the year 2020, 1 089 103 new cases were diagnosed (5.6 % of the total) and 768 793 persons died worldwide (7.7 % of the total) (2). World incidence rates for GC are declining, mainly in developed countries, due to changes in nutrition and better prevention and treatment schemes; nevertheless, the absolute number of new cases is still increasing, mainly because of the aging of the world population (3). Incidence and mortality rates of GC present important variations according to the geographic localization and sex. Over 70% of GC cases occur in developing countries. GC has the highest incidence in Asian southeast countries such as Mongolia, Japan, South Korea, with age-standardized rates close to 30 per 100 000 habitants. Incidence is also high in Eastern Europe, Central and South American countries; on the other hand, Canada, United States, Northern Europe and Africa have the lowest incidences, below to 4 per 100 000 habitants (2). Three Asian countries have the highest mortality rates: Mongolia, Tajikistan and China, with rates of 24.9, 19.7 and 15.9 respectively. Almost 50% of the deaths by CG occurred in China in 2020.

According to the Instituto Nacional de Estadística y Censos of Costa Rica (INEC), in 2020, the cancer in general was the second cause of death in the country, (5792 deaths), slightly behind cardiovascular diseases (5938 deaths). In the same year, GC was ranked as the fourth in incidence and first in mortality (both sexes taken together). According to the latest global statistics, Costa Rica is the 25th in incidence and 21st in mortality rates with regards to GC (2).

In most of the cases, GC is diagnosed in advanced stages, which greatly limits the efficacy of surgery and chemotherapeutic treatment and ultimately results in poor prognosis (4), with a global 5-year survival rates of ~25–30%. The exception to this is Japan and South Korea, where the routine massive early detection efforts and screening strategies translates into a survival rate over 50% (1, 5). Men and women are not affected in the same way, as

incidence rates are about twice as high in men as compared to women; nevertheless, in persons under 40 years the ratio men:women is near the same (6).

2. Classification of gastric adenocarcinomas

Several classification systems have been established for GC, according to its anatomical location, stage, molecular characterization or histological features (3). GC is clinically classified as early or advanced stage, and histologically into subtypes based on microscopic features. The two most used classifications are the World Health Organization (WHO) system, that include the papillary, tubular, poorly cohesive and mucinous; and the Laurén scheme (3, 7).

The Laurén classification (1965) utilizes structural cellular components of the disease to separate patients into three types: intestinal (well differentiated), diffuse (poorly differentiated) and mixed (8). Nearly 50% of all diagnosed adenocarcinomas are intestinal, 35% are diffuse and 15% are mixed. Additionally, intestinal-type GC has a higher incidence in the distal stomach while diffuse-type tumors are more frequently found in proximal regions (9). The Laurén system is the most widely used, since it reflects GC morphology and epidemiology, but not the clinical management and outcome (3).

The intestinal type is generally more prevalent in geographical regions of high-incidence of GC, predominately found in individuals of older age (60 years), who are mostly male (twice more common in men than women); patients often present larger tumor sizes with the formation of intestinal-like glands (4). In 1975, Pelayo Correa postulated that the intestinal type was the end-result of progressive stepwise changes in the gastric mucosa, starting with a superficial or non-atrophic chronic gastritis, followed by a multifocal atrophic gastritis with loss of glands, which in some patients can follow towards intestinal metaplasia, where the gastric epithelium acquires characteristics that resemble small intestine (complete metaplasia) or colon (incomplete metaplasia). The next step is dysplasia, followed by carcinoma *in situ* and eventually it can progress to invasive adenocarcinoma. There is an important body of evidence that the intestinal type is associated with the presence of *H. pylori* infection, which is the most frequent cause of gastritis; indeed, non-atrophic gastritis can be cured by eradication of the infection (3, 5, 10).

The diffuse histological type of GC is characterized by the lack of expression of adhesion molecules and typically shows an infiltrative, poorly differentiated, poorly cohesive appearance (11). The tumor cells diffusely infiltrate the stroma of the stomach and often exhibit deep infiltration of the stomach wall, with little or no gland formation, and scattered signet-ring cell morphology are often observed (12). Diffuse-type GC, is most likely to occur at a younger age, in females; it is mostly found in the body/corpus regions and is associated with a worse prognosis and rapid disease progression (3, 8).

The two histologically distinct forms of GC, diffuse and intestinal, differ not only on their microscopical characteristics, but also in epidemiological, etiological and genetic aspects. According to the TCGA molecular analyses, diffuse and intestinal subtypes of gastric adenocarcinoma have quite different genomic profiles, with intestinal tumors often harboring chromosomal instability while diffuse tumors often being genomically stable (13). Also, intestinal-type GC most frequently metastasizes to the liver, whereas the diffuse type frequently metastasizes to the lung and peritoneum (14).

3. Etiology and risk factors

The etiology of GC is not yet fully understood; the onset of a stomach tumor is a multifactorial, long and complex process. Admittedly, environmental elements (infection with the bacterium *Helicobacter pylori*, diet and lifestyle), as well as genetic determinants and family history of GC are involved throughout the gastric carcinogenic process (15, 16).

3.1. Nutritional factors and lifestyle.

Diets high in fresh vegetables and fruits reduce the risk for GC given that fruits and vegetables containing antioxidants (17). Conversely, the consume of salty, spicy, smoked foods and those containing nitrosamines, is associated with increasing GC risk. It is believed that the N-nitroso compounds, particularly nitrosamines produced endogenously, promote gastric carcinogenesis, while some antioxidants, may block the development of these compounds (18). Other factors as smoking and consumption of foods contaminated with aflatoxins are associated with GC (1).

3.2. Genetic factors and genetic modifications.

Genetic and epigenetic mutations and other DNA abnormalities accumulate during carcinogenesis. This can lead to oncogene activation, inactivation of tumor suppressor genes (TSGs), overexpression of matrix metalloproteases (MMPs), increased activity of growth factors and its receptors, as well as deficiency of DNA repair mechanisms and cell adhesion proteins (19). In CG is common to observe TSGs silencing by loss of heterozygosity, point mutations and epigenetic changes such as CpG island hypermethylation, as well as overexpression of tumor growth promoting genes (*e.g.* anti-apoptotic, pro-angiogenic, growth factors, extracellular matrix remodeling) (20).

Based on identification of key genetic defects and molecular abnormalities (mutations, copy number variations, insertions, deletions or translocations), the Cancer Genome Atlas Consortium (TCGA) divided gastric cancers into four subtypes: EBV-positive tumors (9%), microsatellite unstable tumors (22%), genetically stable tumors (20%), and chromosome unstable tumors (50%) (13). There is considerable intra-patient and inter-patient heterogeneity, therefore the prognostic significance of those subtypes remains to be established (1).

Some genetic variants are considered as risk factors for several cancers, including CG. Cytokines and interleukins are produced during inflammatory responses, including that in response to *H. pylori* infection. Because cytokine production is partially regulated at the transcriptional level, many studies have implicated SNP polymorphisms at the promoter regions of some cytokines as potential determinants of disease susceptibility. Particularly, SNP variants of pro-inflammatory cytokines seem to influence the severity and extent of *H. pylori*−induced gastric inflammation, as well as GC initiation and progression. Several studies have found association between some genetic polymorphisms of *IL-1B, IL-1RN, IL-10* and *TNF-A* genes with risk for GC and precancerous lesions (21-27).

3.3. Pathogenic infections

3.3.1 Epstein Barr virus infection. Epstein Barr virus (EBV) is the main pathogenic factor for nasopharyngeal carcinoma, but it has also been shown to influence GC development in nearly 10% of cases (4, 28). EBV-associated malignancy often occurs in the proximal

stomach (cardia and gastric body), mostly in men, and is associated with good prognosis in patients (28, 29). During infection, EBV-encoded transforming proteins and non-coding RNAs are expressed; the virus can also cause aberrant DNA methylation, which results in the transcriptional inactivation of tumor suppressor genes (1). Indeed, the TCGA reported that the EBV-positive gastric tumors exhibit a higher prevalence of DNA hypermethylation than any other studied cancers (13).

3.3.2. *Helicobacter pylori* infection

H. pylori (Helicobacteraceae, Campylobacterales), first described by Warren and Marshall in 1984 (30), is a Gram-negative bacterium that colonizes the human and other primates gastric mucosa. *Helicobacters* are classified into gastric and enteric according to their niche (31). Since the discovery of *H. pylori* in humans, various other *Helicobacter* species have been identified in the stomach of domesticated and wild birds and mammals, including cats, dogs, pigs, dolphins, ferrets, chickens, among others (32). To date, the *Helicobacter* genus includes 48 distinct, validly published species, among which 17 are gastric and 31 enterohepatic (33). It has been suggested that *H. suis*, *H. felis, H. bizzozeronii*, *H. salomonis* and *H. heilmannii* have zoonotic potential and are possibly clinically relevant in humans (33). There is evidence that *H. pylori* spread from east Africa and coevolved with humans since almost 60,000 years ago (34).

H. pylori infects about 50% of the world's population and is the main cause of gastritis, gastric and duodenal ulcers, gastric lymphomas and adenocarcinomas (35). In Costa Rica, a number of epidemiological studies have been carried out with different samples and strategies reporting an average prevalence of infection of 70%, with variations between 54 to 93% (36-39). Although the transmission mode from one person to another is unknown, it is thought that infection is often acquired in early childhood and transmission between the family members could be fecal–oral or possibly oral–oral (35).

The bacteria colonize the gastric epithelium, where they produce a chronic and persistent infection. Once colonization is established, the infection can last for the lifetime of the host in the absence of treatment (31). *H. pylori* is the most well-known risk factor associated with the development of GC. Indeed, in 1994, the World Health Organization (WHO) and the International Agency for Research on Cancer (IARC) classified *H. pylori* as

a class 1 human carcinogen for GC in humans (40). Among patients with GC, *H. pylori* is believed to be the major causing agent of this malignancy, with in approximately 92% of patients infected; it is also responsible for the induction and progression of gastritis, peptic ulcer disease, dyspepsia and MALT lymphoma (41).

3.3.2.1. Virulence factors.

H. pylori utilizes several strategies for colonizing and surviving in the hostile environment of the stomach. This includes mechanisms of gastric acid resistance and neutralization, as well as others related to motility, chemotaxis, regulation of inflammatory responses, and immune escape (Fig.1). All these strategies lead to improved bacterial survival but, concomitantly, result in the induction of premalignant alterations and severity of *H. pylori*-related diseases (41, 42). Importantly, as not all the bacteria express all of the virulence factors, or to the same extend given the existing genetic variants, some *H. pylori* strains are considered more aggressive and have been associated with a higher risk of GC (35, 43-46).

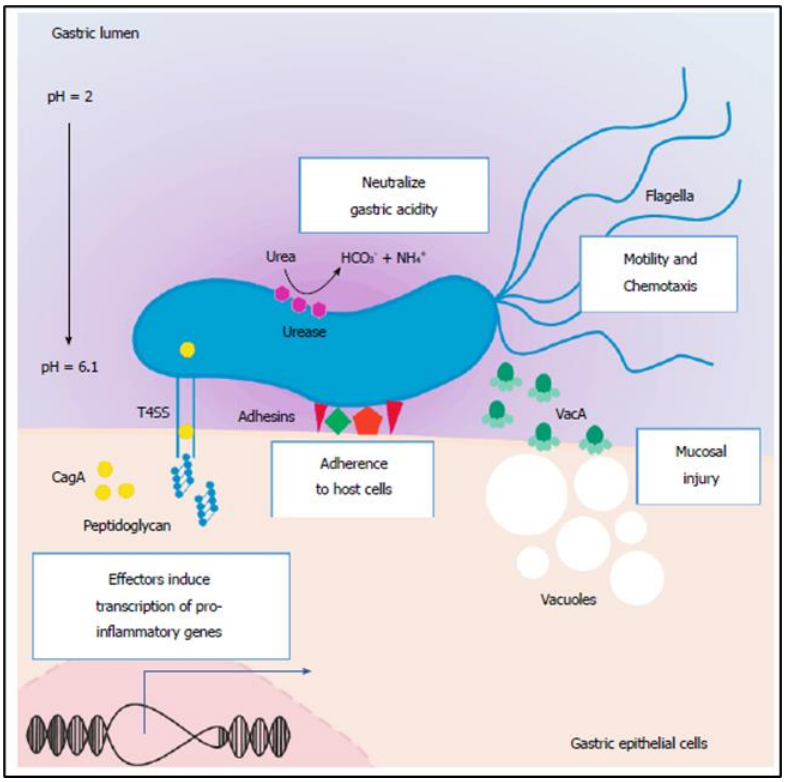

Fig. 1. *H. pylori* virulence factors associated with gastric pathogenic processes. From Molina-Castro et al. (42).

A) CagA (*Cytotoxin-associated gene A*). The *cagA* gene is part of the cag pathogenicity island (cag PAI), a group of about 30 genes, present in some *H. pylori* strains (48). Individuals colonized with cag PAI-positive strains have a higher risk of GC and peptic ulcer compared with individuals with *H. pylori* strains lacking the cag PAI (47). Eighteen genes of cag PAI encode proteins part of a type IV secretion system (T4SS), which acts as a syringe-like structure that translocates CagA protein into the host epithelial cells (48). Based on the presence or absence of *cagA, H. pylori* strains can be divided into *cagA*-positive or *cagA*-negative. The *cagA* gene is found in >95% East Asian strains, and 60-70 % of Western strains (49).

CagA is a 120-145 KDa protein that, so far, is the only identified protein delivered into the host gastric epithelial cells by the T4SS (50). Once in the cytoplasm, CagA is phosphorylated by c-Src and c-Abl tyrosine kinases of the host cell, specifically in repetitive motifs consisting of the amino acids Glu-Pro-Ile-Tyr-Ala (EPIYA) that are present in multiple numbers in the C- terminal region of the protein (43). There is diversity among the strains of *H. pylori* with respect to the number and type of phosphorylation motifs. In fact, there are 4 EPIYA described types: A, B, C and D; these are defined according to the specific amino acid adjacent to the EPIYA motif. Since a single CagA protein never carries both EPIYA-C and EPIYA-D segments, the presence of EPIYA-C is the hallmark of Western CagA, whereas the presence of EPIYA-D is the hallmark of East Asian CagA (31, 48). The Eastern -type CagA strains are reported to be more virulent than the Western type, thus the strains carrying an EPIYA-ABD type have been associated with the higher GC rate in East Asia (43).

Phosphorylated CagA binds to host proteins, such as phosphatase SHP-2, and activates the mitogen-activated protein (MAP) kinase signaling cascade, thus eliciting a cell morphological transformation named the “hummingbird phenotype”, which is typically observed *in vitro* and is characterized by cytoskeletal rearrangements and elongation of epithelial cells (Fig. 2). That morphological change presumably corresponds to an epithelial-mesenchymal transition (EMT), with an increase of mesenchymal markers and downregulation of epithelial markers (31).

CagA is also capable of the activation of nuclear factor kappa B (NF-κB), which in turn induces cancer-promoting responses by stimulating proliferation and inflammation (49). Patients infected with CagA-positive *H. pylori* strains exhibit higher grades of gastric inflammation, atrophic gastritis, and an increased risk of the development of GC (51). The association of *H. pylori* CagA with increased risk of GC was first reported in 1995 by Blaser and colleagues (44).

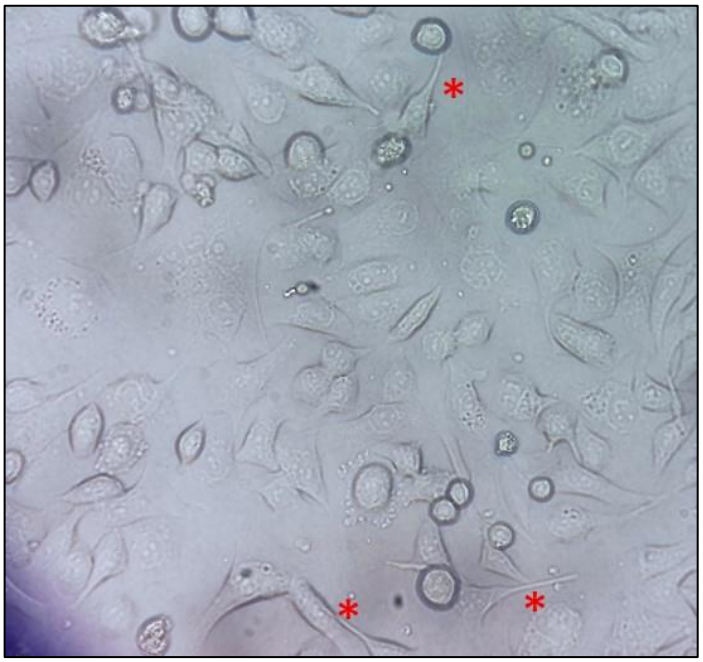

Fig. 2. The hummingbird phenotype induced by *H. pylori* CagA. AGS cells co-cultured 24 h with *H. pylori* 26695. Asteriks point cells with the hummingbird phenotype. Olympus CKX41SF, 100X

B) VacA. Vacuolating cytotoxin A, encoded by the *vacA* gene, which is present in all *H. pylori* strains, can induce the formation of pores in the membrane and intracellular vacuoles that enable the survival of *H. pylori* adhered to the host cells (52). The disruption of the endosomal and lysosomal functions can lead to autophagy; VacA can also alter the mitochondrial functioning by inducing the release of cytochrome c, leading to apoptosis (35, 53, 54). Furthermore, VacA inhibits the activation and proliferation of T and B cells, but induces apoptosis in macrophages (41). VacA plays a vital role in the survival of *H. pylori* by inducing a flow of ions and nutrients from the mucosa toward the stomach lumen (55).

The *vacA* gene has three variable regions (*signal* (s), *intermediate* (i) and *middle* (m) with two major variants in each of the regions (1 or 2) that regulate its activity. Within each of these regions, sequences can be classified into two main types (s1 or s2, i1 or i2, and m1 or m2) (52, 56). There is an association between the type of *vacA* allele present in *H. pylori* strains and the risk of gastric and duodenal disease. *vacA* s1m1 is more frequently found in patients with peptic ulcer disease or with GC than in patients with chronic gastritis only (43). It has been proposed that *H. pylori* with s1/m1 and s1/m2 VacA cause more severe chronic inflammation as compared to the other genotypes (55).

C) Urease. Urease is the most abundantly expressed protein by *H. pylori* and a critical factor that facilitates initial colonization within the acidified compartment of gastric mucus (57), and it is also very important for maintaining chronic infection (58). Despite colonizing the stomach, which has a normal pH of 1.5-2, *H. pylori* is acid-sensitive and, for its optimal survival, requires a pH of 4-5 (31). Urease alkalinizes the microenvironment by catalyzing the conversion of urea into ammonium and carbon dioxide. In addition, urease promotes bacterial acquisition of nutrients from the host and is active in the generation of proton motive force during the hydrolysis of urea (59).

Urease also has several effects in the modulation of host immune responses, facilitating apoptosis, chemotaxis of neutrophils and monocytes, altered opsonization, enhanced release of the pro-inflammatory cytokines (41). Recently, it was demonstrated that urease is an inductor of gastric angiogenesis *in vitro* (60). The detection of urease activity by a breath test is the gold standard for diagnosis of *H. pylori* infection (61).

D) Adhesion molecules. *H. pylori* needs to adhere to the surface of the gastric epithelial cells in order to colonize the gastric mucosa. Blood group antigen-binding adhesin (BabA) enables bacterial adherence and the delivery of toxins by adhesion to H-type 1 and ABO/Lewis b (Le^b) blood group antigens expressed in the gastric epithelium (62). Sialic acid-binding adhesin (SabA), present in approximately 40% of *H. pylori* strains, is an adhesin that facilitates bacterial adherence via binding to the Le^x antigen (63). Duodenal ulcer promoting gene A (DupA) is associated with the risk of gastritis and duodenal ulcers, but is considered to be a GC protective factor (64).

E) Flagella. *H. pylori* possesses a bundle of 2–6 sheathed unipolar flagella that provide bacterial motility and enable the colonization of the gastric mucosa at the first stages of bacterial invasion. Several proteins that compose bacterial flagella such as HpA, FlaA, or FlaB are believed to be involved in *H. pylori*-induced inflammation and immune evasion (41, 65).

4. The inflammatory response against *H. pylori* infection.

The host immune response against *H. pylori* infection is considered as a key factor in the development of gastric pathologies. As stated above, this bacterium colonizes the gastric epithelium, resulting in a chronic and persistent infection that causes gastric mucosal inflammation, which is characterized by the presence of infiltrating neutrophils, macrophages, B and T lymphocytes, and the up-regulation of several proinflammatory cytokines, including tumor necrosis factor-a (TNF-α), interleukin-1 (IL-1), IL-6, and IL-8 (35, 66). The production of cytokines and chemokines is crucial to start and maintain an inflammatory response. Moreover, the activation of immune cells releases free radicals and oxygen reactive species, which can promote malignant transformation by DNA damage and induction of mutations (35, 67).

Although *H. pylori* infection elicits a strong immune response, the immune system is unable to clear the infection; rather, the inflammatory response contributes to the development of more severe pathology (35). More specifically, *H. pylori* evades the host innate and adaptative immune system by a variety of immunomodulatory mechanisms, including the avoidance of recognition by Toll like receptors (TLR's), escaping phagocytosis by macrophages and neutrophiles, inhibiting maturation and function of dendritic cells and the induction of T_{reg} lymphocytes, among other several ways (65).

5. The role of *H. pylori* in the pathogenesis of gastric adenocarcinoma

Since *H. pylori* was first isolated 40 years ago from antral mucosa of patients with chronic gastritis, duodenal ulcer or gastric ulcer, Marshall and Warren proposed that the new uncovered bacterium could be an important factor in the etiology of those diseases (30). From that moment, accumulating evidence has led to an unequivocal and strong link between *H. pylori* and gastrointestinal diseases, including gastric and duodenal ulceration, GC and MALT lymphoma (Fig. 3). Intriguingly, only around 10% of the patients will develop peptic

ulcer disease, and less than 1–2% will develop GC as a result of infection. That means that 85-90% of infected individuals do not develop any evident pathology more than a superficial gastritis (35). Although the development of pathologies in response to the bacterial infection is not totally understood, there is evidence that this depends upon the synergy of bacterial virulence factors, the type and magnitude of the host immune response, the extent and anatomical distribution of inflammation in the stomach, and other modulating factors such as diet and smoking (1, 31). Additionally, the progression towards GC implies deregulated processes such as cell proliferation and differentiation, extracellular matrix degradation and angiogenesis (35, 43, 68, 69).

The role of *H. pylori* in the initiation and progression of intestinal GC is well established by solid evidence, in spite of the Correa's cascade was described before the bacterium was discovered (5, 31, 70). The sequence of events for diffuse GC occurrence is poorly understood. Since intestinal GC has been the most studied type, data from this tumor are usually extrapolated to the diffuse GC. Nevertheless, the aforementioned differences should be considered when trying to understand the specific pathogenesis of both cancer types and the role of *H. pylori* infection (9, 12). The identification of precancerous stages offers the potential opportunity for screening, endoscopic resection, and early detection of neoplasia to thereby reduce disease-related morbidity and mortality (71).

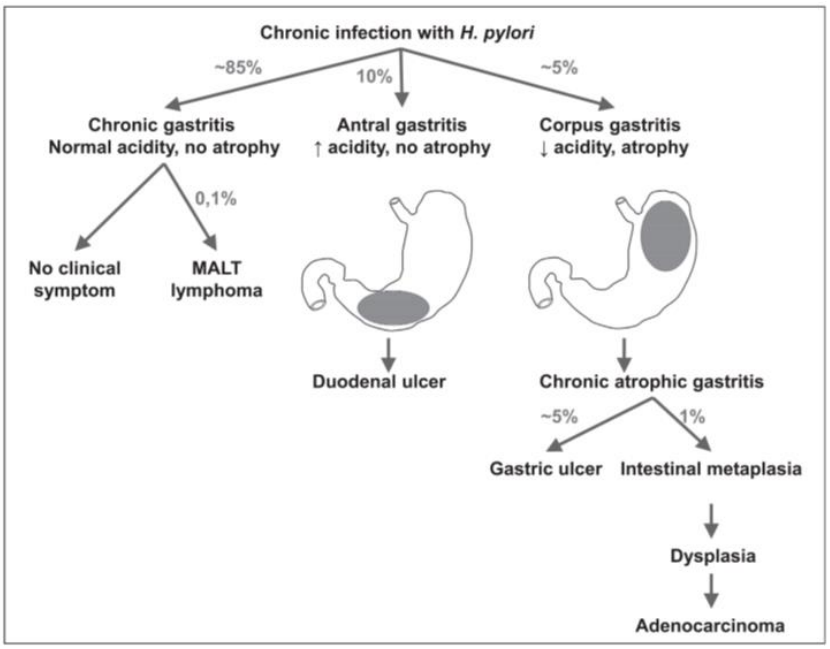

Fig. 3. Consequences of the infection with *H. pylori*. From Mègraud et al. *(31)*

5.1. Pathogenesis of intestinal GC.

It is widely accepted that the intestinal-type GC develops through a sequence of recognized precancerous lesions, and involves the progression from normal gastric mucosa to chronic superficial gastritis, followed by atrophic gastritis and intestinal metaplasia, and finally leading to dysplasia and adenocarcinoma (5). This process usually takes decades after persistent infection with *H. pylori*, which is considered the primary neoplastic stimulus (10, 67). Gastritis is characterized by increased infiltration of the mucosa with neutrophils (acute inflammation) and leukocytes (chronic inflammation) (5). GC is associated with pan-gastritis or corpus-predominant gastritis, which is thought to be at least partially related to decreased acid secretion (35). Gastritis may evolve in two dichotomic ways: either it remains as non-atrophic, or it progresses in severity leading to the loss of gastric glands. The latter results in atrophic gastritis, characterized by the loss of parietal and chief cells in the oxyntic mucosa, that ultimately results in the reduction of gastric acid secretion (72, 73). Atrophic gastritis may be accompanied with iron deficiency anemia; it is believed that the iron malabsorption could be a consequence of reduced gastric acid secretion and intrinsic factor (72, 74). The loss of normal glandular tissue (atrophic mucosa) is the first specific recognizable step in the precancerous cascade (5), and the risk of GC increases to a greater extent the higher is the degree of gastric mucosal atrophy (71). Importantly, the chief cells are the secretors of pepsinogen (PG); as they disappear the serum levels of PGI and the ratio of PGI/PGII decrease significantly, which correlates with the severity of atrophic gastritis (39). It is based on this that these parameters have been proposed as serological markers for the severity of atrophic gastritis (75).

Some of the lost gastric glandular structures can either be replaced by connective tissue (non-metaplastic atrophy) or by intestinal-resembling epithelium (intestinal metaplasia), with the presence of Paneth, goblet and absorptive cells (72, 76). Intestinal metaplasia has been classified into two main types: the complete or small intestine type and the incomplete or colonic type. The relative risk of developing GC is 10 times higher for individuals with intestinal metaplasia than for healthy individuals (77). A small proportion of subjects with intestinal metaplasia will develop dysplasia and ultimately *in situ* GC and invasive adenocarcinoma (31).

The predominant anatomic focus of the *H. pylori* infection determines the degree of impairment and whether acid secretion is increased or decreased. Infection of the gastric antrum is associated with hypergastrinemia, that together with the effects of cytokines derived from the infiltrating proinflammatory leukocytes, drives acid hypersecretion (5, 73). On the other side, infection of the gastric body and fundus is associated with acid hyposecretion, as *H. pylori* induces suppression of H, K-ATPase expression (78). As it has been already mentioned, gastritis of the corpus predisposes towards GC. In contrast, inflammation in the antrum with increased acid production predisposes to duodenal ulcer, which is associated with a decreased risk of GC (79). In people with antral-predominant gastritis, the gastric corpus, which contains the parietal cells, is relatively uninflamed, which allows a high-level acid production that is secreted into the duodenum and favors its ulceration (35).

5.2 Pathogenesis of diffuse GC

Although it is also originated in a chronically inflamed gastric mucosa, the development of diffuse-type GC does not follow the sequential steps described above for the intestinal type. Nevertheless, diffuse GC does have specific molecular profiles that differentiate it from the intestinal type, suggesting the existence of distinct carcinogenic pathways that distinguish each histological subtype (9). The underlying molecular and cellular pathways for diffuse GC, however, are not yet well-studied and understood.

It is thought that at least a subset of diffuse GC is related to several somatic and germline mutations in a number of genes. Some of the most well studied genes related to this cancer subtype include E-cadherin (*CDH1*), Tumor protein 53 (*TP53*) and Catenin beta 1 (*CTNN1B*) (11). Probably, the most important is the loss of expression of *CDH1* gene, by mutation or promoter hypermethylation (80). *CDH1* is a trans-membrane glycoprotein involved in the epithelial cell–cell adhesion (12). The cytoplasmic domain of E-cadherin forms a complex with β- or γ-, p120- and α-catenins; it also plays a critical role in the inhibition of nuclear signaling pathways and tumor-suppression function (12). Approximately in 1–3% of GC germline mutations in *CDH1* gene are linked to an autosomal-dominant predisposition CG syndrome, referred to as hereditary diffuse gastric cancer (HDGC). In fact, 40% of those are associated with germline mutations in *CDH1*, and the risk

of developing cancer is as high as 70% in men and 56% in women by the age of 80 years (8, 11).

Despite the diffuse-type is not related to Correa´s cascade, *H. pylori* infection has also been associated with the development of this CG type (9). CagA has been reported to interact with the E-cadherin (81, 82), destabilizing thus the binding with that protein and β-catenin, which causes accumulation of β-catenin in the cytoplasm and nucleus. Hypermethylation of *CDH1* promotor has been observed in more than 50% of early diffuse-type GC (80). *H. pylori* infection can also induce aberrant hypermethylation of multiple genes, including *CDH1* (12). Oliveira et al. (60) proposed that E-cadherin inactivation could be an initiator event of gastric carcinogenesis, whereas *H. pylori* infection works as a tumor promoter, selecting for survival gastric epithelial cells harboring E-cadherin inactivation, thus enabling further accumulation of oncogenic events.

6 Angiogenesis

6.1 Physiological angiogenesis

Blood vessels allow the immune surveillance, oxygen and nutrients supply, and removal of waste from tissues; they also collaborate with the organization and maintenance of organs (83). Vessels are formed by a monolayer of endothelial cells (ECs), covered by pericytes (mural cells) and smooth muscle layers (83, 84). Adherent and tight junctions are formed between the ECs to keep cells together, through anchorage to cytoskeleton with proteins such as VE-Cadherins, nectins, claudins, occluidins and PECAM-1 (CD31) (85). Vessels are also surrounded by a basement lamina which is a special type of extracellular matrix (ECM); this structure provides a scaffold essential for maintaining the organization of vascular endothelium (83). The physical interaction of ECs with ECM, through adhesion molecules such as integrins regulates EC proliferation, migration, survival, blood vessel stabilization and maturation, all of which are critical for neovascularization (86).

Angiogenesis is the process of formation of new blood vessels from the pre-existing ones, and is a pivotal biological process, not only under normal conditions (*e.g.* embryogenesis, wound healing, endometrium formation), but also in a variety of pathological conditions, including tumor growth and metastasis, rheumatoid arthritis, psoriasis, diabetic

retinopathy, and peripheral vascular disease (87). Therefore, this is a tightly regulated and restricted process (83, 84).

Hypoxia is the main trigger for angiogenesis. Hypoxic tissues secrete pro-angiogenic molecules (HIF, VEGF-A, ANGPT2) which in turn activate the endothelium to proliferate and create new vessels (83, 84). The angiogenic process involves the destabilization of EC junctions, proliferation, sprouting and EC migration, followed by pruning and remodeling of the vascular network (88). In fact, the loss of junctions between ECs is a pivotal early event during angiogenesis as it allows the pericyte detachment ECs differentiate and proliferate under the action of selected growth factors and cytokines, particularly members of the VEGF gene family and angiopoietins (83, 89, 90). Angiogenic activation is followed by vasodilation, which enables the extravasation of MMPs that will eventually degrade the endothelial base membrane. Attracted by proangiogenic signals, ECs become motile, invasive and produce small developing sprouts called filopodia (88). Those cells with protrusions or "tip cells" are followed by "stalk" ECs, which proliferate in order to sprout elongation and mediate lumen formation. Then, anastomosis between neighboring tip cells leads to the connection of sprouts to build the new network. Finally, the basement membrane and cell junctions are re-established, and pericytes recruited to complete the maturation of the nascent vasculature and to allow blood flow. Once formed, blood vessels remain quiescent, which is essential for physiological homeostasis and tissue integrity (83, 87, 88).

6.2 Pro-angiogenic factors

a) Hypoxia Inducible Factor (HIF). HIF is a transcriptional factor that plays a crucial role in the physiologic response to hypoxia. HIF-1 is a heterodimer consisting of oxygen-regulated HIF-1α and HIF-2α subunits, and a constitutively expressed HIF-1β subunit (91). HIF activates genes encoding for proteins involved in increasing oxygen availability and facilitating metabolic adaptation in response to oxygen deprivation, including factors that regulate glycolysis, erythropoiesis and angiogenesis. *VEGFA, ANGPT2* and *NOS* are the most prominent examples of genes that are regulated by HIF, including (92). HIF-1α mRNA expression is upregulated by TNF-α (91).

b) VEGF Family. Vascular Endothelial Growth Factors (VEGFs) are part of a family of ligands (VEGF-A, VEGF-B, VEGF-C and VEGF-D) with specific mitogenic activity on ECs by binding to receptors with tyrosine-kinase activity (VEGFR-1, VEGFR-2 and VEGFR-3). VEGFs are considered as the most important angiogenic inductors in physiological processes and pathological conditions, including cancer (93). VEGFA is the most potent and ubiquitous member of the VEGF family, regarded as the master inductor of physiological and tumor angiogenesis (83). Secreted by macrophages, lymphocytes, fibroblast and tumor cells, VEGFA promotes proliferation, migration and survival of ECs, as well as expression of extracellular matrix proteases, blood vessels permeability; also, it can affect the inflammatory microenvironment by inducing the recruitment of immune cells that in turn secrete extracellular matrix remodeling proteins (83, 84, 94).

c) Angiopoietins. Angiopoietins (ANGPT, ANG) are a family of four extracellular ligands that bind to two receptors with tyrosine-kinase activity in the endothelial cells: Tie1 and Tie2 (encoded by *TIE1* and *TEK* genes respectively). In humans, ANGPT1, ANGPT2 and ANGPT4 have been described. In mice, Angpt1 and Angpt2, as well as the ligand Angpt3, which is considered as an ortholog of ANGPT4, have been reported (86). Tie1 and Tie2 are almost exclusively expressed in the EC, but there is low level expression in hematopoietic cells (95). Although ANGPT1 and ANGPT2 both bind to Tie2 with similar affinities, they seem to act as antagonists of each other, with opposite effects (96) (Fig. 4). Since no ligands for Tie1 have been identified yet, it is considered an orphan receptor (95, 97).

ANGPT-1 is constitutively expressed and secreted by pericytes and acts in a paracrine manner to keep homeostasis of the mature vasculature (95, 98). ANGPT1-Tie2 binding induces Tie2 autophosphorylation and signaling pathways via the serine kinase AKT, which leads to inhibition of the transcription factor Forkhead box protein O1 (FOXO1) and repression of FOXO1 target genes, such as *ANGPT2* and *MMP9* (95, 98, 99) (Fig 4A). Tie2 activation also results in the inhibition of the NF-κB pathway (98) and stabilization of vascular endothelial cadherin (VE-cadherin) (95).

ANGPT2 is mostly produced by ECs, in which it is stored at the cytoplasmatic Weibel-Palade bodies, from where is quickly secreted upon HIF-1 and TNF-α stimulus (100), to act in an autocrine way by blocking Tie2 signaling pathway (95). ANGPT2 is

predominantly expressed by the tip cells of angiogenic sprouts and plays a role as a vessel destabilizing agent that induces permeability, dissociation of cell-cell junctions and pericyte detachment. This in turn favors the ECs to become susceptible to VEGF-A thus increasing proliferation and migration, and also facilitates leukocyte adhesion to the endothelium of newly forming sprouts (98, 101). That means that ANGPT2 can elicit its effects in an environment with the presence of VEGFA (Fig. 4B).

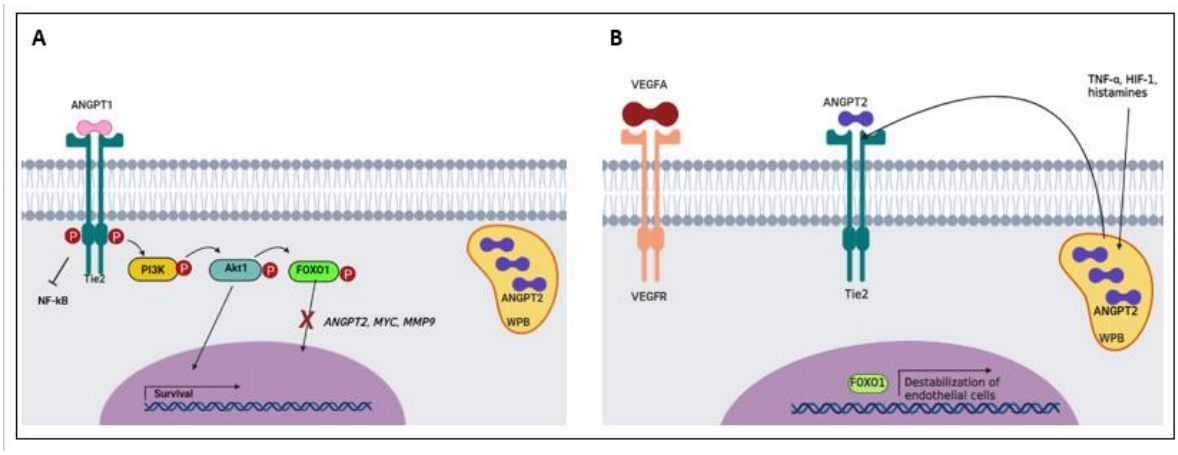

Fig.4. Mechanisms of action of ANGPT1 and ANGPT2 in endothelial cells. A) Quiescent endothelial cells. B) Activated endothelial cells. Created with BioRender.com

d) Other growth factors: FGF, PDGF and TGF-β. Fibroblast growth factors (FGFs) are broad-spectrum mitogens and regulate a wide range of cellular functions, including migration, proliferation, differentiation, and survival. To date, 22 FGF ligands and 4 receptors have been identified (102). Besides their mitogenic properties, they are also involved in stimulating ECs proliferation and migration (94). Platelet-derived growth factor (PDGF) family is comprised of four genes (PDGF-A, B, C, and D), with mitogenic and chemoattractant properties, that have been involved in bone formation, erythropoiesis, wound healing and angiogenesis (103). The Transforming Growth factor-β (TGF-β) family is composed by more than 30 chemokines, with several functions in hematopoiesis, angiogenesis, cell proliferation, differentiation, migration, EMT and apoptosis (104). TGF-β is a potent inducer of angiogenesis *in vivo* by modulating pro- and anti-angiogenic factors

that affect both endothelial and mural cells, and promotes migration and proliferation of endothelial cells (105, 106).

e) Tumor Necrosis Factor-α (TNF- α). TNF-α is a key mediator in the host's response against gram-negative bacteria, such as *H. pylori*. It is produced by lymphocytes and macrophages and promotes leukocyte recruitment (107). TNF-α is a major inflammatory mediator that induces multiple changes in EC gene expression including induction of adhesion molecules, integrins, and MMPs. In the context of angiogenesis, TNF-α activates vascular endothelium and primes ECs for angiogenic sprouting by inducing the tip cell phenotype (108, 109). *In vitro* studies have revealed that TNF-α upregulates the expression of *VEGF-A, ANGPT2,* and *Tie2*, and deregulates *ANGPT1* genes at the mRNA level (110, 111).

In the context of the gastric mucosa, TNF-α expression is substantially elevated in response to *H. pylori* infection, thus inhibiting gastric acid secretion, which in turn facilitates *H. pylori* survival (112). *TNF-A* transcription is induced by NF-κB, which is activated in response *H. pylori* infection in gastric epithelial cells *in vitro* and *in vivo* (113-115), and upregulates VEGFR in endothelial cells (116).

6.3 Tumor angiogenesis

Although essential for tissue growth and regeneration, vessels can also fuel inflammatory and malignant diseases, including cancer. Like normal tissues, tumors require nutrients and oxygen, and need to dispose carbon carbon dioxide waste (19). Because of the rapid cell proliferation of tumor cells, a large amount of nutrients and oxygen are consumed, which creates a hypoxic microenvironment that is not suitable for tumor cell growth (117, 118). Under that selective pressure, tumor cells can undergo metabolic reprogramming that improve their glucose uptake to promote survival and proliferation (119). A tumor mass less than 2-3 mm in diameter may receive oxygen and nutrients by diffusion, so the angiogenesis process is mainly activated when the growing tumor mass is bigger than the maximum volume that can be maintained by diffusion (120).

The change of an avascular to a vascularized tumor, resulting in the recruitment of new blood vessels, called “angiogenic switch”, enables tumor cells survival and provides them access to vasculature, which may result in the escape of malignant cells into circulation

and onset of metastatic disease (117). That means that angiogenesis can stimulate the growth of the primary tumor, but it also plays a critical role in the establishment of metastatic tumors (19). Tumor angiogenesis involves an intricate communication between the tumor cells and its microenvironment, *i.e.* surrounding cells, such as ECs, pericytes, and tumor-associated macrophages and fibroblasts (84).

The tumor vascular network is quite distinct from the normal blood supply system. It is characterized by precocious capillary sprouting, convoluted and excessive vessel branching, distorted and enlarged vessels, sometimes with dead ends, erratic blood flow and abnormal levels of ECs proliferation and apoptosis. Tumor vasculature is also leaky and hemorrhagic, partly due to the overproduction of VEGF and others pro-angiogenic factors. Other characteristic feature of tumor blood vessels is that they fail to become quiescent, which translates into an unrestrained growth of new aberrant blood vessels (19, 121, 122).

6.4 Angiogenesis, inflammation and carcinogenesis

Despite angiogenesis and inflammation are distinct and independent processes, in many cases they are closely intertwined. Both inflammation and angiogenesis share several signaling pathways and common mediators; for instance, they are exacerbated by increased production of chemokines/cytokines, growth factors, proteolytic enzymes, proteoglycans, lipid mediators and prostaglandins (123). Angiogenesis can stimulate and intensify the inflammatory response by providing nutrients and oxygen to the sites of inflammation. In addition, some angiogenic factors exert proinflammatory activity, and in consequence, perpetuate inflammation. Conversely, in chronic inflammation, inflammatory cells produce cytokines and growth factors that may affect endothelial cell functions (98, 124). Inflammation is unequivocally linked to the transformation of cells from benign to malignant (125). Aberrant vessel structure can also induce interstitial hypertension (126), hypoxia, and acidosis, creating a fertile environment for the initiation of inflammation-linked carcinogenesis (127).

The link between and angiogenesis goes beyond cancer initiation. In overt neoplastic tissues, inflammatory cells (lymphocytes, neutrophils, macrophages and mast cells) act together with tumor cells, stromal cells and ECs, to create a microenvironment critical for

the survival, development and spreading f the neoplastic mass (128). Tumor cells secrete pro-angiogenic factors; those cells are surrounded by an infiltrate of inflammatory and stromal cells, which cooperate with malignant cells in stimulating EC proliferation and blood vessel formation (89). In consequence, the newly formed vascular supply contributes to the perpetuation of inflammation by promoting the migration of inflammatory cells to the site of inflammation. These synergies may represent important mechanisms for tumor development, progression and metastasis by providing efficient vascular supply and an easy pathway for cancer cells to disseminate to distant sites (19, 129, 130).

6.5 Angiogenesis, H. pylori and gastric carcinogenesis

Besides its role in cancer progression, angiogenesis plays a major role in the multi-step gastric (131)carcinogenesis process due to the fact that the gastric mucosa undergoes important histological changes, which may require gaining access to the vasculature in order to receive an adequate supply of nutrients and oxygen (90). In the stepwise series of lesions that comprise atrophy, hyperplasia, metaplasia and dysplasia, gastric cells accumulate genetic and epigenetic alterations that may change their gene expression programs thus leading to alteration in their phenotypes.

Angiogenesis is already present in early GC, but as the cancer progresses toward more advanced stages, angiogenesis becomes more pronounced (132). Feng et al. (133) reported positive immunostaining for p53, iNOS and VEGF in gastric epithelial cells and cancer cells, showing a growing tendency in the degree of expression and a linear correlation with the progression of above-mentioned lesions. This suggests that accumulation of p53 protein and increased expression of iNOS and VEGF represent may be of importance in gastric carcinogenesis.

Besides the inflammatory cell infiltration, a major component of gastrointestinal inflammation is the alteration of vascular structure and function, including erythema, edema, and vasodilation (89). Several studies have indicated that *H. pylori* infection can alter the gastric vasculature by inducing proinflammatory and proangiogenic factors, such as VEGFA, uPA and MMP-9 and stimulation of collagenase activity *in vitro* (115, 134, 135). Other *H. pylori*-induced inflammatory factors related with angiogenesis include IL-6 and IL-8, which increase angiogenesis via expression of VEGF; COX-2 catalyzes the production of

prostaglandins, which increase vascular permeability and promote cellular migration to the inflamed tissue; and the production of reactive oxygen species (68).

Various *in vitro* studies have unraveled the role of *H. pylori* in the induction of angiogenic response. Leite *et al*. (136) showed that MKN74 gastric cancer cells infected with *H. pylori* 60190 strain have increased expression of numerous pro-angiogenic proteins, including ANGPT2, interleukin-8, VEGFA and VEGFC, platelet-derived growth factor, fibroblast growth factor-1, -2, and -4, and urokinase plasminogen activator (uPA), among others. Olivera-Severo *et al.* (60) showed an increase in ANGPT2 protein and other pro-angiogenic factors upon exposure of AGS cells to purified *H. pylori* urease from strain 26695. They further demonstrated that *H. pylori* urease induced formation of tube-like structures by HUVECs and intense neo-vascularization in the CAM assay model.

When it comes to *in vivo* settings, data from murine models on the role of *H. pylori* induction of angiogenesis is scarce, but there are some reports about *H. pylori*-induced angiogenesis in human gastric samples. It has been shown that neovascularization of the gastric mucosa is significantly higher in the antrum of *H. pylori*-positive than in *H. pylori*-negative cases in dyspeptic patients (137). Different studies have reported upregulated expression of inflammatory and pro-angiogenic genes. More specifically, there is a higher expression of HIF-1a, VEFG-A and higher density of CD31-positive blood vessels in *H. pylori*-positive patients, compared to not infected persons (137-139). These data imply that *H. pylori* is capable of inducing angiogenesis in its host, which may play a critical role in the development and progression of cancer. However, the process by which the bacterium induces neovascularization in precancerous lesions and early GC development is not fully understood.

7 MicroRNAs

7.1 Biology

MicroRNAs (miRNAs, miRs) are a class of small non-coding RNAs with 19-25 nucleotides length that are derived from hairpin pre-miRNA precursors, transcribed by RNA polymerase II. miRNAs maturation process requires the enzyme Drosha, which cleaves the pre-miRNA to yield an approximately 70-100 nucleotide pre-miRNA. The precursor is then

translocated to the cytoplasm by exportin 5; then a second cleavage takes place, involving the action of a complex containing the enzyme Dicer (140).

MiRs regulate gene expression either by inhibiting mRNA translation or by inducing its degradation, by binding by sequence complementarity. miRNAs post-transcriptionally repress gene expression by recognizing complementary target sites in the 3′ untranslated region (UTR) of target mRNAs (141). These sequences are now known to be evolutionarily conserved across species, which suggest their importance in essential biological processes (142). In mammals it is estimated that microRNAs control the activity of 50% of all protein-coding genes, and that they participate in regulation of almost all cellular processes (141). One miRNA molecule can regulate many target mRNAs. In turn, one mRNA molecule can be inhibited by different miRNAs (143).

Several algorithms have been designed to predict the possible target genes regulated by specific microRNAs. Computational methods such as TargetScan, mirBase (144), miRDB (145) and DIANA-Tarbase (146) are available online, and are based on the complementarily of sequences between miRNAs and 3' non-coding regions of the mRNA targets, as well as on the thermodynamics of this union.

7.2 Role of microRNAs in Cancer

MicroRNAs regulate several biological processes including carcinogenesis. In cancer cells, miRNAs have been found to be dysregulated and have been shown to affect the hallmarks of cancer, including sustaining proliferative signaling, evading growth suppressors, resisting apoptosis, activating invasion and metastasis, and inducing angiogenesis (147). Additionally, as miRNAs regulate the expression of their target mRNAs, the changes in expression of miRNAs are expected to result in down- or upregulation, respectively, of the protein product of the target mRNAs. Consequently, a microRNA can act as a TSG or as an oncogene, as its loss of function may initiate or contribute to the malignant transformation of a normal cell (Fig. 5). Decreased or null expression of TSG miRNAs results in upregulation of genes important for tumor progression, including antiapoptotic proteins or transcription factors (143). Loss of function of a microRNA could occur by various mechanisms, including deletions, mutations, epigenetic silencing or alterations in its processing and maturation (148, 149). Combined with the above, different

transcription factors, such as p53 and c-Myc, and also proto-oncogenes as RAS, control miRNA expression and are often associated with tumorigenesis (150).

Abnormal microRNAs expression has been reported in various types of human cancers. The first report was made by Calin and colleagues (2002) in lymphocytic chronic leukemia (151). Subsequently, several groups have found changes in the expression of microRNAs in various tumors, including colorectal (152), hepatocellular (153), meningiomas (154), breast (155) and stomach (156-160) cancer. Nowadays, the evidence is rapidly and increasingly growing, as miRNA profiles have been reported in clinical specimens from various cancers, and are seen as potential biomarkers of diagnosis, prognosis and for the development of new therapeutic strategies (143, 147).

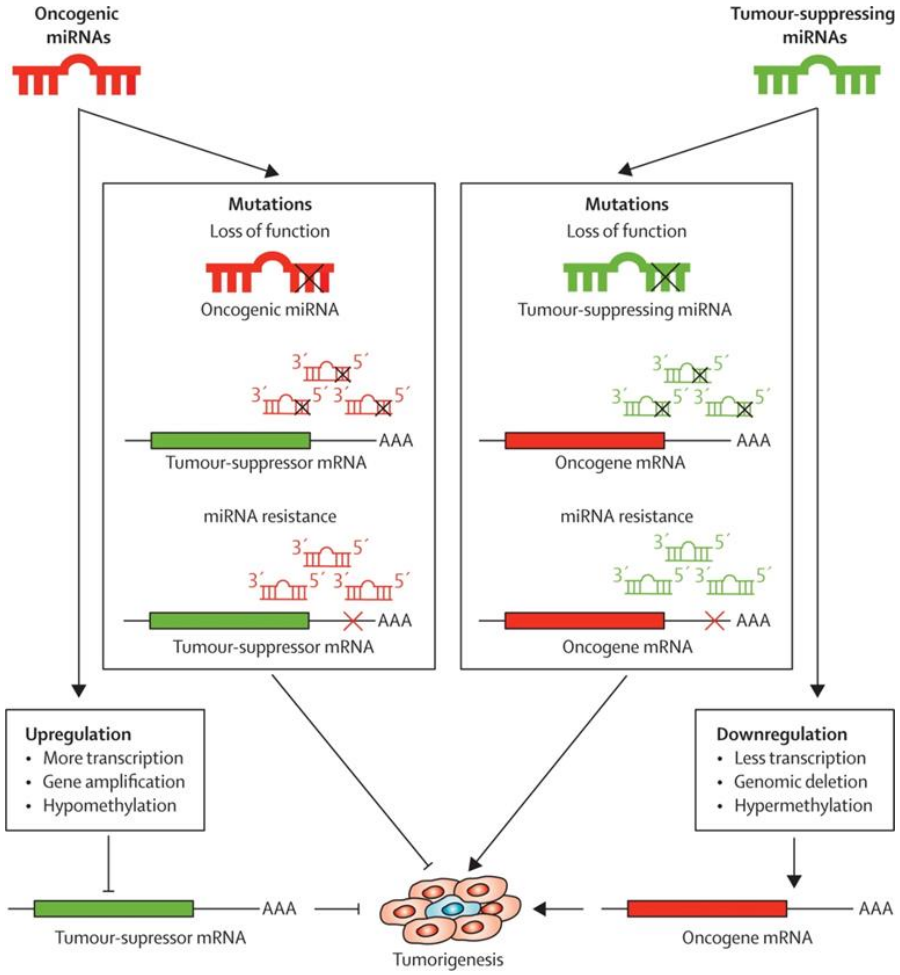

Fig. 5. Regulation of tumorigenesis by miRNAs. Upregulation of oncogenic miRNAs reduces expression of tumor-suppressor protein, but downregulation of tumor-suppressing miRNAs results in an increased production of oncogenic protein. From Kong 2012 (149).

7.3 Role of microRNAs in H. pylori infection, GC and angiogenesis

In the context of GC, the number of publications investigating the relationship between microRNAs, *H. pylori* and carcinogenesis has been increasing each year (161), suggesting that microRNAs act as key regulators of gastric carcinogenesis. Several investigations have reported the effect of *H. pylori* infection in the deregulation of miRNAs expression in both GC cell lines and human gastric mucosal tissue. Zhang and colleagues (160) reported the over-expression of miR-21 in the gastric mucosa infected by *H. pylori*, both *in vitro* and *in vivo*, which suggests the role of the bacterium in the manipulation of the signaling pathways regulated by miR-21. They also noted that increased expression of miR-21 led to a significant increase in the proliferation rate and invasion capacity of the cells in the *in vitro* model. Matsushima *et al*. (158) reported a set of 30 miRNAs differentially expressed (*e.g*. miR-141, miR-200a, miR-203a, downregulated, and miR-200b, miR-223, upregulated) in *H. pylori*-positive noncancerous human gastric mucosa, compared to uninfected individuals. Demiryas *et al*. (162) studied the miRNA expression profiles in GC patients, and detected several upregulated (miR34c, miR-194, miR-22, miR-223) and downregulated (miR-203a, miR-378, miR-145, miR-375, miR-335) microRNAs in *H. pylori* positive patients compared to *H. pylori* negative patients. The authors proposed that the virulence of *H. pylori* strains may also be involved in the up- or downregulation of miRNA expression levels. Further analysis of those and many other microRNAs and their target genes may clarify their roles in the context of *H. pylori* infections.

Tumor angiogenesis involves the overexpression of pro-angiogenic factors by multiple mechanisms; these factors and the molecules acting downstream their signaling pathways are potential targets for regulation by microRNAs. In fact, several microRNAs have been reported as angiogenic regulators during normal physiological processes, such as wound healing, and the aberrant expression of miRNAs can promote and inhibit angiogenesis during tumor progression (150, 163). miRNAs controlling the angiogenic mechanisms are collectively known as angiomiRs (164). AngiomiRs can work targeting the negative regulators of angiogenesis and so promoting angiogenesis (pro-angiomiRs) or by targeting positive regulators of angiogenesis and so inhibiting it (anti-angiomiRs) (150).

Several studies have related microRNAs with tumor angiogenesis in many cancer types. miR-205 was upregulated in ovarian cancer tissue, adjacent endothelial cells and in

serum and circulating exosomes of the patients (165). Hypoxia-associated angiomiRs, such as miR-21 and miR-200c are known to be dysregulated in pancreatic (166, 167) and bladder cancer (168). miR-145 was found to suppress the expression of p70S6K1 (Ribosomal protein S6 kinase beta-1), and to upregulate HIF-1 and VEGF, which are the downstream mediators of p70S6K1 in colorectal cancer cells (169). miR-616-3p facilitates tumor angiogenesis *in vitro* by elevating the expression level of VEGF-A/VEGFR2 via the PTEN/AKT/mTOR pathway in GC cells, and was also up-regulated in cancer tissues (170). MiR-27b, miR-101 and miR-128 were shown to inhibit angiogenesis by downregulating VEGF-C expression in GC cells (171).

AIMS OF THE STUDY

There is evidence suggesting that *H. pylori* is capable of inducing angiogenesis in its host, which may play a critical role in GC initiation and progression. However, the molecules and mechanisms by which *H. pylori* induces early neovascularization in the gastric mucosa, and how this influences the precancerous series of events that precede GC are not completely understood. Therefore, the main aim of this thesis was to investigate the role of *H. pylori* infection in the induction of microRNAs and inflammatory pro-angiogenic factors, using *in vitro* infections of human gastric cell lines, a mouse model of *H. pylori*-induced gastritis and DNA samples from patients with chronic gastritis, duodenal ulcer or CG. Three specific objectives were formulated:

1. To investigate the expression of the pro-angiogenic genes *ANGPT1*, *ANGPT2*, and their receptor *TEK*, and microRNAs miR-203a, miR-200a and miR-135, using human gastric cancer cell lines co-cultured with *H. pylori*.
2. To characterize the expression of *Tnf-A*, Angpt1, Angpt2 and VegfA in the gastric mucosa in response to *H. pylori* infection and to explore their relationship with inflammation, vascularization and gastric pathology in a mouse model.
3. To ascertain whether genetic polymorphisms of the genes encoding *TNF-A*, *IFNGR1*, *IL-6* and *IL-8* are associated with gastric pathologies in a Costa Rican high-risk population.

BIOLOGICAL SAMPLES, MODELS OF STUDY AND METHODOLOGICAL ASPECTS

A brief overview of the methodology is presented below. The detailed description of materials and methods is presented in the three articles that constitute the main body of this thesis.

1. In vitro models of infection with *H. pylori* (Paper I)

The following gastric cancer cell lines were used.

Cell line	**Age, sex**	**Source of culture**	**Characteristics**
AGS (ATCC, CRL-1739)	54, F	Primary tumor	Poorly differentiated. Lacks E-Cadherin expression at protein level.
MKN28 (JCRB0253)	70, F	Lymph node metastasis	Moderately differentiated
MKN45(RCB1001RIKEN, BRC Cell Engineering Division, Japan)	62, F	Liver metastasis	Poorly differentiated

The following *H. pylori* strains were used for infections:

***H. pylori* strain**	**Characteristics**
7.13 (kindly provided by Christine Varon, INSERM, University of Bordeaux)	*cagA*+, *vacA* s1m1 Gerbil-adapted strain, derived from clinical strain B128 (172)
26695 (ATCC700392)	*cagA*+, *VacA* s1m1
60190 (ATCC49503)	*cagA*+, *vacA* s1m1
84183 (ATCC53726)	*cagA*+, *vacA* s1m1
84183ΔcagA	Mutant deficient in *cagA*
84183ΔcagE	Mutant deficient in *cagE*

Co-cultures of 24 h with AGS and MKN28 cells and 26695, 62190 and 84183 strains were performed at IPATIMUP, in Portugal. Of note, it was in the context of the present research that the assays with *in vitro* infections of gastric cancer cells were done at INISA laboratories for the first time. This study began with the acquisition and establishment of the culture of the GC cell lines and the reference strains of *H. pylori*, in order to perform the infections. Co-cultures of 24 h with AGS and MKN45 cells, with *H. pylori* 26695 and 7.13 strains, were performed at INISA-UCR. Additionally, time-course experiments were performed with AGS cells, which were co-cultured for 3, 6, 12, 18, 24, and 36 h with *H. pylori* 26695.

2. Chick Chorioallantoic Membrane (CAM) assay (Paper I)

CAM is a highly vascularized extra-embryonic membrane with multiple functions during embryonic development, such as gas exchange (173). It is one of the most widely used *in vivo* assay for studying angiogenesis and its regulators in the context of tumor biology (174). CAM has a fully developed lymphatic system that holds remarkable functional and molecular similarities to mammalian lymphatics. The CAM assays for the present study were performed at IPATIMUP, with conditioned media from AGS cells co-cultured with *H. pylori* 26695.

***3. In vivo* infection murine model (Paper II)**

There are several animal models that can be used to study the biology of GC (175, 176), *however the m*ouse is the most widely used. When it comes to study *Helicobacter*-induced gastric pathology in the mouse, only a limited number of *Helicobacter* strains successfully colonize the mouse stomach. In this regard, the most robust and useful mouse model to date have been *H. pylori* SS1 and *H. felis*-infected mice (177). This murine model largely recapitulates the human stepwise process leading to GC, with some specific differences. Unlike humans, the corpus rather than the antrum is the primary target of disease, and pseudopyloric metaplasia is much more common than intestinal metaplasia, but has the same preneoplastic significance in both species (178).

For their Th1-biased inflammatory response, C57BL/6 mice have been the mouse strain of choice to model *in vivo* gastric carcinogenesis induced by *H. pylori* infection (175). The present study, as many others, uses only female mice because these are more tractable and pose fewer problems from an animal housing perspective than male animals (179). The *H. pylori* Sydney strain (SS1) is a mouse-adapted strain useful in murine model systems. C57BL/6 mice infected with *H. pylori* SS1 develop severe gastritis and atrophy, which may progress to high-grade dysplasia, although adenocarcinomas do not usually appear (176). The SS1 bacterial strain has a non-functional T4SS, which may explain to some extent the limited virulence of SS1 in mice (177).

For this study, a total of thirty-nine C57BL/6N female mice certified as *Helicobacter* spp. free and inoculated with *H. pylori* SS1 strain. Infected groups were euthanized by cervical dislocation after 5-,10-, 20-, 30-, 40- and 50- weeks post-infection (w.p.i.). The control group was euthanized at 50 w.p.i. The entire stomachs were opened along the greater curvature, washed with PBS, and cut longitudinally into four stripes, extending from the squamous forestomach through the duodenum. The stripes were used as follows: one for confirmation of bacterial colonization (culture), one was cut in halves for RNA and protein isolation, and two for immunohistochemistry assays. Animals were housed at the Laboratory for Biological assays (LEBI), UCR. The bacteria, RNA, protein and gastric tissue samples were processed at INISA and at the Centre for Research on Microscopic Structures (CIEMic).

4. Human DNA samples for studies of SNPs (Paper III)

The host immune response against *H. pylori* infection is considered a key factor in the development of gastric pathologies. The infection up-regulates the expression of several proinflammatory cytokines, including tumor necrosis factor-a (TNF-α), interleukin-1 (IL-1), IL-6, and IL-8 (112, 180). Because cytokine production is partially regulated at the transcriptional level, many studies have implicated polymorphisms at the gene promoter regions as potential determinants of disease susceptibility. Particularly, SNP variants of pro-inflammatory cytokines seem to influence the severity and extent of *H. pylori*−induced gastric inflammation, as well as GC initiation and progression (21-23, 180). In the present study, five SNPs of the genes *TNF-A, IFNGR, IL-6* and *IL-8* were chosen; since they had not

been studied before in Costa Rica. Blood samples were collected as part of a larger investigation conducted between 1996−2000, with 1300 participants at the Gastric Cancer Early Detection Center at the Max Peralta Hospital in Cartago, which is a province in Costa Rica with a high risk of GC, as previously described by Rosero-Bixby & Sierra (181). DNA was isolated from the blood samples using standard proteinase K digestion and phenol/chloroform extraction and stored at -70˚C since then. *H. pylori*, CagA status and blood PG levels were previously determined by ELISA**.**

5. PCR and RT-qPCR

The genotyping of *TNF-A-308G/A, TNF-A-857C/T, IL-6-174G/C* and *IFNGR1-56C/T* SNPs polymorphisms was performed using the 5'-nuclease Taqman PCR assays. *IL-8*-251*T/A genotyping was performed by PCR-RFLP (Paper III). Taqman probes were also utilized for all of the gene expression analysis for coding genes and microRNAs in human cells (Paper I) and mouse tissue (Paper II).

RESULTS

Summary of the papers

Paper I: *Helicobacter pylori* infection induces abnormal expression of pro-angiogenic gene ANGPT2 and miR-203a in AGS gastric cell line

In this study, we investigate the expression level of pro-angiogenic genes (*ANGPT2*, *ANGPT1*, receptor *TEK*), and microRNAs (miR-135a, miR-200a, miR-203a) predicted to regulate those genes, using *H. pylori* co-cultures with gastric cancer cell lines. *In vitro* infections of different gastric cancer cell lines with *H. pylori* strains were performed, and the expression of ANGPT1, ANGPT2, and TEK genes, and miR-135a, miR-200a, and miR-203a, was quantified after 24 h post infection (h.p.i.). We performed a time-course experiment of *H. pylori* 26695 infections in AGS cells at 6 different time points (3, 6, 12, 18, 24, and 36 h.p.i.). The angiogenic response induced by supernatants of non-infected and infected cells at 24 h.p.i. was evaluated *in vivo*, using the CAM assay. In response to infection, ANGPT2 mRNA was upregulated at 24 h.p.i, and miR-203a was downregulated in AGS cells co-cultured with different *H. pylori* strains. The time-course of *H. pylori* 26695 infection in AGS cells showed a gradual decrease of miR-203a expression, concomitant with an increase of ANGPT2 mRNA and protein expression. Expression of ANGPT1 and TEK mRNA or protein could not be detected in any of the infected or non-infected cells. CAM assays showed that the supernatants of AGS cells infected with *H. pylori* 26695 strain induced a significantly higher angiogenic and inflammatory response. The results suggest that *H. pylori* could contribute to the process of carcinogenesis by downregulating miR-203a, which further promotes angiogenesis in the gastric mucosa by increasing ANGPT2 expression.

Paper II: *Helicobacter pylori* infection induces gastric precancerous lesions and persistent expression of Angpt2, Vegf-A and *Tnf-A* in a mouse model

Using a mouse model of *H. pylori*-induced gastritis, we evaluated the mRNA and protein expression levels of pro-inflammatory and pro-angiogenic factors, as well as the histopathological changes in gastric mucosa in response to the infection. Five- to six-week-old female C57BL/6N mice were challenged with *H. pylori* SS1 strain. Animals were euthanized after 5-, 10-, 20-, 30-, 40- and 50- weeks post infection. mRNA and protein

expression of Angpt1, Angpt2, VegfA, *Tnf-A*, bacterial colonization, inflammatory response and gastric lesions were evaluated.

Robust bacterial colonization was observed in 30 to 50 weeks-infected mice, which was accompanied by immune cell infiltration in the gastric mucosa. Compared to non-infected animals, *H. pylori*-colonized animals showed an upregulation in the expression of Tnf-A, Angpt2, and VegfA at the mRNA and protein levels. In contrast, Angpt1 mRNA and protein expression was downregulated in *H. pylori*-colonized mice. The data show that *H. pylori* infection induces the expression of *Tnf-A* and Vegf-A in the murine gastric epithelium. This may contribute to the pathogenesis of *H. pylori*-associated gastritis, however the significance of this should be further addressed.

Paper III: The *TNF-A*–857*T Polymorphism is Associated with Gastric Adenocarcinoma Risk in a Costa Rican Population

In this study, we assessed associations of the polymorphisms *IL-6*-174 G/C, *IFNGR1*-56 C/T, *IL-8-251* T/A and *TNF-A* (-857 C/T, -308 A/G) with gastric pathologies in a high-risk population of GC. DNA samples of 47 patients with GC, 53 with chronic gastritis, 56 with duodenal ulcer, and 94 healthy controls, were genotyped for the five mentioned SNPs. All participants were ≥50-years-old. Genotyping was performed by PCR-RFLP and 5'-nuclease PCR assay. *H. pylori* infection, CagA status, pepsinogen (PG) I and II blood levels were determined by ELISA. Logistic regression analysis was used to determine possible associations of the polymorphisms with cancer, gastritis and duodenal ulcer, and linear regression analysis to determine associations with blood PG levels.

A total of 86.6% of the population was positive for *H. pylori*; of them, 51.6% were CagA+. Patients with the *TNF-A*- 857*T allele had an increased risk for gastritis (OR:3.67, $p = 0.015$) and GC (OR: 6.15, $p = 0.001$). Associations between other polymorphisms and gastric diseases, or PG levels, were not found. The results indicate that the *TNF-A-857**T SNP is associated with the increased risk of GC in Costa Rica.

Paper I

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CLINICAL MICROBIOLOGY - RESEARCH PAPER

Helicobacter pylori infection induces abnormal expression of pro-angiogenic gene *ANGPT2* and miR-203a in AGS gastric cell line

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Abstract

Helicobacter pylori colonizes the stomach and induces an inflammatory response that can develop into gastric pathologies including cancer. The infection can alter the gastric vasculature by the deregulation of angiogenic factors and microRNAs. In this study, we investigate the expression level of pro-angiogenic genes (*ANGPT2*, *ANGPT1*, receptor *TEK*), and microRNAs (miR-135a, miR-200a, miR-203a) predicted to regulate those genes, using *H. pylori* co-cultures with gastric cancer cell lines. In vitro infections of different gastric cancer cell lines with *H. pylori* strains were performed, and the expression of *ANGPT1*, *ANGPT2*, and *TEK* genes, and miR-135a, miR-200a, and miR-203a, was quantified after 24 h of infection (h.p.i.). We performed a time course experiment of *H. pylori* 26695 infections in AGS cells at 6 different time points (3, 6, 12, 28, 24, and 36 h.p.i.). The angiogenic response induced by supernatants of non-infected and infected cells at 24 h.p.i. was evaluated in vivo, using the chicken chorioallantoic membrane (CAM) assay. In response to infection, ANGPT2 mRNA was upregulated at 24 h.p.i, and miR-203a was downregulated in AGS cells co-cultured with different *H. pylori* strains. The time course of *H. pylori* 26695 infection in AGS cells showed a gradual decrease of miR-203a expression concomitant with an increase of *ANGPT2* mRNA and protein expression. Expression of *ANGPT1* and *TEK* mRNA or protein could not be detected in any of the infected or non-infected cells. CAM assays showed that the supernatants of AGS-infected cells with 26695 strain induced a significantly higher angiogenic and inflammatory response. Our results suggest that *H. pylori* could contribute to the process of carcinogenesis by downregulating miR-203a, which further promotes angiogenesis in gastric mucosa by increasing *ANGPT2* expression. Further investigation is needed to elucidate the underlying molecular mechanisms.

Keywords *Helicobacter pylori* · *ANGPT2* · miR-203a · Angiogenesis · *ANGPT1*

Introduction

Helicobacter pylori is a Gram-negative microaerophilic bacterium that colonizes the human stomach. The infection causes inflammation, whose characteristics depend on the type of strain and on the host response against the infection

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[1]. Infection by *H. pylori* is initially presented as chronic gastritis in the antrum, which in some patients can proceed to chronic atrophic gastritis and, in some cases, result in gastric cancer (GC). The progression towards GC involves multiple alterations, including cellular proliferation and differentiation processes, degradation of the extracellular matrix, and angiogenesis [1–3].

Inflammation caused by *H. pylori* infection is initially characterized by the recruitment of neutrophils. This process is facilitated by an increase in the permeability of the vascular endothelium, allowing extravasation of inflammatory cells, proteins, and fluids. *H. pylori* is also a potent activator of nuclear factor-kB (NF-kB) in gastric epithelial cells [4]. Activation of NF-kB by *H. pylori* infection induces a variety of cytokines, angiogenic factors, and matrix metalloproteinases (MMPs) [5].

A major component of gastrointestinal inflammation is the disturbance of the vascular structure and function, as supported by endoscopic and histologic findings related to *H. pylori* infection, including erythema, edema, and vasodilation, as well as inflammatory cell infiltration [6]. Several studies have indicated that *H. pylori* infection can alter the structure and function of the gastric vasculature, and induce angiogenic growth factors, such as vascular endothelial growth factor A (VEGFA) and MMP-9 [7–9]. Neovascularization of the gastric mucosa was shown to be significantly higher in the antrum of *H. pylori*-positive than in *H. pylori*-negative cases in dyspeptic patients, and significantly upregulated expression of inflammatory and pro-angiogenic genes in gastric epithelial cells has been reported in different studies [10–12]. These data imply that *H. pylori* is capable of inducing angiogenesis in its host, which may play a critical role in the development and progression of cancer. However, the process by which the bacterium induces neovascularization in precancerous lesions and early GC development is not fully understood.

Angiogenesis is already present in early GC, but as the cancer progresses toward more advanced stages, angiogenesis becomes more pronounced [13]. Neovascularization not only provides nutrients and oxygen to the tumor cells, and carries away metabolic waste, but it also stimulates tumor growth through autocrine or paracrine modes of action [14, 15]. Endothelial cells differentiate and proliferate under the action of selected growth factors and cytokines, particularly VEGF and angiopoietins (ANGPT, ANG), which are also produced by cancer cells and macrophages (6). ANGPT-1 and ANGPT-2 share the Tie2 (*TEK* gene) receptor in endothelial cells, with opposite effects. ANGPT1 induces maturation and quiescence of vessels, whereas ANGPT2 destabilizes blood vessels for the sprouting of new capillaries [16].

MicroRNAs (miRNAs) are small, non-coding RNAs of 19–25 nucleotides in length that negatively regulate gene expression interacting with the 3′ untranslated region (3′ UTR) of target mRNAs to induce mRNA degradation and translational repression [17]. Consequently, a downregulation of the miRNA produces an upregulation in its target gene; therefore, the upregulation of the miRNA implies the downregulation of the target gene. MicroRNAs modulate multiple cellular activities, including cellular differentiation, cell-cycle control, angiogenesis, and migration. Also, the investigation of tumor-specific miRNA expression profiles has shown their deregulation in diverse cancers [18]. miRNAs have been identified to act as tumor suppressors or as oncogenes based on their modulating effect on the expression of their target genes [19]. miR-203a, miR-200a, and miR-135 are predicted to regulate *ANGPT2*. miR-203a has been reported as tumor suppressor [20–22]; miR-200a and miR-135 have been reported to have tumor suppressor or oncogene function depending on the context [23–25]. So far, there are no reports about the regulation of the *ANGPT2* gene by microRNAs in the context of *H. pylori* infection. In this study, we investigate the expression level of the pro-angiogenic genes *ANGPT1*, *ANGPT2*, and their receptor *TEK*, and microRNAs miR-203a, miR-200a, and miR-135, using GC-cell lines co-cultured with *H. pylori*.

Materials and methods

Cell culture

The gastric cancer cell lines AGS (ATCC, CRL-1739), MKN28 (JCRB0253), and MKN45 (RCB1001RIKEN, BRC Cell Engineering Division, Japan) were maintained in RPMI 1640 or Dulbecco's modified Eagle medium/F12-Glutamax growth media, supplied with 10% fetal bovine serum, 50 IU/mL penicillin, and 50 ug/mL streptomycin (All Gibco). Cells were kept at 37 °C in a humidified incubator with 5% CO_2. The experiments were performed with 400, 000 AGS, 600, 000 MKN28, and 200, 000 MKN45 cells, seeded in 6-well plates and cultured for 48 h before infection, until reaching 90% confluence.

H. pylori culture and *in vitro* infection model

The following *H. pylori* strains were used: 7.13 (kindly provided by Christine Varon, INSERM, University of Bordeaux, France), 26695 (ATCC 700392), and 60190 (ATCC 49503). Insertion mutants with inactivation of cagA (84183ΔcagA) or cagE (84183ΔcagE) were used together with the wild-type strain 84183 (ATCC 53726). These were a kind gift from Professor John Atherton (University of Nottinham, UK) and were constructed as described previously [26]. Strains were inoculated on Columbia agar plates containing

5% sheep blood and incubated at 37 °C for 2–5 days under microaerophilic conditions in a humidified CO2 incubator.

For co-culture experiments, bacteria were added to the gastric cells at a multiplicity of infection (MOI) of 100 bacteria per gastric cell. Cells were infected for 24 h. Additionally, AGS cells were infected for 3, 6, 12, 18, 24, and 36 h with *H. pylori* strain 26695. Prior to infections, cells were washed twice in PBS, and incubated for at least 3 h in antibiotics-free medium with no serum, and co-cultured until RNA and protein were extracted after the induction. As negative controls, monolayers without *H. pylori* were processed in the same way.

Studied genes and microRNAs

The results of a whole-transcriptomic microarray previously performed at IPATIMUP (deposited at Gene Expression Omnibus under accession number GSE70394) [27] to characterize changes in the gene expression of the human gastric carcinoma cell line AGS in response to *H. pylori* infection showed increased expression of *ANGPT2* gene. We chose to study miR-135a, miR-200a, and miR-203a as they showed to be downregulated in human gastric mucosa infected by *H. pylori* in previous reports [28], and are predicted to regulate the *ANGPT2* gene by algorithms as TargetScan [29] and miRbase [30]. We also chose to study ANGPT1 and Tie2 (*TEK*), as they are also related with ANGPT2 pathway.

Quantitative PCR for miR-135a, miR-200a, miR-203a, *ANGPT1*, *ANGPT2* and *TEK*

Total RNA was isolated from cells using miRvana kit (Ambion) or Trizol (Invitrogen) in accordance with the manufacturer's instructions. RNA from each sample was transcribed into cDNA using random primer or specific miRNA primers. TaqMan assays (Thermo Scientific) were used to quantify the expression of miRNAs and the other genes (Table 1). A total of 500 ng of cDNA was used for qRT-PCR amplification using the StepOne Plus thermal cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95 °C for 10 s, 95 °C for 5 s, and 60 °C for 30 s, run for 40 cycles. Levels of cDNA for the microRNAs were normalized by *RNU48* small RNA; for other genes, *GAPDH* was used as endogenous control. Ct values were obtained from independent triplicate co-cultures with three technical replicas of each sample. Fold-difference for infected vs. non-infected cells was estimated by the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Cells were lysed using lysis buffer solution (PBS 1×, 1% Triton X-100, 1% NP-40, Roche Complete™ Mini EDTA-free protease inhibitor cocktail and phosphatase inhibitor cocktail) (Merck, Darmstadt, Germany). Following centrifugation at 14, 000 *g* for 30 min, the proteins in the cell lysates and supernatants were quantified using the protein assay dye reagent concentrate (BioRad), and 30 µg of protein was separated by 7.5% SDS-PAGE, prior to being transferred onto a nitrocellulose membrane (BioRad). Following blocking with 5% skim milk in PBS buffer in 0.05% Tween 20 for 1 h at room temperature, the membranes were then separately incubated overnight at 4 ˚C with the following monoclonal antibodies: Rabbit ANGPT1 (1:500; Abcam ab102015) and Rabbit ANGPT2 (1:500, Abcam ab8452) and Rabbit GAPDH (1:1000, Abcam, ab9485). The secondary antibodies (dilution 1:10 000, horseradish peroxidase-conjugated anti-rabbit, Sigma-Aldrich A9169) were applied at room temperature for 1 h. Labeled bands were detected by enhanced chemiluminescence (Clarity ECL, BioRad, USA) and analyzed with the Chemidoc Imaging System (Bio-Rad). Protein levels were quantified using the Image Lab software, version 4.1 (Bio-Rad, USA).

Table 1 List of TaqMan assays for analyzing gene and microRNA expression by RT-qPCR

Gene/microRNA	Taqman assay ID
ANGPT1	Hs00919202_m1
ANGPT2	Hs001048042_m1
GAPDH	Hs02786624_g1
TEK (Tie2)	Hs00945150_m1
miR-135a	002232
miR-200a	001011
miR-203a	000507
RNU48	001006

In vivo chicken embryo chorioallantoic membrane (CAM) assay

The CAM assay was used to evaluate in vivo, the angiogenic activity of *H. pylori*-treated cells in comparison with non-infected cells. Filtrated supernatants of three independent infections of AGS cells with 26695 strain were tested in two CAM independent experiments, as previously described [31]. Briefly, fertilized chicken (*Gallus gallus*) eggs obtained from commercial sources (Pintobar, Braga, Portugal) were incubated horizontally at 37.8 °C in a humidified atmosphere and referred to embryonic day (E). On E3, a square window was opened in the shell after removal of 2–2.5 ml of albumen to allow detachment of the developing CAM. The window was sealed with transparent adhesive tape, and the eggs returned to the incubator. The experimental setting was designed to decrease intra-animal variability; thus, both test conditions were applied into the same egg. Accordingly,

at E10 and under sterile conditions, two silicon rings were placed in distinct areas of the same CAM, and 10 µl of supernatants, derived from infected and non-infected cells, was applied in each of the inoculation sites, delimited by the rings. The eggs were re-sealed and returned to the incubator for an additional 3 days. At E13, after removing the rings, the CAM containing the inoculation area was excised from the embryos, photographed ex ovo under a stereoscope, at 20 × magnification (Olympus, SZX16 coupled with a DP71 camera). The number of new vessels (less than 20 µm diameter) growing radially towards the ring area was counted blindly to the tested condition. All experiments using chick embryos were carried out in accordance with the Directive 2010/63/EU of the European Parliament and of the Council (22 September 2010) on the protection of animals used for scientific purposes, as well as the National Regulations (Decreto-Lein.º113/2013). Accordingly, experiments with chick embryos do not require approval from any licensing committee.

Statistical analysis

The Mann–Whitney test was used to compare between 2 groups (non-infected vs. infected) and the Kruskal–Wallis test was used to compare between groups. For analysis of the CAM assay experiments, the paired Student *t* test was used. Data is presented as mean ± standard deviation (SD). The analysis was performed using the GraphPad Prism 9 software (San Diego, CA, USA).

Results

H. pylori infection enhances expression levels of *ANGPT2* mRNA in AGS, MKN28, and MKN45 cells

A previous whole-transcriptomic microarray to characterize changes in the gene expression of AGS cells in response to *H. pylori* an upregulation of ANGPT2 upon infection [27]. Based on those results, here, we chose to study the *ANGPT1/2* and Tie2 pathway in gastric cell lines in the context of *H. pylori* infection.

First, the effect of *H. pylori* infection on *ANGPT2* expression in vitro after 24 h of co-culture was addressed in GC cells. In AGS and MKN28 cells, *ANGPT2* expression was upregulated in response to infection with distinct *H. pylori* strains, namely, 26695, 60190, 84183, and 7.13 strains ($p < 0.001$, Fig. 1a; $p < 0.005$, Fig. 1b). We additionally tested the effect of *H. pylori* 26695 and 7.13 strains on MKN45 cells, and it was found that *ANGPT2* mRNA was also upregulated in this cell line 24 h.p.i ($p < 0.001$; Fig. 1c).

ANGPT2 induction is CagA-independent

To assess if the induction of *ANGPT2* depends on the bacterial virulence factor CagA, we performed infections with mutants deficient in *cagA* (not expressing CagA) or in *cagE* (not expressing a functional type IV secretion system – T4SS). There were no differences in the induction of *ANGPT2* expression between the wild-type and both 84183 mutants (Fig. 1a, $p = 0.933$; 1b, $p = 0.800$).

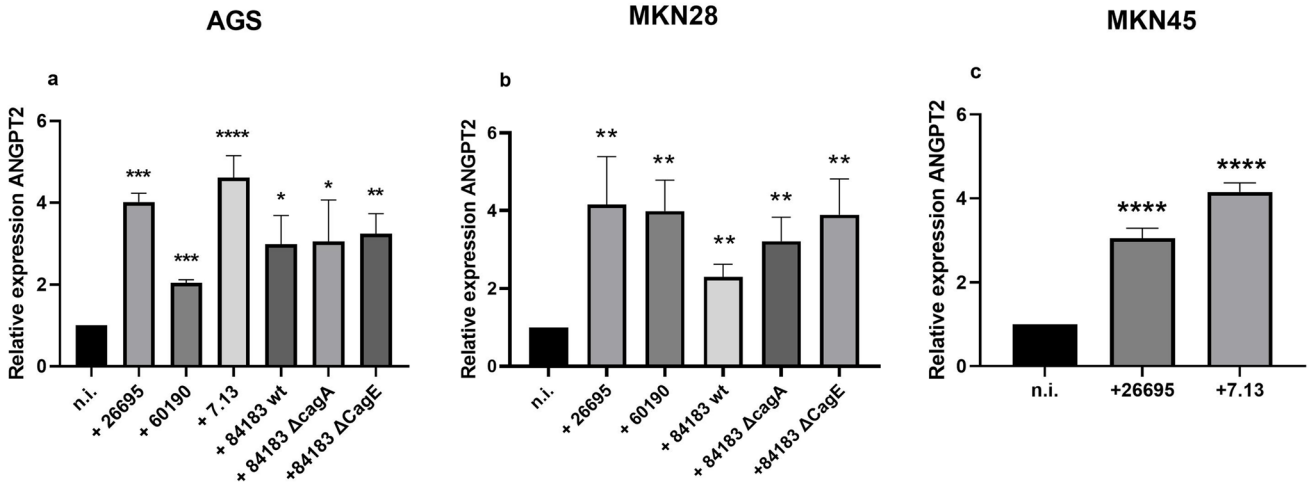

Fig. 1 *ANGPT2* mRNA expression increased in *H. pylori*-treated AGS (**a**), MKN28 (**b**), and MKN45 (**c**) gastric cell lines in a CagA-independent pathway. AGS, MKN28, and MKN45 gastric cells were incubated with *H. pylori* at a MOI of 100 for 24 h, with *H. pylori* 26695, 60190, 7.13, 84183wt, 84183ΔcagA, and 84183ΔcagE strains. Cells were analyzed to measure the relative expression levels of *ANGPT2* mRNA compared with *GAPDH* mRNA, in infected cells compared with non-infected AGS cells.****$p < 0.0001$; ***$p < 0.001$; **$p < 0.005$; *$p < 0.05$, compared to non-infected cells

ANGPT1 and TEK expression was not detected in gastric cell lines

The expression of *ANGPT1* and *TEK* in AGS, MKN28, and MKN45 cells was also assessed. In AGS and MKN28 cells, *ANGPT1* and *TEK* expression was detected at extremely low levels, and there was no change upon *H. pylori* infection. In MKN45 cells, the expression of *ANGPT1* and *TEK* was not detected at all in infected or non-infected cells, 24 h.p.i (data not shown). The protein ANGPT1 was not detected by Western blot analysis in cell lysates or in supernatants of AGS cells, on infected nor non-infected cells, at any time of infection with *H. pylori* 26695 (data not shown). These results suggest that AGS, MKN28, and MKN45 cells do not express the *ANGPT1* and *TEK* genes.

H. pylori infection alters expression levels of microRNAs in AGS and MKN28 cells

In concordance with the biological function of microRNAs, if a microRNA is the regulator of a gene, its downregulation implies the upregulation of its target gene, or *vice versa* [18]. According to this, as we found that *ANGPT2* is upregulated in response to *H. pylori* infection, miR-135a, miR-200a, and miR-203a must be expected to be downregulated. In MKN28 and AGS cells, miR-135a and miR-200a were upregulated in response to infection by *H. pylori* (Fig. 2a and b) (data not shown for miR-135a). Also, in MKN28 cells, *H. pylori* infection upregulated miR-203a (Fig. 2c). On the other hand, *H. pylori* infection downregulated miR-203a and upregulated *ANGPT2* expression in AGS cells (Fig. 2d). Consequently, it was decided to continue studying miR-203a and *ANGPT2* in AGS cells infected with 26695 strains.

Kinetics of expression of *ANGPT2* and *miR-203a* in *H. pylori* infected cells

In order to analyze the dynamics of the expression levels of *ANGPT2* and miR-203a, we performed a time course experiment of AGS infected with *H. pylori*. The increase in *ANGPT2* mRNA expression started at 18 h.p.i., and stabilized at 24 h, when the expression of *ANGPT2* reached a fold difference of almost 5 × relative to non-infected cells ($p < 0.0001$) (Fig. 3a and d). On the contrary, the expression of miR-203a decreased gradually, becoming evident at 12-h post-infection (h.p.i.) (Fig. 3c and d). To assess whether *H.*

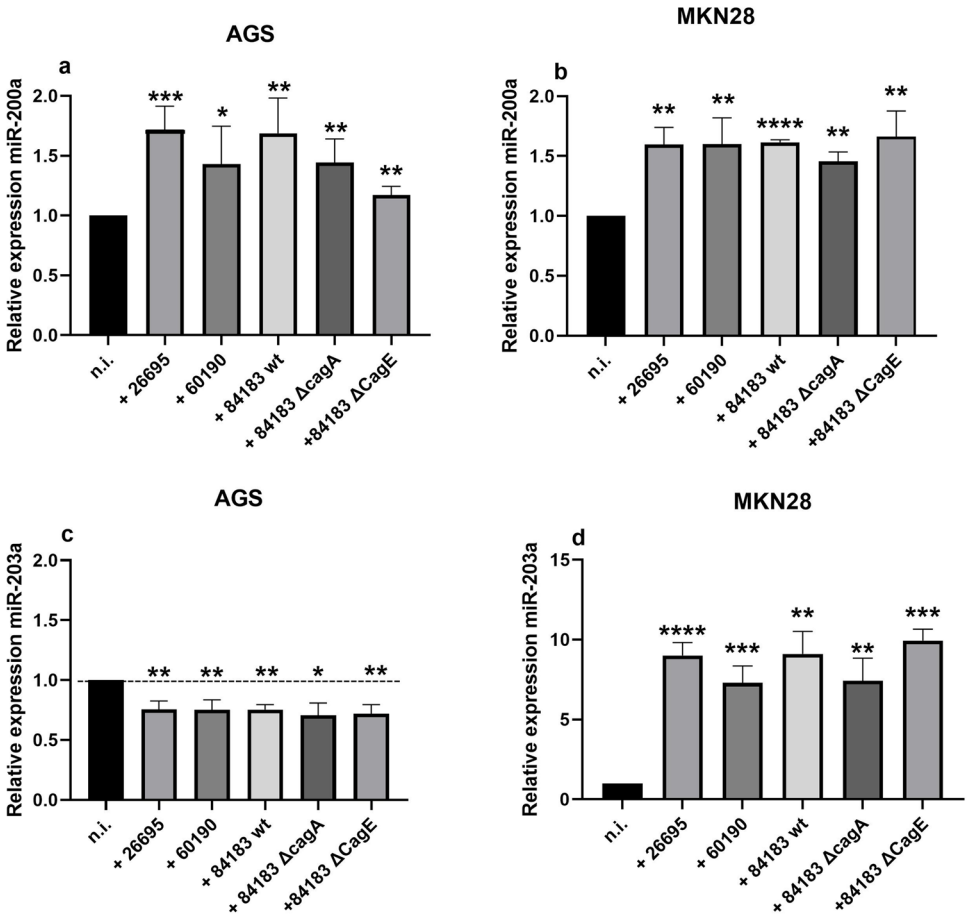

Fig. 2 *H. pylori* alters the expression of microRNAs *in vitro*. **a** and **b** miR-200a expression is increased in *H. pylori*-treated MKN28 and AGS cell lines. **c** and **d** miR-203a is upregulated in MKN28 and downregulated in AGS cell lines. AGS and MKN28 gastric cells were incubated with *H. pylori* at a MOI of 100 for 24 h, with 26695, 60190, 84183wt, 84183ΔcagA, and 84183ΔcagE strains. Cells were analyzed to measure the relative expression levels of microRNAs compared with RNU48 RNA, in infected cells compared with non-infected cells. ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.005$; *$p < 0.05$, compared to non-infected cells

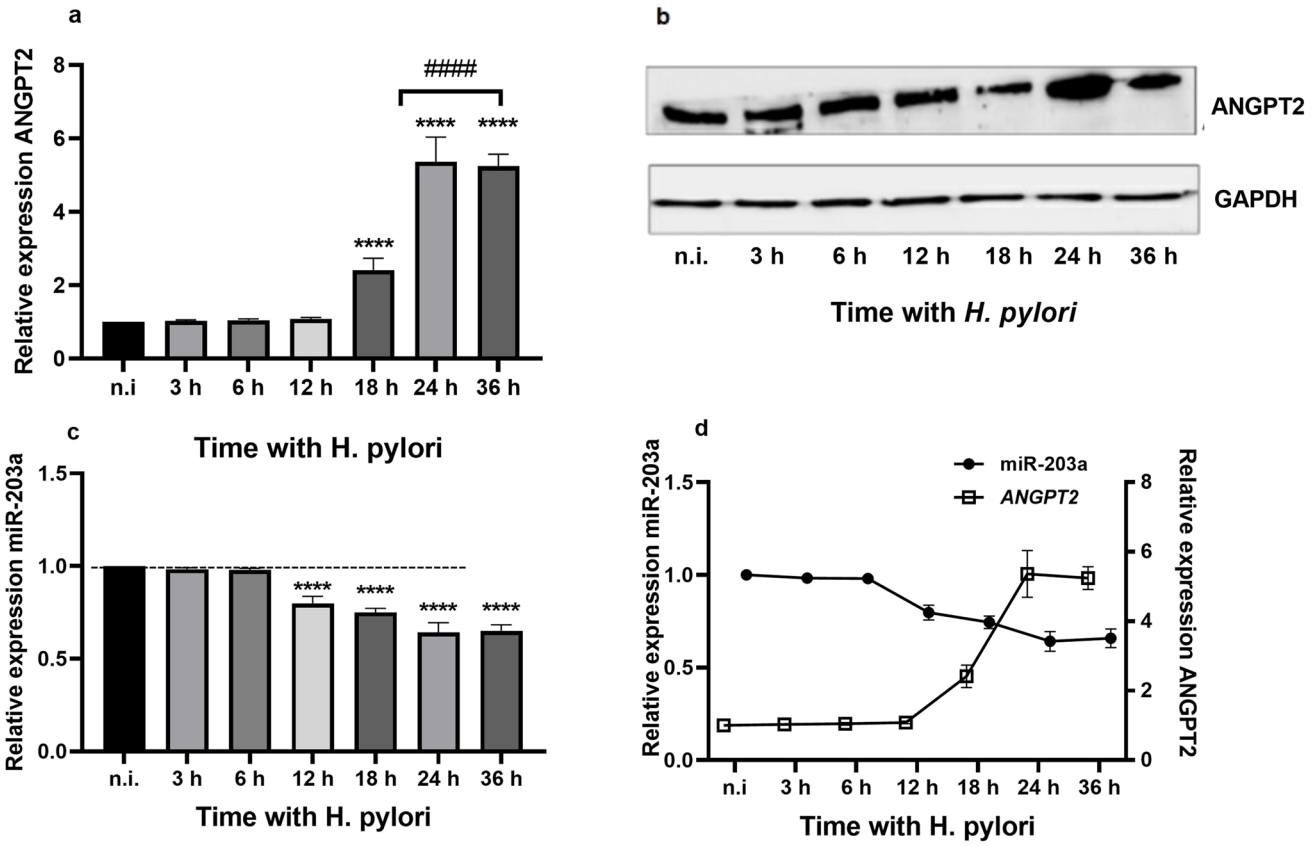

Fig. 3 Time-dependent induction of *ANGPT2* mRNA and protein levels, and miR-203a, by *H. pylori* 26695 in AGS cells. **a** *ANGPT2* mRNA expression increased overtime in *H. pylori*-treated cells, reaching significance after 18 h in comparison to the initial time point (0 h). **b** Western blot analysis of *H. pylori* induced ANGPT2 protein expression in AGS cells over time. **c** miR-203a expression was downregulated over time in *H. pylori*-treated cells, reaching significance after 12 h.p.i. until 36 h.p.i. **d** Concurrently with the downregulation of miR-203a by *H. pylori*, there was an increase in the expression of ANGPT2. AGS gastric cells at 90% confluence were incubated with *H. pylori* at a MOI of 100 for 3, 6, 12, 18, 24, and 36 h. Cells were analyzed to measure the relative expression levels of *ANGPT2* mRNA compared with *GAPDH* mRNA, in infected cells compared with non-infected AGS cells at the same time points. #### or ****$p < 0.0001$. Protein levels were determined in total cellular protein extracts by Western blotting

pylori upregulation of *ANGPT2* also occurred at the protein level, its expression was studied by Western blot, where a significant increase of the ANGPT2 protein was detected at 24 h.p.i (Fig. 3b).

H. pylori infection increases angiogenesis *in vivo*

To evaluate the effect of *H. pylori* infection in the potential angiogenic response of AGS cells, the in vivo chick embryo chorioallantoic membrane (CAM) model was used. The angiogenic response of supernatants from AGS cells infected for 24 h with *H. pylori* 26695, versus uninfected cells, was quantified by counting the number of neovessels recruited to the inoculation site. Conditioned media derived from *H. pylori*-infected AGS cells elicited a significantly higher angiogenic response than supernatants of non-infected cells ($p = 0.004$; Fig. 4a). Although not defined as a primary readout of the CAM assay, it was also possible to evaluate the inflammatory/reaction area of the CAM inoculation sites. Using the Cell Sens Olympus software, the reaction areas were quantified, and CAM sites treated with supernatants derived from *H. pylori*-infected AGS cells showed a significantly higher response than uninfected cells ($p < 0.0001$; Fig. 4b).

Discussion

The gastric carcinogenic process is characterized by numerous genetic and epigenetic changes, including oncogene activation, loss of tumor suppressor and DNA repair genes, and overexpression of angiogenic, inflammatory, and growth factors [1, 6, 32–34]. miRNAs may play a role in this process, by inhibiting the expression of target genes involved

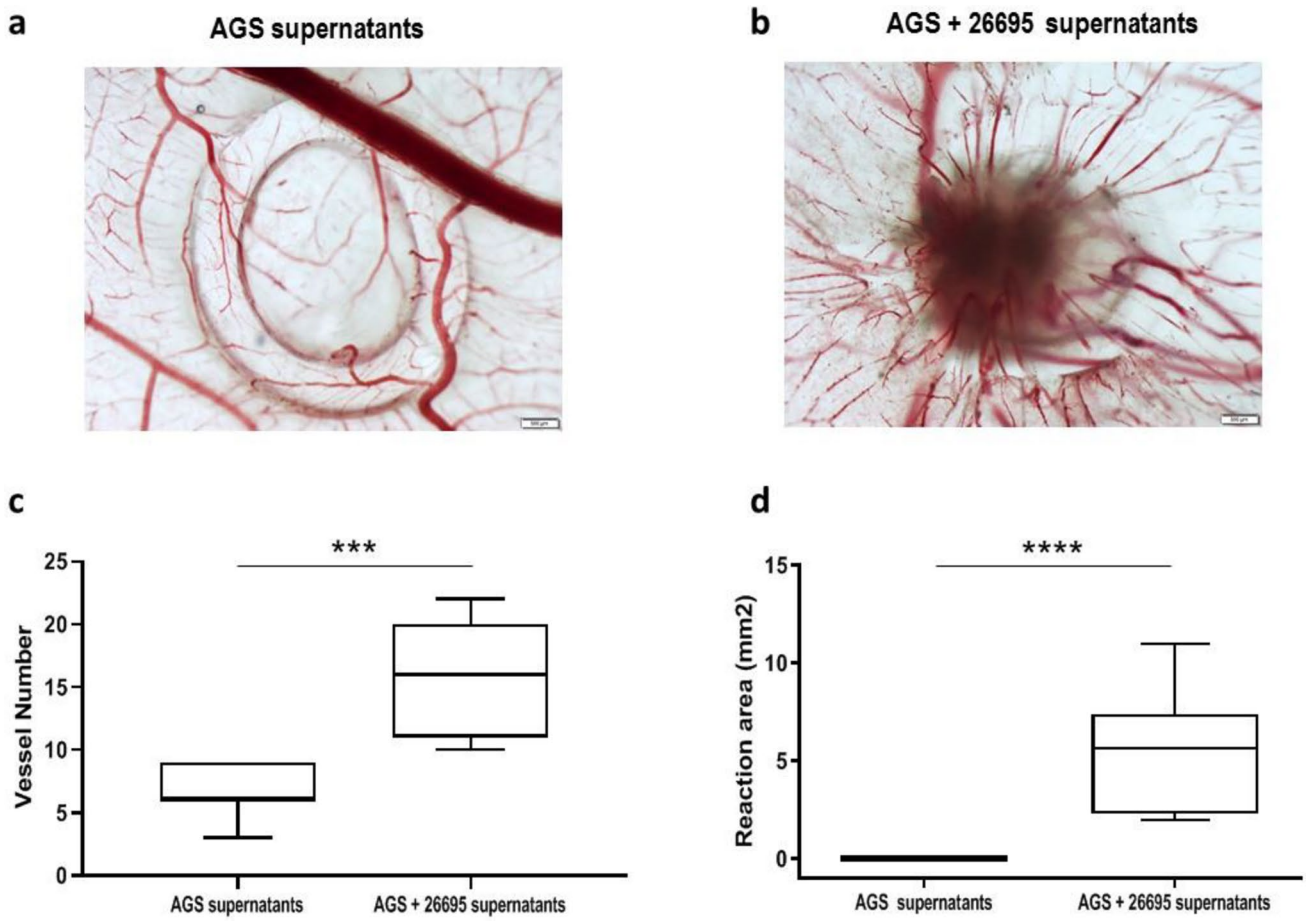

Fig. 4 Chicken embryo choriollantoic membrane (CAM) assay. **a**, **b** Stereomicroscope images of excised wounded CAMs representative of the two conditions tested pairwise, in the same egg, supernatants of *H. pylori* 26695-infected AGS cells and supernatants of uninfected AGS cells, 3 days after inoculation (scale bar = 100 µm); **c** quantification of newly formed blood vessels (< 20 µm in diameter) and **d** reaction areas at the inoculation sites. Supernatants of *H. pylori* 26695-infected AGS cells induce a significantly increased angiogenic (**c**) and inflammatory (**d**) responses in the CAM assay. Data regards 12 eggs (12 pairs of samples) from two independent experiments. ***$p < 0.001$, ****$p < 0.0001$ (pair *t* test)

in the signaling pathways. Previous studies using different in vitro models have indicated that the expression of some pro-angiogenic factors is altered by *H. pylori* infection, in gastric cancer cells and in endothelial cells [8, 10, 11, 31, 35]. Using an in vitro co-culture system, this study describes the changes in the expression of the pro-angiogenic gene *ANGPT2* and its predicted regulator miR-203a, in response to infection by *H. pylori*.

In the presence of VEGFA, ANGPT2 destabilizes established blood vessels through the interruption of Tie2 signaling, inducing removal of pericytes, and triggering the permeability of endothelial tubes [36, 37]. ANGPT2 is expressed in endothelial cells and macrophages, but also in cancer cells in vitro and in vivo. *ANGPT2* mRNA and protein have been reported as upregulated in several gastric cancer cell lines such as AZ521, SOH [38], NCI-87 [39], AGS, and SGC7901 [40]. Here, we consistently observed an upregulation of *ANGPT2* mRNA in AGS, MKN28, and MKN45 cells, upon infection with different *H. pylori* strains, showing that this effect is cell line and bacterial strain is independent. Furthermore, upregulation of *ANGPT2* by *H. pylori* is independent of the bacterial virulence factors CagA and T4SS. We additionally observed that *H. pylori* induces increasing *ANGPT2* mRNA and protein expression levels in time course experiments performed in AGS cells.

In agreement with our findings, Leite et al. [31] showed that MKN74 gastric cells infected with *H. pylori* 60190 triggered a strong angiogenic response, increasing the expression of numerous pro-angiogenic proteins, including ANGPT2, interleukin-8, VEGFA and VEGFC, platelet-derived growth factor, fibroblast growth factor-1, -2, and -4, and plasminogen activator urokinase (uPa), among

others. In contrast to our findings, Kim et al. [41] reported that *ANGPT2* mRNA was constitutively expressed in AGS cells but was not regulated by *H. pylori*, although data on *ANGPT2* expression upon infection was not shown. Such differences may be related to various aspects of the experimental setting, including differences in the *H. pylori* strain used.

The CagA oncoprotein, which is delivered into the host cell by the T4SS, is among the best-studied *H. pylori* virulence factors with impact on multiple host cellular responses. Our data suggest that *ANGPT2* upregulation by *H. pylori* is mediated by factors other than CagA or the T4SS. *H. pylori* has numerous other virulence factors that could be involved in the induction of ANGPT2, some of which are factors associated with gastric pathology, including the cytotoxin VacA, the adhesins BabA and SabA, and the outer membrane protein OipA, reviewed elsewhere [42]. Actually, Olivera-Severo et al. [35] showed an increase in ANGPT2 protein and in other pro-angiogenic factors upon exposure of AGS cells to purified *H. pylori* urease from strain 26695. They further demonstrated that *H. pylori* urease induced formation of tube-like structures by HUVECs and intense neovascularization in the in vivo CAM assay model. Similarly, when we evaluated the angiogenic potential and inflammatory response of conditioned medium of *H. pylori*-infected cells in the CAM assay, we verified significant increases in the number of newly formed vessels and in the inflammatory reaction area. This suggests that pro-angiogenic factors are secreted to the medium directly by *H. pylori* or indirectly by the host cells upon infection.

As we found an interesting upregulation of *ANGPT2* gene and protein in response to *H. pylori* infection, we hypothesized that in our model it could be possible that other angiogenesis-related factors could be altered by the infection. In contrast with the results of Wang et al., who reported the expression of both *ANGPT1* and *TEK* mRNA and protein in AGS, MKN45, and other gastric cells [40], we could not detect the expression of *ANGPT1* or *TEK* mRNAs in any of the studied gastric cell lines. ANGPT1 is an activator of tyrosine kinase receptor Tie2 responsible for a quiescent vascular phenotype and is known as an endothelial survival and vascular stabilization factor, necessary for the maturation of newly formed vessels [43]. It is produced by pericytes and immune cells [37, 44] and is also expressed in several cancer cell lines and tissues [45, 46]. As the ANGPT2 and ANGPT1 proteins antagonize for the same receptor, our observation that in the same gastric cell line there is an upregulation of ANGPT2, but no expression of ANGPT1, suggests that a gain in ANGPT2 activity over ANGPT1 might be an initiating factor for tumor angiogenesis. The tumor angiogenic environment in vivo is characterized by a deregulation of ANGPT1/ANGPT2 ratio, with a bias towards ANGPT2 [40]. Overproduction of ANGPT2 drives the destabilization of existent vasculature to create new vessels [36]. ANGPT1 is also overproduced but is insufficient for the adequate maturation of new abnormal vessels, which have few pericytes and become tortuous and leaky [36, 44, 47]. Elevated plasma levels of ANGPT2, but not ANGPT1, have been associated with metastasis and worse prognosis in cancer patients, as well as with resistance to anti-VEFG therapy [48]. This suggests that an early high production of ANGPT2 rather than ANGPT1 may favor tumoral fitness.

Previous reports, and our bioinformatics analyses, predicted miR-203a as a regulator of the *ANGPT2* gene. Our work is the first report for a significant downregulation of miR-203a in response to *H. pylori* infection in AGS cells, which coincided with the upregulation of *ANGPT2* gene, in a time-dependent manner. miR-203a is expressed specifically in epithelial cells and is considered as a tumor suppressor, as it has been shown to be downregulated in vitro and in vivo in several cancers, including colorectal, lung, and esophageal cancer [20, 21, 49]. Significant downregulation and hypermethylation of miR-203a gene promoter were found in patients with advanced GC, associated with poor patient survival [50]. miR-203a was also shown to be aberrantly downregulated in *H. pylori*-positive tissues and cancer-derived cell lines, including AGS and MKN45, also regulating the *CASK* oncogene [51]. Furthermore, the reduced expression of miR-203a was shown to increase angiogenesis in vitro and in vivo in human hepatocellular carcinoma, via the *HOXD3* gene, through the upregulation of the VEGFR signaling pathway [52]. These data, together with ours suggest that miR-203a, as other microRNAs, have numerous targets and participate in several carcinogenic pathways, and could therefore be a key master regulator of angiogenesis, invasion, and metastasis.

The results presented here suggest that *H. pylori* could contribute to the process of carcinogenesis by downregulating miR-203a, which further promotes angiogenesis in gastric mucosa by increasing *ANGPT2* expression, favoring the process of chronic inflammation that has important functional implications for the etiology of GC. The molecular mechanism by which miR-203a could regulate the development of *H. pylori*-associated GC, by *ANGPT2* deregulation, is not yet clear. It has been demonstrated that NF-κB can promote angiogenesis, invasion, and metastasis in several types of cancer [53]. It has been observed that miR-203a is downregulated in a NF-κB-dependent mechanism in Epstein Barr virus-related nasopharynx cancer [49]. Although in this study we did not examine the pathways or mechanisms involved, it can be hypothesized that *H. pylori*-dependent activation of NF-κB could deregulate miR-203a and upregulate *ANGPT2* (Fig. 5). Further investigation is needed to clarify this question.

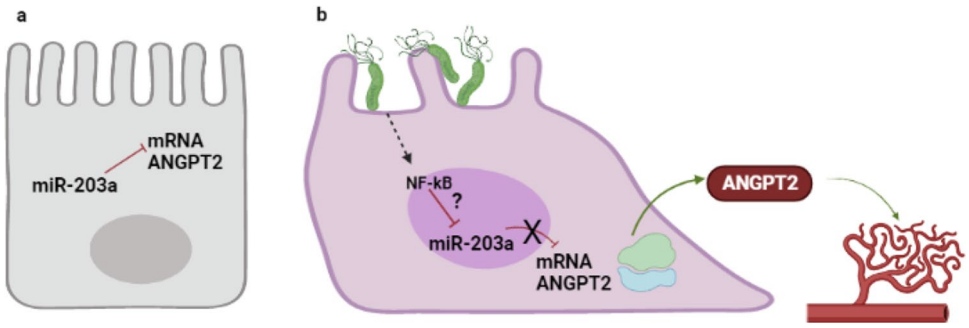

Fig. 5 Model for *H. pylori*-induced downregulation of miR-203a and upregulation of *ANGPT2* in AGS cells. **a** Uninfected AGS cells show a basal expression of ANGPT2 and miR-203a. **b** In an analogous way to the EBV infection model [49], *H. pylori* infection may activate the NF-κB pathway that could then downregulate miR-203a which would allow the translation of ANGPT2 mRNA, by its destabilizing effect in near vasculature. Created with BioRender.com

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Author contribution Wendy Malespín-Bendaña: Conceptualization, Methodology, Investigation, Formal analysis, Writing-original draft. Rui M. Ferreira: Methodology, Writing-review and editing, Funding acquisition. Marta T. Pinto: Methodology, Formal analysis, Writing-review and editing. Ceu Figueiredo: Conceptualization, Funding acquisition, Project administration, Writing-original draft. Warner Alpízar-Alpízar: Conceptualization, Writing-review and editing. Clas Une: Conceptualization, Writing-review and editing. Lucía Figueroa Protti: Methodology, Writing-review and editing. Vanessa Ramírez: Conceptualization, Funding acquisition, Project administration, Formal analysis, Writing-original draft.

Data Availability Datasets are available on request.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Conflict of interest The authors declare no competing interests.

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Helicobacter pylori infection induces gastric precancerous lesions and persistent expression of Angpt2, Vegf-A and *Tnf-A* in a mouse model

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Introduction: *Helicobacter pylori* colonizes the gastric mucosa and induces chronic inflammation.

Methods: Using a mouse model of *H. pylori*-induced gastritis, we evaluated the mRNA and protein expression levels of proinflammatory and proangiogenic factors, as well as the histopathological changes in gastric mucosa in response to infection. Five- to six-week-old female C57BL/6N mice were challenged with *H. pylori* SS1 strain. Animals were euthanized after 5-, 10-, 20-, 30-, 40- and 50-weeks post infection. mRNA and protein expression of Angpt1, Angpt2, VegfA, Tnf-α, bacterial colonization, inflammatory response and gastric lesions were evaluated.

Results: A robust bacterial colonization was observed in 30 to 50 weeks-infected mice, which was accompanied by immune cell infiltration in the gastric mucosa. Compared to non-infected animals, *H. pylori*-colonized animals showed an upregulation in the expression of *Tnf-A*, *Angpt2* and *VegfA* at the mRNA and protein levels. In contrast, *Angpt1* mRNA and protein expression was downregulated in *H. pylori*-colonized mice.

Conclusion: Our data show that *H. pylori* infection induces the expression of Angpt2, *Tnf-A* and Vegf-A in murine gastric epithelium. This may contribute to the pathogenesis of *H. pylori*-associated gastritis, however the significance of this should be further addressed.

KEYWORDS

Helicobacter pylori*, angiogenesis, ANGPT1, ANGPT2, VEGFA, mouse model, *TNF-A

1 Introduction

Angiogenesis, the process of formation of new blood vessels from the pre-existing, involves proliferation, sprouting and migration of endothelial cells, as well as degradation of the basement membrane. After the re-establishment of cell junctions and coverage of pericytes, the newly formed vessels mature and remain quiescent (1). Importantly, endothelial cells start proliferating to initiate angiogenesis only after stimulation by several growth factors and inflammatory mediators, mainly members of vascular endothelial growth factor (VEGF) family and angiopoietins.

Angiogenesis and tumor promoting inflammation are closely interconnected and are crucial events during cancer progression and metastasis; in fact, they are both considered as hallmarks of cancer (2). Both tumor and stromal cells produce angiogenic factors that ultimately lead to endothelial cell proliferation. These cancer-associated endothelial cells cooperate in the perpetuation of inflammation, which reciprocally promotes angiogenesis by secretion of cytokines, proteases, growth and proangiogenic factors, thus creating a positive feedback loop (1, 2). Aberrant vascular structures can also induce hypoxia, acidosis and DNA damage (3), which contribute to the establishment of a tumor-promoting microenvironment since very early in the carcinogenesis. This could be particularly relevant in cancers in which chronic and persistent inflammation is the main driving force for the malignant transformation, for example in gastric carcinogenesis.

VEGFA is the most potent and ubiquitous member of VEGF family; it is considered as the master inductor of physiological and tumor angiogenesis (4). Secreted by macrophages, lymphocytes, fibroblast and tumor cells, it promotes proliferation, migration and survival of endothelial cells, as well as expression of extracellular matrix proteases (5). Angiopoietin 1 (ANGPT1) and ANGPT2 bind to TIE1-TIE2 (tyrosine kinase with immunoglobulin-like and epidermal growth factor–like domains 1 and 2) receptor complex and $\alpha5\beta1$ integrin in endothelial cells, thus activating signaling pathways that lead to blood and lymphatic vessel formation. ANGPT1 is secreted by pericytes and acts in a paracrine manner to keep homeostasis of the mature vasculature. ANGPT2 is mostly produced by endothelial cells, in which it is stored at the cytoplasmatic Weibel-Palade bodies, and released after stimulation, to act in an autocrine way (6). ANGPT2 plays a role as a vessel destabilizing agent that induces permeability and leads to dissociation of cell-cell contacts, allowing the sprouting of new vessels (1, 7). The expression of ANGPT2 in normal tissues is low or absent, but it is upregulated in many cancers in which it is mainly expressed by tumor-associated macrophages (6). In humans, alternative splicing generates a smaller isoform, $ANGPT2_{443}$, which has been reported as upregulated in tumor cell lines and human tumor tissue (8, 9).

TNF-α is a key mediator in a host's response against gram-negative bacteria, such as *H. pylori*. It is produced by lymphocytes and macrophages and promotes leukocyte recruitment. In the context of angiogenesis, TNF-α primes endothelial cells for angiogenic sprouting by inducing a tip cell phenotype (10).

Helicobacter pylori colonization of the gastric mucosa induces chronic and persistent inflammation. This is associated with several clinical outcomes, such as peptic ulcer, MALT-lymphoma and gastric cancer (GC). The latter results from a stepwise cascade of preneoplastic lesions known as Correa's cascade (11, 12). The persistent inflammation mounted in response to this bacterial infection is characterized by the infiltration in the gastric mucosa of neutrophils, lymphocytes and macrophages. Increased permeability of the endothelium is a pivotal event for the extravasation of immune cells, proteins and fluids (13).

Several reports indicate that *H. pylori* can induce the production of proangiogenic factors *in vitro* and *in vivo* (14–18). Specifically, the expression of HIF-1a, VEFGA and the density of CD31+ blood vessels is higher in *H. pylori*-positive patients, compared to negative persons (19, 20). Those findings suggest that the bacterium is capable of inducing a proangiogenic response after colonizing its host. This could be of pivotal importance during gastric cancer initiation and development. However, the molecules and mechanisms by which *H. pylori* induces early neovascularization in gastric mucosa, and how this influences the precancerous series of events that precede GC is not completely understood.

The present study, we used a mouse model in order to characterize the expression kinetics of *Tnf-A, Angpt1, Angpt2* and *VegfA* in the gastric mucosa in response to *H. pylori* infection and to explore the correlation to inflammation, vascularization and gastric pathology.

2 Methods

2.1 Animals

Thirty-nine C57BL/6N female mice certified as *Helicobacter spp*-free were acquired from Harlan Laboratories® and housed at the Laboratorio de Ensayos Biológicos (LEBi), University of Costa Rica, at standard temperature (25.5°C ± 1.20°C) and humidity (50–70%), and maintained on a 12-h light/dark cycle (lights off at 6:00 p.m). Water and food (LabDiet 5010.) were provided *ad libitum*. Mice were distributed into 7 experimental groups, each consisting of 5-7 animals per cage (Figure 1) and were moved to an experimental section one week before they were inoculated with *H. pylori*. Experimental procedures and methods were carried out in accordance with the guidelines of the Costa Rican Ministry of Science and Technology and were approved by the Institutional Committee for Animal Care and Use of Animals (CICUA) of the University of Costa Rica (permission number CICUA-031-17).

2.2 Bacterial culture and inoculation

The inoculation was performed with SS1 strain (mouse-adapted cagA+, vacA+ strain with no functionality of the cag pathogenicity island; kindly donated by Dr. James Fox, MIT, USA). Bacteria were grown on Skirrow plates and incubated at 37°C for 5-7 days under microaerophilic conditions. The SS1 strain was harvested and mice were inoculated by oral gavage with a single dose of approximately 10^9 bacteria (CFU) in 0.2 mL of 0.9% NaCl. Control mice received 0.2 mL of the saline solution only. The bacteria used for inoculations were isolated from previously colonized mice from our bioterium.

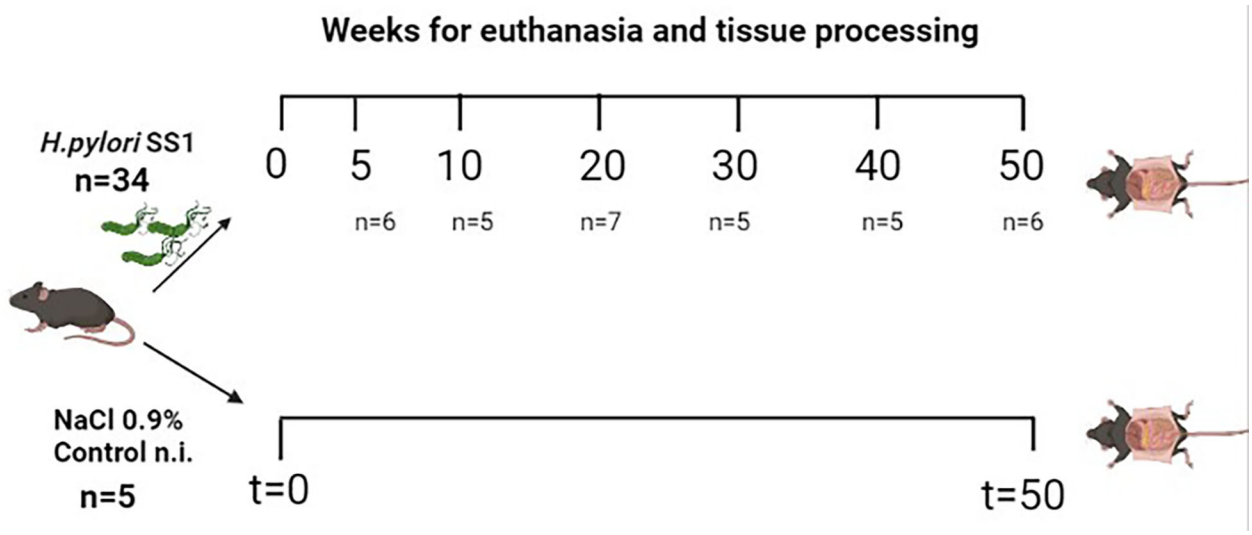

FIGURE 1
Schematic view of the experimental groups of the mouse model of *Helicobacter pylori* SS1 infection used in the study. A total of 39 female C57BL/6N mice were divided into 7 groups (n per group indicated between parentheses), 6 of these groups were infected with *H. pylori* and one group received NaCl 0.9% only (control group, n=5). Mice were euthanized between 5-50 w.p.i. Control non-infected mice were sacrificed at 50 w.p.i. Created with BioRender.com.

2.3 Resection and processing of gastric tissue for histology

Infected groups were euthanized by cervical dislocation after 5-, 10-, 20-, 30-, 40- and 50- weeks post-infection (w.p.i.). The control group was euthanized at 50 w.p.i. Entire stomachs were opened along the greater curvature, washed with PBS, and cut longitudinally into four stripes, extending from the squamous forestomach through the duodenum. The stripes were used as follows: one for confirmation of bacterial colonization (culture), one was cut in halves for RNA and protein isolation, and two for immunohistochemistry assays.

2.4 Culture for confirmation of *H. pylori* colonization

The stripe was macerated with saline solution, vortexed 5 s, then 40 µl of the suspension was seeded in both Skirrow and agar-blood BHI plates and incubated at 37°C for 5-7 days under microaerophilic conditions.

2.5 Quantitative real time PCR

The stomach tissue was suspended in RNAlater (Invitrogen) until utilization. Total RNA was isolated with Trizol (Invitrogen) in accordance with the manufacturer's instructions. RNA from each sample was transcribed into cDNA using High-capacity cDNA reverse transcription kit (ThermoFisher Scientific). A total of 0,5 µl cDNA was used for qRT-PCR amplification using TaqMan Gene Expression Assays (Thermo Fisher Scientific) for *Angpt1* (Mm00456503_m1), *Angpt2* (Mm00545822_m1), *Tnf-A* (Mm00443258_m1) and *VegfA* (Mm00437306_m1) with *Gapdh* (Mm99999915_g1) as endogenous control, using the StepOne Plus thermal cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95°C for 10 sec, 95°C for 5 sec, and 60°C for 30 sec, run for 40 cycles. Fold-difference for infected *vs.* non infected mice was estimated by the $2^{-\Delta\Delta Ct}$ method.

2.6 Western blot analysis

Stomach tissues were macerated and lysed using lysis buffer solution (PBS 1X, 1% Triton X-100, 1% NP-40, proteases and phosphatases cocktail inhibitors, pH 7.4). Following centrifugation at 14 000 g for 30 min, the proteins were quantified using the Protein Assay Dye Reagent Concentrate (BioRad) and 50 µg of protein were separated by 10% SDS–PAGE, prior to being transferred onto a nitrocellulose membrane (BioRad). Following blocking with 5% fat –free milk in PBS buffer in 0.05% Tween 20 for 1 h at room temperature, the membranes were then separately incubated overnight at 4˚C with the following monoclonal antibodies: Rabbit ANGPT1 (1:500; Abcam ab102015), Rabbit ANGPT2 (1:500, Abcam ab8452) and Rabbit GAPDH (1:1000, Abcam, ab9485). The secondary antibodies were applied (dilution 1:10 000, horseradish peroxidase-conjugated anti-rabbit, Sigma-Aldrich A9169) at room temperature for 1 h. Labeled bands were detected by enhanced chemiluminescence (Clarity ECL, BioRad, USA) and analyzed by Chemidoc Imaging System (Bio-Rad). Protein levels were quantified using the Image Lab software, version 4.1 (Bio-Rad, USA).

2.7 Immunohistochemical detection of Angpt1, Angpt2, VegfA, CD31 and *H. pylori*

The stinging protocols were a modification of Alpízar-Alpízar et al. (21). Two stomach stripes were fixed 24 hours in 4% paraformaldehyde and paraffin embedded. Then, 4 µm tissue

sections were deparaffinized in xylene and hydrated in a gradual series of ethanol-water dilutions. For *H. pylori*, sections were pretreated with Proteinase K (10 µg/mL) for 15 min, 37°C. For CD31, Angpt1, Angpt2 and VegfA, sections were pretreated at 98°C for 15 min in 10 mM sodium citrate pH 6.0. In all cases, endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 solution for 15 min. The primary antibodies were diluted in Antibody Diluent (Dako, code S3022) and incubated overnight at 4°C in Shandon racks (Thermo Shandon, Pittsburg, PA, USA) at the following dilutions: Rabbit-anti *H. pylori* 1:150 (Dako, code B0471), Rabbit anti-Angpt1 1:500, Rabbit anti-Angpt2 1:1000, Rabbit anti-CD31 1:250, Rabbit anti-VegfA 1:200. All primary antibodies were detected with EnVision reagent anti-rabbit IgG horseradish peroxidase-conjugated polymers (Dako Code: K3468). Each incubation step was followed by washes in TBS containing 0.5% (v/v) Triton X-100. Finally, the reactions were visualized by incubating the sections with Liquid DAB+ substrate chromogen system (Dako, Code S3022) and counterstained with Mayer's hematoxylin.

2.8 *H. pylori* colonization, histopathology and immunohistochemistry evaluations

H. pylori colonization was evaluated according to the number of gastric glands containing bacteria and their density, as follows: 0: No observed bacteria; 1: Occasional pits and/or glands with individual bacteria; 2: frequent pits and/or glands with individual bacteria; 3, infrequent pits and/or gland with dense bacterial colonies; and 4, frequent pits and/or glands with dense bacterial colonies (22). Sections were stained with hematoxylin and eosin (H&E) for histopathological evaluations of the mouse gastric mucosa describing inflammation, infiltration, metaplasia, and anatomical localization of the lesions, using published guidelines (23): 0, no infiltration, 1, patchy or multifocal small islands of inflammatory cells in the mucosa and/or submucosa; 2, coalescing aggregates of inflammatory cells in submucosa or mucosa; 3, organizing nodules of lymphocytes and other inflammatory cells in submucosa and mucosa; 4, follicles and/or sheets of inflammatory cells extending into or through muscularis propria adventitia. Photomicrographs were captured using the bright field microscope Motic BA400 and Motic Images Plus 3.0 software (China).

2.9 Statistical analysis

Data for gene expression is represented as the mean ± standard deviation for every experimental group. Data for inflammation and *H. pylori* scores are represented with median values. The association between variables on an ordinal scale is presented by the Spearman rank correlation. Experimental data were compared among the groups using the Kruskal-Wallis test. p-values less than 0.05 were considered significant. ***$p < 0.001$. Data presented as mean ± SD of each experimental group. All data were analyzed using the GraphPad Prism 9 software.

3 Results

3.1 Histopathological changes induced by *H. pylori* infection

In the present study, we used a mouse model of *H. pylori*-induced gastritis to explore the relationship between the bacterium and the induction of proangiogenic and inflammatory mediators *in vivo*. We evaluated the histopathological changes and lesions induced by the *H. pylori* infection in the groups of challenged and unchallenged mice. Non infected mice showed a normal mucosal architecture (Figure 2A). Five weeks after colonization, *H. pylori*-infected mice showed scattered immune cells infiltrating mucosa and submucosa, at antrum, corpus and the squamo-columnar transition. Two of the animals showed patches of mucous metaplasia in corpus (Figure 2B). Ten weeks after challenge, mice showed minor immune cell infiltration at the mucosa, especially at the squamo-columnar and corpus-antrum transitions, with some mucous metaplastic patches (Figure 2C). Twenty-week-colonized mice showed minor immune cell foci in antral mucosa, at the base of the corpus glands and squamo-columnar transition (not shown). Thirty weeks after inoculation, mice presented a stronger and widespread infiltration of immune cells in mucosa and submucosa of corpus and corpus-antrum transition, with hyperplasia and multiple foci of mucous metaplasia (Figure 2D). Forty- and fifty-week challenged animals also showed an abundant and extensive immune cell infiltration in mucosa and submucosa of the corpus, with large areas of hyperplasia and mucous metaplasia (Figures 2E, F).

3.2 Description of the *H. pylori* colonization score on gastric mucosa

We assessed the *H. pylori* colonization status by culture and immunohistochemistry for each animal and found *H. pylori*-positive mice in all time points. In accordance with the bacterial score, the most robust infiltration of immune cells and more severe lesions were found at 30, 40 and 50 w.p.i (Figures 2G, H), p=0.0187).

3.3 *H. pylori* infection induces concomitant expression of *Tnf-A, Angpt2* and *VegfA*

We assessed the expression at the mRNA and protein level of several proangiogenic factors in the gastric mucosa in our mouse model. In the *H. pylori*-infected animals *Tnf-A*, *VegfA* and *Angpt2* mRNAs normalized to *Gapdh* showed an increased expression in time, showing the lowest ΔCt values at 30 to 50 w.p.i (Figures 3A–C). When comparing unchallenged animals with the 50 w.p.i. group, a significant upregulation was observed for *Tnf-A, VegfA* and *Angpt2* mRNAs expression (Figures 3E–G)

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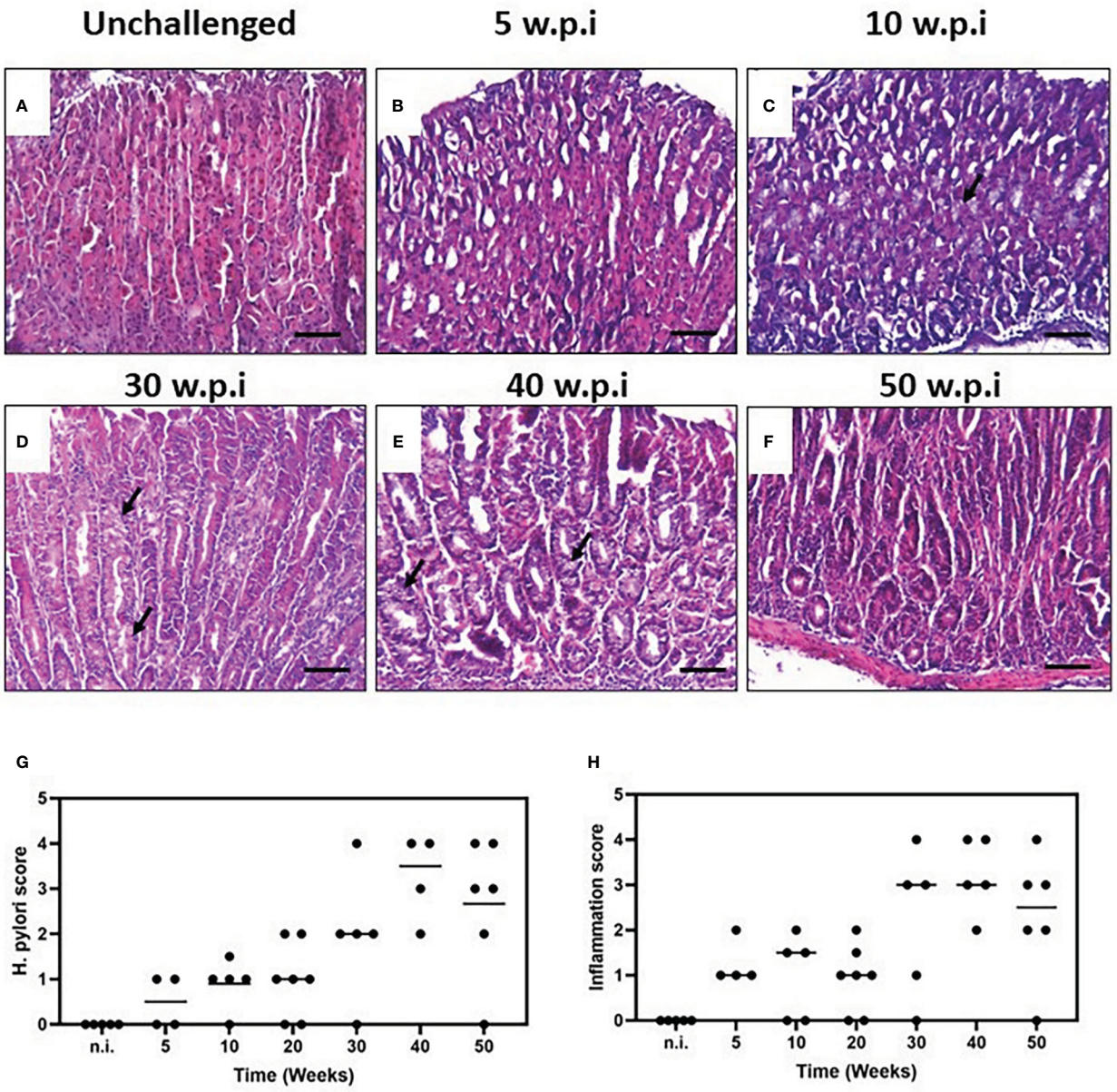

FIGURE 2
Histopathology of the murine gastric corpus mucosa in unchallenged and *H. pylori*-challenged mice. Tissue sections from resected gastric mucosa of unchallenged and H. pylori-inoculated mice were stained by H&E. **(A)** Unchallenged mice show a normal mucosal architecture. **(B)** Five weeks after *H. pylori* inoculation, minor multifocal infiltration of immune cells is observed. **(C)** Minor infiltration and some metaplastic patches are observed ten weeks after challenge. **(D)** The pathology and infiltration are intensified after thirty weeks of infection, with hyperplasia and multiple foci of mucous metaplasia. **(E)** Animals infected for forty and fifty **(F)** weeks also showed an extensive infiltration of immune cells with areas of hyperplasia and metaplasia (black arrows). *H. pylori* colonization status and histopathological assessment of inflammation. **(G)** *H. pylori* score at different time-points. **(H)**. Inflammation score of the murine gastric mucosa with *H. pylori* infection at different time-points (scored according to the scheme proposed by Wang et al. (22) and Rogers (23), one experiment; n = 5-7 mice per point; black line represents median values). Formalin-fixed, paraffin-embedded gastric tissue were stained with hematoxylin and eosin and examined. Scale bars: 50 µm (20x).

3.4 *Angpt1* mRNA exhibited a peculiar expression

In *H. pylori*-infected mice from 5 to 20 w.p.i., there was a slight increment that reached its top level of expression at 30 w.p.i., but at 40 and 50 w.p.i. the mRNA levels were drastically downregulated (Figure 3D). At 50 w.p.i *Angpt1* mRNA levels were significantly different from the not infected animals (Figure 3H). When analyzing the expression of *Tnf-A, VegfA, Angpt2* and *Angpt1* altogether, it is worth nothing that *Tnf-A, Vegf-A* and *Angpt2* mRNAs showed a significant upregulation starting at 30 w.p.i, that coincides with the downregulation of *Angpt1* at the 40 and 50 w.p.i. This is important since all these molecules act in concert to trigger the angiogenic switch.

The Western blot analysis showed an upregulation of VegfA and Angpt2 proteins at 30 to 50 w.p.i (Figures 3F, G, I). In contrast, Angpt1 protein was downregulated (Figures 4F, H). Of note, two bands for Angpt2 protein with weights between 50-57 KDa, were

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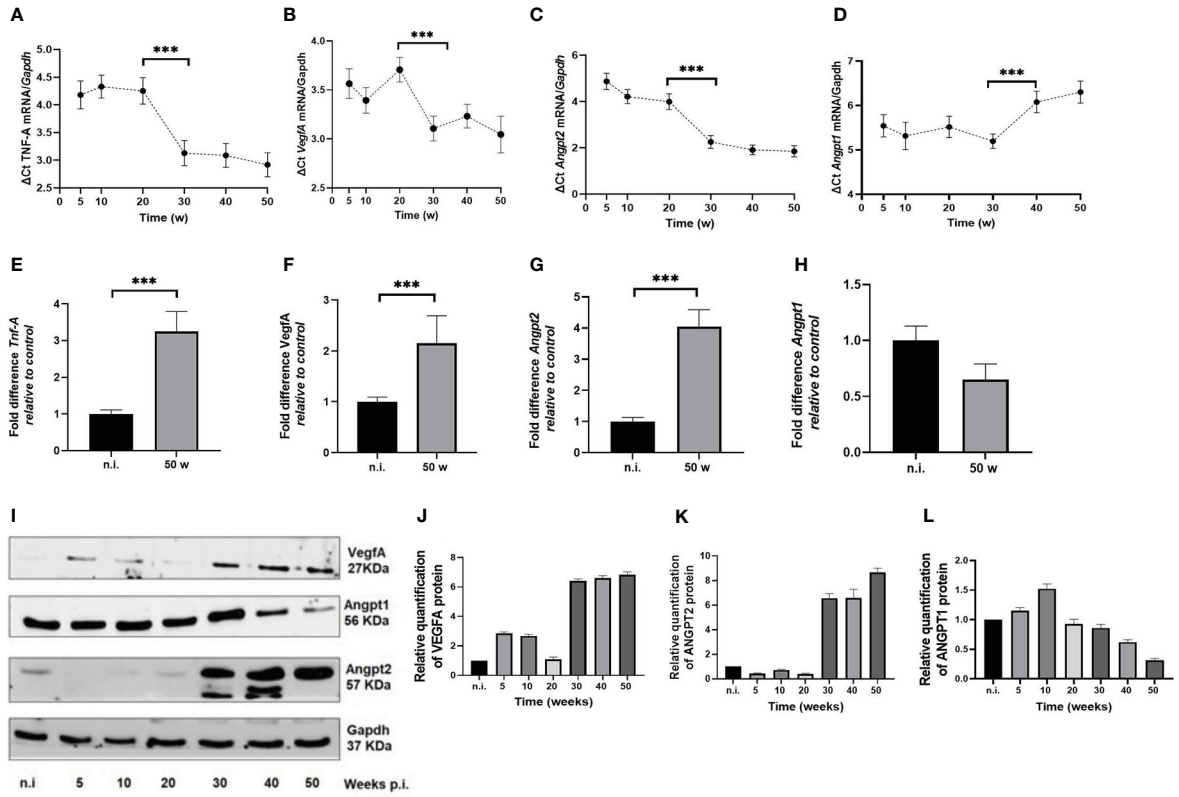

FIGURE 3
H. pylori-SS1 upregulates the expression of pro-angiogenic factors in murine gastric mucosa. mRNA expression was analyzed by real-time PCR, and represented as Delta (Δ) Ct values. The higher ΔCt values represents the lower expression of genes. **(A)** *Tnf-A*, **(B)** *VegfA* and **(C)** *Angpt2* mRNAs show a concomitant upregulation in time, becoming more evident from 30 w.p.i, whereas **(D)** *Angpt1* mRNA is downregulated at 40 and 50 w.p.i. When comparing not infected animals with the 50 w.p.i. group, a significant upregulation was observed for **(E)** *Tnf-A*, **(F)** *VegfA* and **(G)** *Angpt2* mRNAs expression, but a significant deregulation for **(H)** *Angpt1*mRNA. **(I-L)** Western blot and concordant relative quantification of VegfA, Angpt1 and Angpt2 proteins in mouse gastric mucosa of *H. pylori*-SS1 infected and uninfected mice are shown. VegfA and Angpt2 are upregulated and Angpt1 is downregulated in time. Data presented as mean ± SD of each experimental group. ***p < 0.001.

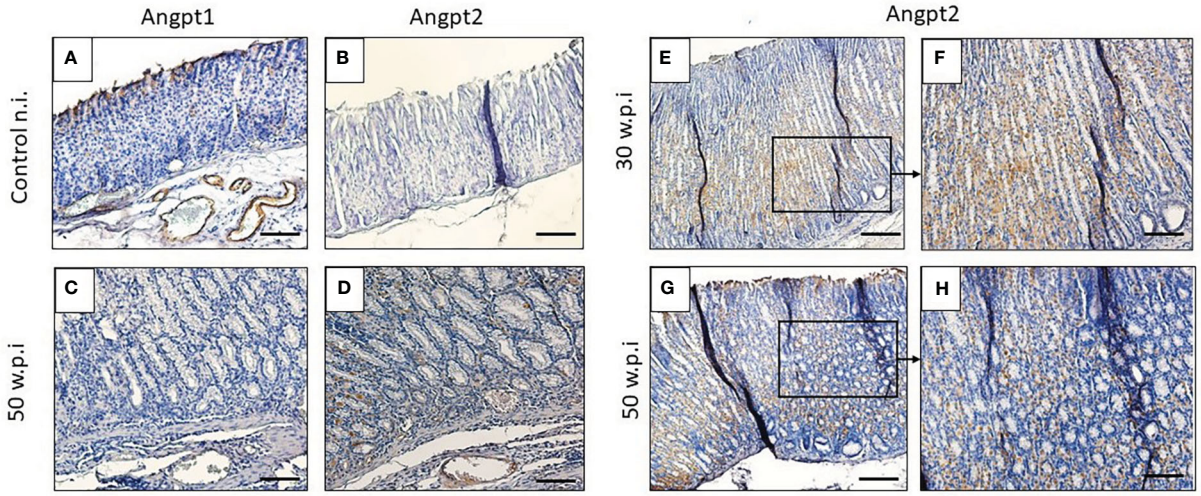

FIGURE 4
H. pylori upregulates Angpt2 expression in murine gastric mucosa. **(A, B)** IHC staining in blood vessels is positive (brown color) for Angpt1 and negative for Angpt2 in unchallenged mice. **(C, D)** IHC staining of Angpt1 compared to Angpt2 in lamina propria and blood vessels of infected mice 50 w.p.i. IHC staining with Angpt2 in infected mice 30 w.p.i. compared to 50 w.p.i. mice. Scale bars: **(A, B, E, G)** ≈ 50 μm; **(C, D, F, H)** ≈ 100 μm.

detected in 30 and 40 w.p.i mice. This was not observed at 50 w.p.i. (Figure 3F).

We also performed immunohistochemistry to assess the expression pattern and localization of Angpt1 and Angpt2 in murine tissue. *H. pylori* infection induced Angpt2 expression in gastric epithelial cells. Unchallenged mice showed a strong staining for Angpt1 in blood vessels, but Angpt2 was not detected (Figures 4A, B). Angpt2 immunoreactivity was observed in gastric mucosa of infected mice, with an intense expression in metaplastic areas at the corpus mucosa in 30- and 50-w.p.i. challenged mice (Figures 4D–G), but no signal for Angpt1 (Figure 4C).

4 Discussion

Pathological angiogenesis is a process that takes place during chronic inflammation and cancer. It involves activation of endothelial and immune cells that secrete pro-angiogenic factors such as VEGF-A, ANGPT2, TNF-α and MMPs, thus triggering proliferation of quiescent endothelium to make new blood vessels. This has been intensively studied in several cancer types, including GC. Although some evidence from *in vitro* models and studies in human gastric mucosa suggest that *H. pylori* infection may be involved in the induction of pro-angiogenic factors as VEGFA and HIF-1 (17–19, 24–26), to the best of our knowledge, no studies have addressed the role of *H. pylori* infection in the induction of proangiogenic proteins as Angpt1 and Angpt2 in the gastric mucosa. Therefore, we used a mouse model of *H. pylori*-induced gastritis to explore the kinetics of the expression of Angpt1, Angpt2, Tnf-α and Vegf-A *in vivo*.

We found a significant upregulation of *Angpt2* mRNA and protein, whereas *Angpt1* mRNA was downregulated. The ANGPT2/ANGPT1 balance determines the fate of the endothelium. ANGPT2 has opposing role to ANGPT1 since it promotes blood vessel wall destabilization. This is achieved by competitively inhibiting the binding of ANGPT1 to Tie-2 and reducing Tie-2 activation and phosphorylation (1). ANGPT2 is produced by endothelial cells and macrophages, and plays a key role in the promotion of vessel sprouting, pericyte detachment and basement membrane remodeling. ANGPT1 is produced by pericytes and is associated with endothelium quiescence. ANGPT2/ANGPT1 imbalance parallels capillary destabilization; as the inflammatory response mounted against *H. pylori* infection persists, activated endothelial and infiltrating immune cells produce increasing ANGPT2 levels while ANGPT1 is downregulated, ultimately leading to vessel destabilization (1, 6).

In mouse and human, the *Angpt2/ANGPT2* gene encodes a 496 aa protein of 57 KDa. Hypoxia is an inducer of alternative splicing in cancer and endothelial cells that frequently display a temporal and tissue-specific expression (27). In humans, alternative splicing generates the $ANGPT2_{443}$ isoform lacking exon 2, which produces a smaller (443 aa) 51 KDa protein that is found in activated endothelial cells and macrophages. Reports indicate that functions as a more potent competitive inhibitor of ANGPT1 (8) and is upregulated in cancer cell lines, breast tumor tissues, and canine adrenocortical tumor tissue (27, 28). In the performed Western blots we observed the presence of two bands with weights between 50-57 KDa for the infected mice of groups of 30 and 40 w.p.i. (Figure 3F), using an antibody (ab8452, Abcam) predicted to recognize both isoforms of Angpt2. We hypothesize that the observed bands could be $Angpt2_{443}$. Nevertheless, as there are no previous reports for the expression of the isoform $Angpt2_{443}$ in mice in normal or pathological conditions, further transcriptomic and protein assays are necessary to check whether the observation of the present study corresponds to that isoform.

We observed a concomitant upregulation of *Tnf-A, Vegf-A, Angpt2* mRNAs at 30, 40 and 50 w.p.i. *In vitro* studies have revealed that TNF-α upregulates the expression of *VEGF-A*, *ANGPT2*, and *Tie2*, and deregulates *ANGPT1* genes at mRNA level (29, 30). After translation, the ANGPT2 protein is stored in Weibel-Palade bodies at endothelial cells. The main factor causing its secretion is TNF-α, which ultimately results in autocrine inhibition of the Tie-2 receptor (31). TNF-α is mainly produced by activated macrophages and T lymphocytes, and is a potent activator of endothelium by inducing vasodilatation, increase of vascular permeability and the recruitment of immune cells (32). TNF-α expression is substantially elevated in response to *H. pylori* infection thus inhibiting gastric acid secretion, which in turn facilitates *H. pylori* survival (32). *TNF-A* transcription is induced by nuclear factor κappa B (NF-κB), which is activated in response *H. pylori* infection in gastric epithelial cells *in vitro* and *in vivo* (15, 33, 34), and upregulates VEGFR in endothelial cells (16).

Besides its role in cancer progression, angiogenesis plays a major role in the multi-step carcinogenesis process due to the fact that the gastric mucosa undergoes important histological changes, which may require gaining access to the vasculature in order to receive an adequate supply of nutrients and oxygen (35). Tuccillo et al. (36) found elevated levels of VEGFA in *H. pylori*-associated human gastritis. VEGFA and iNOS levels are also high in patients with chronic atrophic gastritis as well as in metaplastic and dysplastic areas (37). These observations, and ours, support the evidence pointing to the role of *H. pylori* as a promotor of angiogenesis.

The normal gastric epithelium does not express angiogenic factors; those are secreted by endothelial and some immune cells under stimulus. Several *in vitro* studies, including our own observations (manuscript submitted), show that in co-cultures *H. pylori* induces the expression of *ANGPT2*, *VEGFA* and other angiogenesis-related factors in gastric adenocarcinoma cell lines (17, 26, 38). Atrophic, hyperplastic, metaplastic or dysplastic cells in *H. pylori*-infected gastric mucosa have accumulated genetic and epigenetic alterations that may change their gene expression programs thus leading to alteration in their phenotypes (39). In this context, it could be possible that the gastric epithelial cells themselves secrete some angiogenic factors, in response to signals from the inflammatory microenvironment, bacterial virulence factors (e.g. CagA, VacA, LPS, urease), or both (Figure 5). In the present study we challenged mice with *H. pylori* SS1, which has a nonfunctional CagA protein. Therefore, the mechanisms by which the bacterium induces angiogenic responses may be CagA-independent. Other virulence factor, such as urease, may be implicated since it has been demonstrated that this enzyme induces ANGPT2 and other angiogenic factors in human gastric cell lines (17).

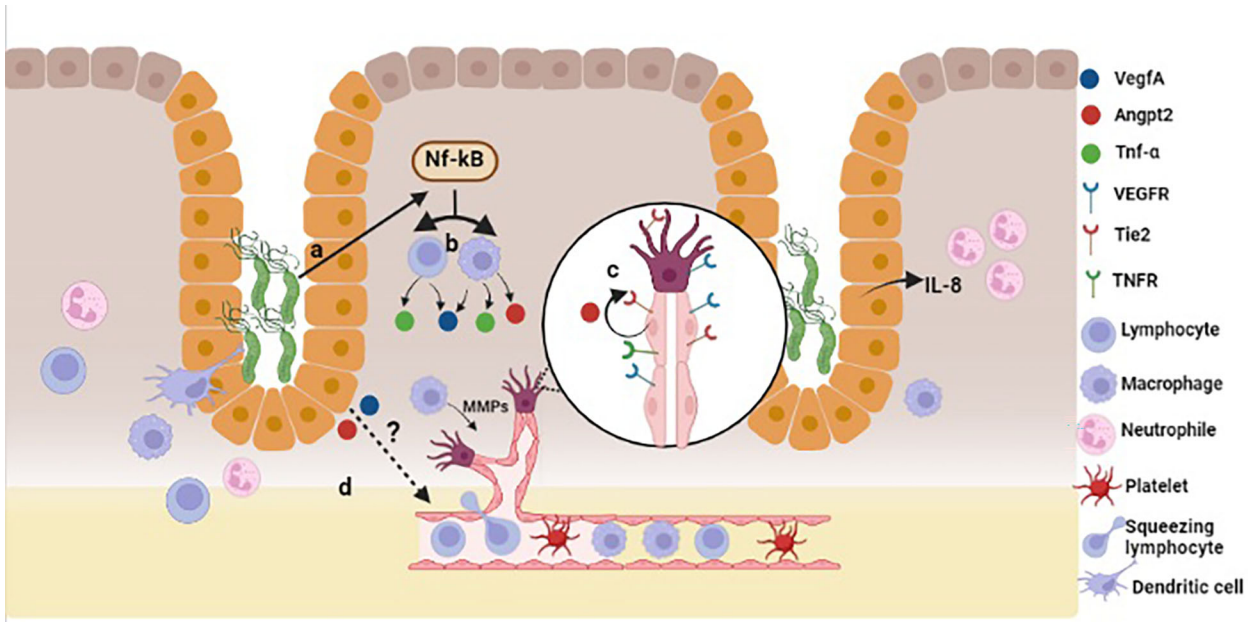

FIGURE 5
Model for *H. pylori*-induced angiogenesis in gastric mucosa. In a CagA-independent pathway, *H. pylori* infection may trigger the activation of NF-κB **(A)**, which in turn induces TNF-α production **(B)** in lymphocytes and macrophages. TNF-α activates endothelial cells, which promotes Angpt2 release **(C)** from Weibel-Palade bodies. *H. pylori* induces the expression of *Angpt2* and *VegfA* from epithelial cells in a CagA-independent pathway, and also the activation and proliferation of endothelial cells **(D)**. Created with BioRender.com.

In conclusion, in this study we have found that *H. pylori* infection concurrently modulates the expression of several angiogenic mediators in the murine stomach. Specifically, this bacterial infection induces the expression of Angpt2, *Tnf-A* and VegfA at the mRNA and protein levels. Concomitantly, it downregulates Angpt1 expression in mouse gastric mucosa in a time-dependent manner. Nevertheless, our study has two major limitations; first, we had only one group of non-infected mice for the experiment that was euthanized at 50 weeks, but no control groups for early time points of infected animals. Hence, we show only the evolution of the expression in infected animals, and the comparison with the control group is shown only for the 50 w.p.i. mice. Second; the presented evidence describes the role of *H. pylori* in the modulation of the studied angiogenic factors, therefore, subsequent mechanistic and functional studies are necessary to establish the impact to *H. pylori* infection to angiogenesis *in vivo*.

Data availability statement

Datasets are available on request: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Institutional Committee for Animal Care and Use of Animals (CICUA) of the University of Costa Rica (permission number CICUA-031-17).

Author contributions

VR-M, WA-A, WM-B, CU, SM-C and LR designed the study. WM-B, WA-A and LF-P performed the experiments. WM-B, WA-A, VR and SM-C analyzed the data. WM-B, VR and WA-A interpreted the data. WM-B, WA-A and VR wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Paper III

The TNF-A–857*T Polymorphism is Associated with Gastric Adenocarcinoma Risk in a Costa Rican Population

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ABSTRACT

Background: Costa Rica is ranked as one of the countries with highest incidence of gastric cancer worldwide. Previous studies in Costa Rican populations have revealed associations between gastric cancer risk and several cytokine polymorphisms that seem to play a role in the regulation of the expression of these proteins. In this study, we assessed associations of the polymorphisms IL-6-174 G/C, IFNGR1-56 C/T, IL-8-251 T/A and TNF-A (-857 C/T, -308 A/G) with gastric pathologies in a high-risk population of Latin America.

Methods: DNA samples of 47 patients with gastric adenocarcinoma, 53 with chronic gastritis, 56 with duodenal ulcer and 94 healthy controls, were genotyped for the five mentioned SNPs. All participants were ≥50-years-old. Genotyping was performed by PCR-RFLP and 5'-nuclease PCR assay. *H. pylori* infection, CagA status, pepsinogen (PG) I and II blood levels were determined by ELISA. Logistic regression analysis was used to determine possible associations of the polymorphisms with cancer, gastritis and duodenal ulcer, and linear regression analysis to determine associations with blood PG levels.

Results: A total of 86.6% of the population was positive for *H. pylori*; of them, 51.6% was CagA+. Patients with the TNF-A-857*T allele had an increased risk for gastritis (OR: 3.67, $p = 0.015$) and gastric adenocarcinoma (OR:6.15, $p = 0.001$). Associations between other polymorphisms and gastric diseases, or PG levels, were not found.

Conclusions: Our results indicate that the TNF-A-857*T SNP is among the risk factors associated with the risk of gastric cancer in Costa Rica. **[Am J Med Sci 2021;362(2):182–187.]**

INTRODUCTION

Gastric adenocarcinoma (GC) is the fifth most common type of cancer worldwide, and the fourth cause of death by cancer in both sexes.[1] World incidence rates for GC are declining, mainly in developed countries, but the absolute number of new cases is still increasing, due to aging of the world population. Over 70% of GC cases occur in developing countries. According to the latest global statistics, Costa Rica ranks at 13th place by incidence of GC.

Infection with *Helicobacter pylori* increases the risk of developing GC and other gastric diseases, including chronic gastritis (ChG), duodenal ulcer (DU) and MALT lymphoma.[2–4] Strains harbouring the *cagA* gene and the s1m1 allelic form of *vacA* are considered more aggressive, and infection with such strains is associated with an increased risk of peptic ulceration and GC.[4,5] Whether a person will develop a disease depends upon bacterial virulence factors, the type and magnitude of the host immune response, the distribution of inflammation in the stomach, and other modulating factors such as diet and smoking.[3,6,7] Of note, prevalence of *H. pylori* infection in the Costa Rican population is over 50%.[8–10]

The host immune response against *H. pylori* infection is considered a key factor in the development of gastric pathologies. This bacterium colonizes the gastric epithelium resulting in a chronic and persistent infection, that cause gastric mucosal inflammation characterized by the presence of infiltrating neutrophils, macrophages, B and T lymphocytes and up-regulation of several proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, and IL-8.[11] Because cytokine

production is partially regulated at the transcriptional level, many studies have implicated SNP polymorphisms at the promoter regions of some cytokines as potential determinants of disease susceptibility. Particularly, SNP variants of pro-inflammatory cytokines seem to influence the severity and extent of *H. pylori*–induced gastric inflammation, as well as GC initiation and progression. The cytokine gene polymorphisms most consistently associated with the occurrence of GC are *IL-1B, IL-1RN, IL-8* and *TNF-A* variants.[12–16] Despite this, several studies have failed to replicate these results in some particular populations, which highlights the relevance of further investigating the potential relation between cytokine polymorphisms and GC in different geographic regions and populations.

Previous studies in Costa Rican populations have shown that some polymorphisms increase the risk of developing GC or precancerous lesions.[9,17,18] The aim of the present study was to ascertain whether genetic polymorphisms of the genes encoding for IFNGR1, IL-6, IL-8 and TNF-A are associated with gastric pathologies in a Costa Rican high-risk population.

MATERIALS & METHODS

Study population

The work described has been carried out in accordance with Declaration of Helsinki. This is a case-control study, part of a larger investigation conducted during 1996–2000 with 1300 participants, performed at the Gastric Cancer Early Detection Center at the Max Peralta Hospital in Cartago, which is a province in Costa Rica with a high risk of gastric cancer. As previously described by Rosero-Bixby & Sierra,[19] participants 50 years or older were invited to participate in an X-ray mass screening of the upper gastrointestinal tract. Cases with a suspected abnormality were referred to endoscopic examination and gastric biopsies were taken if endoscopy showed a lesion suspected of gastric cancer. As our study was made in a model of screening for cancer by X-rays, the diagnosis by histology was available only for cases with suspected cancer and not for other findings. Out of this population, 249 patients, 50 to 84 years old, were selected and grouped as follows: (A) 47 GC patients according to the Japanese histological classification system; (B) 94 individuals, age- and sex matched to the GC cases, that according to X-ray examination (double contrast gastroduodenal series) were considered as healthy; (C) 56 patients diagnosed with DU by endoscopy; (D) 52 patients endoscopically diagnosed with ChG. Informed consent was obtained from all individual participants included in the study.

DNA extraction and genotyping

DNA was isolated from the blood samples using standard proteinase K digestion and phenol/chloroform extraction. *IL-8-251*T/A* genotyping was performed by PCR-RFLP. DNA (100 ng) was amplified using primers sequences and PCR conditions previously reported.[20] *IFNGR1*-56C/T, *IL-6*-174G/C *TNF-A*-308G/A and *TNF-A-857C/T* genotyping was performed by the 5'-nuclease PCR assay (Taqman, Life Technologies, California, USA). Taqman probes and conditions are available upon request.

H. *pylori* and PGs measurements

H. pylori *and CagA status as well as blood PG levels were determined by ELISA, as previously described.*[21]

Statistical analysis

Genotype frequencies were tested for Hardy-Weinberg equilibrium proportions using a Chi squared test. Statistically significant differences between groups were determined by Fisher's test. Logistic and lineal regression analyses were performed with the STATA 8.0 (Stata Corporation, 2003, Texas USA). Odds ratios (OR) with 95% confidence intervals (95% CI) were estimated to assess possible associations of the studied polymorphisms with pathology by comparing every case group (gastric pathologies) against the normal control group, with adjustment for age, sex, H. pylori infection and CagA status. Multiple lineal regression analysis was used to determine possible associations of polymorphisms with blood pepsinogen concentrations. p values ≤ 0.05 were considered statistically significant.

Table 1. Characteristics of the studied population

	Controls	ChG	DU	GC	Total
N	94	56	52	47	249
Males/Females	70/24	33/23	35/17	35/12	173/76
Age (mean ± SD)	64.91 ± 6.88	61.79 ± 6.97	58.85 ± 6.08	66.85 ± 8.96	63. 31 ± 7.69
H. pylori + (%)	84.0	89.3	88.5	68.1	86.6
CagA + (%)	46.8	53.6	53.8	42.6	51.7
CagA+/*H.pylori* + (%)	53.2	60.0	60.9	56.2	57.0
Mean PGI (μg/L) ± SD	100.62 ± 61.29	77.26 ± 44.87	134.33 ± 48.32	45.03 ± 36.75	97.72 ± 58.29
Mean PGII (μg/L) ± SD	36.33 ± 23.71	48.04 ± 21.68	18.1 ± 11.38	34.35 ± 22.37	35.07 ± 23.23
Mean PGI/PGII ± SD	3.53 ± 2.58	1.75 ± 1.00	8.22 ± 2.41	1.91 ± 2.47	4.00 ± 3.27

Abbreviations: ChG, chronic gastritis; DU, duodenal ulcer; GC, gastic cancer.

RESULTS

Population characteristics

Table 1 shows demographic and clinical characteristics of the studied population. The distribution of males and females was similar in the different groups ($p = 0.194$). Patients diagnosed with duodenal ulcer and gastritis were significantly younger than healthy and GC patients ($p < 0.0001$). For the purpose of analysis, all studied groups (control and gastric diseases) were divided into three age categories: 50−59 years; 60−69 years and over 69 years.

207 out of the 239 characterized (86.6%) participants were positive for *H. pylori*. Infection prevalence tended to be higher among patients with gastritis and lower in cancer patients, although this was not significantly different ($p = 0.086$). When groups were compared for CagA status, no significant differences were found ($p = 0.607$), although the proportion tended to be higher in *H. pylori* positive DU and ChG patients, compared with controls (60.9% and 60.0%, respectively).

Association of genetic polymorphisms with gastric pathology

The distribution of the five SNPs genotypes and allele frequencies are shown in Table 2. The studied polymorphisms were in Hardy-Weinberg equilibrium in all four groups.

The homozygotes of *IL-6-174*CC, *TNF-A*-308AA and *TNF-A*-857TT were extremely rare (4.0%, 1.2% and 2%, respectively) and could therefore not be considered as separate genotypes for the purpose of statistical analysis. Instead, they were grouped together with heterozygotes and regarded as "C carriers", "A carriers" and "T carriers", respectively.

Table 2. Genotypic and allele frequencies of the studied population

	Controls n (%)	ChG n (%)	DU n (%)	GC n (%)	Total n (%)
IFNGR1-56	85	55	51	45	236
CC	15 (17.64)	7 (12.73)	11 (21.57)	5 (11.11)	38 (16.10)
CT	43 (50.59)	27 (49.09)	28 (54.90)	28 (62.22)	126 (53.39)
TT	27 (31.77)	21 (38.18)	12 (23.53)	12 (26.67)	72 (30.51)
Allelic frequency of T	0.57	0.63	0.51	0.59	0.57
IL-6-174	92	56	52	45	245
GG	60 (65.22)	35 (62.50)	36 (69.23)	31 (68.89)	162 (66.12)
C carriers	32 (34.78)	21 (37.50)	16 (30.77)	14 (31.11)	83 (33.88)
Allelic frequency of C	0.19	0.22	0.17	0.17	0.19
IL-8-251	81	52	50	46	229
TT	30 (37.04)	21 (40.38)	17 (34.00)	20 (43.48)	88 (38.43)
AT	39 (48.15)	22 (42.31)	20 (40.00)	21 (45.65)	102 (44.54)
AA	12 (14.81)	9 (17.31)	13 (26.00)	5 (10.87)	39 (17.03)
Allelic frequency of A	0.39	0.38	0.46	0.34	0.39
TNF-A-308	93	56	51	46	244
GG	74 (79.57)	46 (82.14)	37 (72.55)	35 (76.09)	192 (78.69)
A carriers	19 (20.43)	10 (17.86)	14 (27.45)	11 (23.91)	52 (21.31)
Allelic frequency of A	0.10	0.09	0.14	0.13	0.11
TNF-A-857	84	49	49	40	222
CC	77 (91.67)	35 (71.43)	42 (85.71)	25 (62.50)	179 (80.63)
T carriers	7 (8.33)	14 (28.57)	7 (14.29)	15 (37.50)	43 (19.37)
Allelic frequency of T	0.04	0.15	0.11	0.20	0.12

Abbreviations: ChG, chronic gastritis; DU, duodenal ulcer; GC, gastic cancer.

Table 3. Odds ratios and 95% confidence intervals of TNF-A 857 polymorphisms and other factors for the studied population

	ChG			DU			GC		
	OR	p	95% C.I.	OR	p	95% C.I.	OR ratio	p	95% C.I.
60-69 years	0.38	0.034	0.16−0.93	0.19	0.000	0.08−0.46	–	–	–
>69 years	0.16	0.020	0.05−0.51	0.03	0.000	0.06−0.16	–	–	–
Male	0.37	0.027	0.15−0.89	0.35	0.039	0.13−0.95	–	–	–
CagA+	1.33	0.480	0.60−2.94	1.20	0.670	0.52−2.79	0.95	0.900	0.41−2.18
TNFA 857 T carriers	3.67	0.015	1.28−10.53	1.33	0.682	0.34−5.29	6.07	0.001	2.16−17.02

Abbreviations: ChG, chronic gastritis; DU, duodenal ulcer; GC, gastic cancer; OR odds ratio.

Because the statistical models (logistic and linear) gave equivalent results in the whole population (*H. pylori*$^+$ plus non-infected cases) and in *H. pylori*$^+$ populations, only the results of *H. pylori*$^+$ population are shown. Also, pro-inflammatory polymorphisms would not be relevant in the absence of *H. pylori*, unless there is another triggering factor.

According to our statistical analysis, TNF-A-857*T-carriers have a significantly increased risk of developing gastritis and GC as compared to healthy individuals (Table 3). No association of *IFNGR1, IL-6-174, IL-8-251* or *TNF-A-308* alleles with the risk of developing any of the gastric pathologies was found (data not shown). Neither of the polymorphisms showed association with serum PG levels or the PGI/PGII ratio (data not shown).

DISCUSSION

The purpose of this study was to test whether pro-inflammatory genotypes increase the risk of ChG, DU or GC in a high-risk population from Costa Rica that has not been previously assessed for these polymorphisms. These three pathologies were chosen as GC and DU are divergent clinical outcomes of ChG, and patients with DU rarely develop GC. This apparent "protection" is a consequence of differences in the pattern and extent of *H. pylori* induced gastritis/gastric atrophy and stomach pH.[22] DU occurs in gastric acid hypersecretors with non-atrophic antral gastritis, whereas GC is associated with atrophic gastritis, that reduces acidity.[23]

The clinical outcome of the inflammation provoked by *H. pylori* infection depends on environmental factors, bacterial virulence genes and host genetic background. This study focused on polymorphisms of host pro-inflammatory cytokines, which regulate the immune response against *H. pylori* infection by altering the expression levels of cytokines and their membrane receptors.[12,14,24]

The frequencies for IL-8-251*A in our sample are similar to those reported in Mexican,[25] Brazilian[26] and Taiwanese[27] populations with a prevalence close to 40%. The data are partly in accordance with results reported in Portuguese,[28] Italian,[29] and Finnish populations.[30]

IL-8 is among the proinflammatory cytokines induced in response to *H. pylori* infection, particularly by CagA-positive strains.[26] Given this link, some studies have addressed the possible relation between the IL-8-251*A allele and risk for gastric disease. Although some of these have revealed an association between this genetic variant and GC,[20,25,26,29,31–33] others have not.[28] In the present study we found no evidence for the existence of an association between the IL8-251*T/A polymorphisms and risk for developing ChG, DU or GC. A meta-analysis found significantly elevated risks in all comparison models in the Asian group but not in the European group[34] suggesting that -251*A allele could be an ethnicity-dependent risk factor for GC.

A study in Portuguese and Italian patients have shown an association with IFNGR1-56*T/T homozygous genotype and increased risk for early-onset GC (40 years or less).[35] In our investigation, the allele frequencies for IFNGR1-56*T are very similar to those described for the Portuguese population, but we did not find any significant association between IFNGR1-56 polymorphisms and gastric lesions. On the other hand, in a Venezuelan population, homozygotes for the C allele of this SNP were at increased risk of atrophic gastritis.[36] To our knowledge, ours and the two aforementioned studies are the only three published data sets on associations between *IFNGR1-56* polymorphisms and precancerous or cancerous gastric lesions.

IL-6 is an inflammatory cytokine that is synthesized by many cell types, including T-cells, macrophages and stromal cells, in response to stimulation from TNF-α and IL-1. No significant association between IL-6 polymorphisms and GC was found in our population. Similar results were reported in Finnish, American, Colombian, Japanese and Korean studies.[30,37,38] On the contrary, a Brazilian study found that the G allele at -174 was significantly more common among GC patients than in patients with ChG or controls.[39] More studies in different populations are needed to clarify a potential role for this polymorphism in the development of GC.

The *TNF-A* G/A transition in -308 position of the promoter is the most studied SNP for this gene. We did not find any association between the allele A and the risk of GC, ChG or DU. These results are similar to what has been reported in a Honduran population[40] and partially in accordance with those of a Mexican population,[41] where a significant association of allele A was reported with DU but not with GC, when compared to the non-atrophic gastritis group. Another Mexican study[13] also showed no association between -308*A with the risk for GC. In contrast, a study in a Portuguese population showed that TNFA-308*A allele carriers have an increased risk of GC.[16] For this population, the-308*A allele had a frequency of 18,9% in controls and 25,2% in GC patients, which is considerably higher than in the present Costa Rican population. We report that the *TNF-A-308*A* allele has a prevalence of 13% for both GC and duodenal ulcer patients, and 11% for the total sample. This is considerably lower than Portugal but similar to what was found in a Mexican population, with a frequency of 8% for the -308A allele, and a little higher than the prevalence reported in Honduras.[40] We conclude that the TNF-α-308*A allele, given its scarcity, is not a robust determinant of GC susceptibility in these three Hispanic populations.

Compared to other populations, we report marked differences in allelic frequencies for *TNF-A-857* variant. More specifically, the frequency of *TNF-A-857*T* allele in our study is lower than that reported in an Italian population,[14] but higher than the one in a Han Chinese population in which *TNF-A−857*T* allele conferred a 3-fold decreased risk in MALT-lymphoma.[42]

Our results show that subjects carrying the TNF-A-857*T allele had a 6-fold increased risk of developing GC when compared with CC genotype. Our findings are in accordance with other studies that have reported similar

association in other geographic regions, including two meta-analysis.[43,44] The *TNF-A-857*T* allele was also associated with increased risk of chronic gastritis. This is not surprising as this pathology is one of the steps leading to GC. However, DU, which is a distinct outcome of *H. pylori* infection, was not associated with this polymorphism in the population studied.

Functional consequences of TNF-A promoter polymorphisms have been investigated; it has been concluded that the transcriptional activity is affected by this polymorphism. The consensus in previously published reports is that the presence of the T allele is associated with higher expression of the *TNF-A* gene, due to an increased binding of the OCT1 transcription factor to the gene promoter.[45,46] However, one study by van Heel *et al*.[47] reported that the C allele is related to a higher production of TNF-α. Our results tend to support the first findings.

H. pylori *infection activates the Nuclear Factor -κB (NF-κB), a pivotal regulator of immune response, inflammation, cell proliferation and apoptosis.*[48] *Interestingly, there is a NF-κB binding site on the* TNF-A *promoter region that is in close proximity to -857 position, and it has been demonstrated that OCT1 physically interacts with NF-κB.*[47] *Based on these observations, it is tempting to speculate that during* H. pylori *infection, OCT-1 and NF-κB contribute to the modulation of* TNF-A *expression, which in* TNF-A-857*T *allele carriers may result in increased TNF-α levels.*

It has been previously demonstrated that TNF-α plays a critical role in initiating and amplifying the inflammatory responses to *H. pylori* infection, and it is also a potent inhibitor of gastric acid secretion.[11] In response to *H. pylori* infection, *TNF-A-857*T* allele carriers would produce an excess of TNF-α, promoting hypochlorhydria, which in turn can be linked to higher bacterial load in the stomach and a change in the anatomical distribution of the bacteria.

In conclusion, we have demonstrated that carriers of *TNF-A-857*T* allele had an increased risk of the development of ChG and GC when compared to those homozygous for C allele. This emphasizes the important role of this cytokine in the pathogenesis of *H. pylori*-related CG. Positive associations between pro-inflammatory genotypes and higher risk of GC and other gastric diseases, previously reported in other populations, could not be replicated in this Costa Rican population except for *TNF-A*. Costa Rica is considered an admixed population, product of contact of different ethnic groups at the beginning of the Spanish colonization in S. XVI. Campos-Sánchez *et al.* calculated Costa Rica's genetic mean admixture of 45.6% European, 33.5% Native American, 11.7% African and 9.2% Chinese.[49]

Larger studies with these and other polymorphisms, in different populations, are necessary to clarify the contribution of individual SNPs in GC risk, and how they may modulate the response to *H. pylori* infection and subsequent phases of gastric disease. The increasing survival and decreased mortality of GC patients in developed countries are attributed to the combination of earlier detection, better access to care and improved treatment.[50] A pilot study realized in Costa Rica with double-contrast X-ray screening showed to reduce stomach cancer mortality by early detection in a high-risk population, but the high cost of the intervention makes it difficult to apply in a developing country like ours.[19] Our results provide a contribution in the search for economically and logistically feasible methods for detection of molecular markers in blood, with the aim of defining a genetic profile which could help identify people at risk for GC.

CONFLICTS OF INTEREST

Authors declare no conflicts of interest.

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MAIN RESULTS AND GENERAL DISCUSSION

H. pylori infection triggers a persistent inflammatory reaction whose clinical outcome depend on environmental factors, bacterial virulence genes, and host genetic background. The infection may exert either direct or indirect effects in the gastric mucosa. The direct contact of the bacteria with epithelial cells leads to the translocation of some of their virulence factors that ultimately alter the cell behavior in several ways (48, 54, 82, 113, 182). The indirect effect is the result of the sustained inflammatory response that eventually becomes a chronic inflammation, which is accompanied by angiogenesis. The magnitude of the inflammatory response is partially regulated at the transcriptional level by SNPs in the promotor regions of some cytokines (26, 27, 112, 183).

In vivo models of *H. pylori* infection, *e.g*. mouse models, can recapitulate fairly well the different stages of the human precancerous lesions leading to GC, which make these models a useful tool to study gastric carcinogenesis at the histological, immunological and molecular level. On the other hand, *in vitro* infection models are widely used for *H. pylori* infection research, as they allow the investigation of cancer pathogenesis by studying the close interactions between the bacteria and gastric cells (176, 184); *in vitro* models have been fundamental in showing *H. pylori*-host interactions during colonization (113, 135, 139, 185).

In the present study, *in vitro* and *in vivo* systems, as well as human biological material were used in order to unravel the role of *H. pylori* infection in the induction of some inflammatory and pro-angiogenic factors. The presented findings contribute to elucidate how the inflammation driven by *H. pylori* may trigger changes on ECs of the gastric mucosa, which alter their physiological function and modify the vascularization, thus favoring gastric carcinogenesis.

Angiogenesis and tumor promoting inflammation are closely interconnected and are crucial events during cancer progression and metastasis; in fact, they are both considered as hallmarks of cancer (19). Both tumor and stromal cells produce angiogenic factors that ultimately lead to endothelial cell proliferation. The cancer associated ECs cooperate in the

perpetuation of inflammation, which reciprocally promotes angiogenesis by secretion of cytokines, proteases, growth and proangiogenic factors, thus creating a positive feedback loop (98). Aberrant vascular structures can also induce hypoxia, acidosis and DNA damage (124), which contribute to the establishment of a tumor-promoting microenvironment very early in the carcinogenic process. This could be particularly relevant in cancers in which chronic and persistent inflammation is the main driving force for the malignant transformation, as in the case of gastric carcinogenesis.

By infecting gastric cancer cell lines AGS, MKN28 and MKN45 with *H. pylori*, it was observed an upregulation of the *ANGPT2* gene at the mRNA and protein level. This molecule plays important roles in angiogenesis and inflammation, both in normal physiology and in carcinogenesis. In the presence of VEGFA, ANGPT2 destabilizes the mature blood vessels, inducing the removal of pericytes, thus increasing the endothelial permeability (84, 94). The *in vitro* infections were performed in the absence of immune cells; this implies that only the link between epithelial cells and bacteria is studied. In consequence, those results suggest that maybe the pro-angiogenic factors could be secreted by the host cells upon infection to the medium, or could be directly produced by *H. pylori* and have effect on the nearby endothelium. Additionally, the supernatants of *H. pylori*-infected cells induced an angiogenic and inflammatory response in the *in vivo* CAM assay. Angpt2 was also upregulated in the mouse model in response to infection, and the highest levels of expression were observed in the most severe lesions.

An upregulation of ANGPT2 in response to *H. pylori* infection was observed in both human gastric cancer cell lines and murine gastric mucosa. Regarding the cell lines, the levels of *ANGPT2* mRNA were similar between cells co-cultured with neither CagA-positive or mutant CagA-negative strains. The results were supported by those obtained in the animal model, since the mice were infected with *H. pylori* SS1, a strain with no functional CagA. Taken together, *in vitro* and *in vivo* models suggest that the induction of *ANGPT2* is a CagA-independent mechanism, and that upregulation of this proangiogenic gene by *H. pylori* is mediated by factors other than CagA. The CagA oncoprotein, which is delivered into the host cell by the T4SS, alters multiple host cellular responses and is among the best-studied *H. pylori* virulence factors. Nevertheless, *H. pylori* has several other virulence factors that could

be involved in the induction of ANGPT2, some of which are associated with gastric pathology, including the cytotoxin VacA, the adhesins BabA and SabA, and the outer membrane protein OipA, reviewed elsewhere (35, 53, 65). Actually, Olivera-Severo *et al*. (60) showed an increase in ANGPT2 protein and other pro-angiogenic factors upon exposure of AGS cells to purified *H. pylori* urease from strain 26695.

In AGS cells infected with *H. pylori* 26695, a time-dependent downregulation of miR-203a was observed, which parallels the upregulation of *ANGPT2* gene. miR-203a is predicted to regulate *ANGPT2* gene *in silico* and is expressed specifically in epithelial cells (186-188). This microRNA is regarded as tumor suppressor, and several *in vitro* and *in vivo* studies have found it downregulated in a number of cancers, including colorectal, lung, and esophageal cancer (189-191), and has shown to promote EMT transition in pancreatic and colorectal cancer cells, enabling tumor cells to acquire invasive metastatic features (186, 192). miR-203a has also been shown to be aberrantly down-regulated in *H. pylori*-positive gastric tissue and cancer-derived cell lines, including AGS and MKN45 (193), and showed to be the most downregulated microRNA in *H. pylori* positive GC tissue specimens, compared to non-tumor mucosa tissue specimens of *H. pylori* negative GC patients (162). Also, Craig *et al.* described that the progression from *H. pylori*-associated gastritis to low-grade MALT lymphoma is epigenetically regulated by methylation of the miR-203 promoter region (194). Interestingly, the reduced expression of miR-203a has been linked to increased tumor angiogenesis *in vitro* and *in vivo* (195). A possible way of deregulation is through a NF-κB-dependent mechanism (190). However, more studies are needed to elucidate the role of *H. pylori* infection in the downregulation of miR-203a, the mechanistic insights and its relevance of this in the angiogenic process.

In contrast to the above-mentioned results for ANGPT2, the expression of *ANGPT1* was not detected in any of the studied cancer gastric cell lines. In the mouse model, Angpt1 mRNA and protein levels were downregulated after 30 weeks of colonization. This contrasts the already mentioned finding for murine Angpt2, which was upregulated at the mRNA and protein levels at the same time-point. The ANGPT2/ANGPT1 balance determines the fate of the endothelium. These two proteins have antagonistic effects and compete for the same receptor; ANGPT2 promotes blood vessel wall destabilization, while ANGPT1 is responsible

for a quiescent vascular phenotype. Therefore, ANGPT1 is known as an endothelial survival and vascular stabilization factor, necessary for the maturation of newly formed vessels. As the chronic inflammatory response mounted against *H. pylori* infection persists, activated endothelial and infiltrating immune cells produce increasing ANGPT2 levels and ANGPT1 is downregulated, ultimately leading to vessel destabilization (95, 98). This suggests that high production of ANGPT2 rather than ANGPT1 early in carcinogenesis may favor preneoplastic and tumoral fitness.

TNF-α has an important role in the pathophysiology of gastric carcinogenesis (107, 108, 196). TNF-α induces multiple changes in EC gene expression including induction of adhesion molecules, integrins, and MMPs, and the endothelial tip cell phenotype *in vitro* (109). In the present study, the *TNF-A* gene, coding for the TNF-α protein, was significantly upregulated in the mouse model, particularly in animals colonized for 30, 40 and 50 weeks. TNF-α is produced by macrophages and other immune cell types, including lymphoid cells, mast cells, and by non-immune cells, such as endothelial cells, fibroblasts, and smooth muscle cells (107). It plays a critical role in initiating and amplifying the inflammatory responses to *H. pylori* infection; it is also a potent inhibitor of gastric acid secretion (66), which in turn can be linked to higher bacterial load in the stomach and a change in the anatomical distribution of the bacteria, thus facilitating bacterial survival (112). Concomitantly with *TNF-A* upregulation, *VegfA* and *Angpt2*mRNAs and proteins were upregulated in the same experimental groups. It has been reported that TNF-α protein induces *ANGPT2* mRNA in HUVEC cells in a time and dose-dependent way (197), and also VEGFA, VEGFR, ANGPT2 and Tie2 *in vitro* (116, 198).

Expression levels of TNF-α can change according to specific SNPs present in the promotor or coding region of *TNF-A* gene. Therefore, the link between one of those SNPs and the risk of gastric pathology was determined in the present study in a Costa Rican population with high-risk of GC. It was found that subjects carrying the *TNF-A-857*T* allele had a 6-fold (OR=6.07; p=0.001) increased risk of developing GC when compared with those carrying the CC genotype. The *TNF-A*-857*T allele was also associated with increased risk of chronic gastritis (OR=3.67; p= 0.015). However, DU, which is a distinct outcome of *H. pylori* infection, was not associated with this polymorphism in the population studied. In response to *H. pylori* infection, *TNF-A*-857*T allele carriers would produce an excess of

TNF-α, promoting hypochlorhydria. *TNF-A* transcription is induced by NF-κB, which is activated in response *H. pylori* infection in gastric epithelial cells *in vitro* and *in vivo* (113-115). Interestingly, there is a NF-kB binding site on the *TNF-A* promoter region that is in close proximity to -857 position; the presence of the T allele is associated with higher expression of the *TNF-A* gene due to an increased binding of the OCT1 transcription factor to the gene promoter (199, 200). For this population no association of *IFNGR1, IL-6-174, IL-8-251* or *TNF-A*-308 alleles with the risk of developing any of the gastric pathologies was identified. None of the polymorphisms were associated with serum PG levels or the PGI/PGII ratio of patients.

When combined with data from the gastric cancer cell lines and from mouse model, it is tempting to speculate that *H. pylori* infection may trigger the activation of NF-κB in a CagA-independent way, which together with OCT1 contribute to the modulation of *TNF-A* gene expression in *TNF-A*-857*T allele carriers, and this may result in increased TNF-α protein levels. Besides promoting hypochlorhydria, TNF-α activates ECs, upregulates *VEGFA* and *ANGPT2* genes and promotes ANGPT2 protein release from Weibel-Palade bodies, which leads to destabilization, proliferation and sprouting of ECs (109, 110, 197). Normal gastric epithelium does not express angiogenic factors, but previous *in vitro* reports (60, 136, 201, 202) and the present work show that in vitro, *H. pylori* induces the expression of *ANGPT2*, *VEGFA* and other angiogenesis-related factors in GC cells. Consequently, epithelial gastric cells may produce *ANGPT2 in vivo*, after activation of NF-κB, by miR-203a downregulation and act upon endothelium in response to bacterial factors (Fig. 6). Additional studies are necessary to unravel the specific mechanisms of induction in response to *H. pylori* infection *in vivo*, and its relation to pre-malignant gastric lesions. One potential approach would be to study fresh human gastric tissue specimens from precancerous lesions (*e.g.* atrophy and incomplete intestinal metaplasia), that may accumulate genetic and epigenetic alterations and may change their gene expression programs thus leading to alteration in their phenotypes (203).

CONCLUSIONS

1. The results presented here suggest that *H. pylori* infection contributes to the process of carcinogenesis by promoting angiogenesis in the gastric mucosa, which may in turn favor the perpetuation of chronic inflammation. This could have important functional implications for the process of gastric carcinogenesis.

2. Combining the results from the *in vitro* infections, the mouse model, and the human SNPs studies, the presented evidence describes the role of *H. pylori* and host genetic factors in the modulation of some angiogenic and inflammatory factors. Therefore, subsequent mechanistic and functional studies are necessary to further elucidate the impact to *H. pylori* infection in the induction of angiogenesis *in vivo*, and its relation to pre-malignant gastric lesion development.

3. The present experimental data showed that CagA negative strains induced the upregulation of ANGPT2 (and *TNF-A* and VEGFA in mice), both in the *in vitro* cell infection and the mouse model. This suggests that the induction of the studied proangiogenic genes could be also in response to virulence factors other than CagA, or a general mechanism of *H. pylori* infection. The *H. pylori*-driven angiogenesis may be CagA-independent, but it is probably linked to other bacterial virulence factors (e.g. VacA, LPS, Urease), and signals from the inflammatory microenvironment, such as the cytokine production (TNF-α, interleukins) that is partially regulated by host genetic polymorphisms.

4. Carriers of the *TNF-A*-857*T allele had an increased risk of the development of chronic gastritis and GC when compared to those homozygous for the C allele. This emphasizes the important role of this cytokine in the pathogenesis of *H. pylori*-related CG. It is important to clarify the contribution of individual SNPs in GC risk, and how they may modulate the response to *H. pylori* infection and precancerous lesions in different populations.

5. The presented evidence describes the role of *H. pylori* in the modulation of the studied angiogenic factors, therefore, subsequent mechanistic and functional *in vivo* studies are necessary to establish the impact to *H. pylori* infection in the angiogenic process.

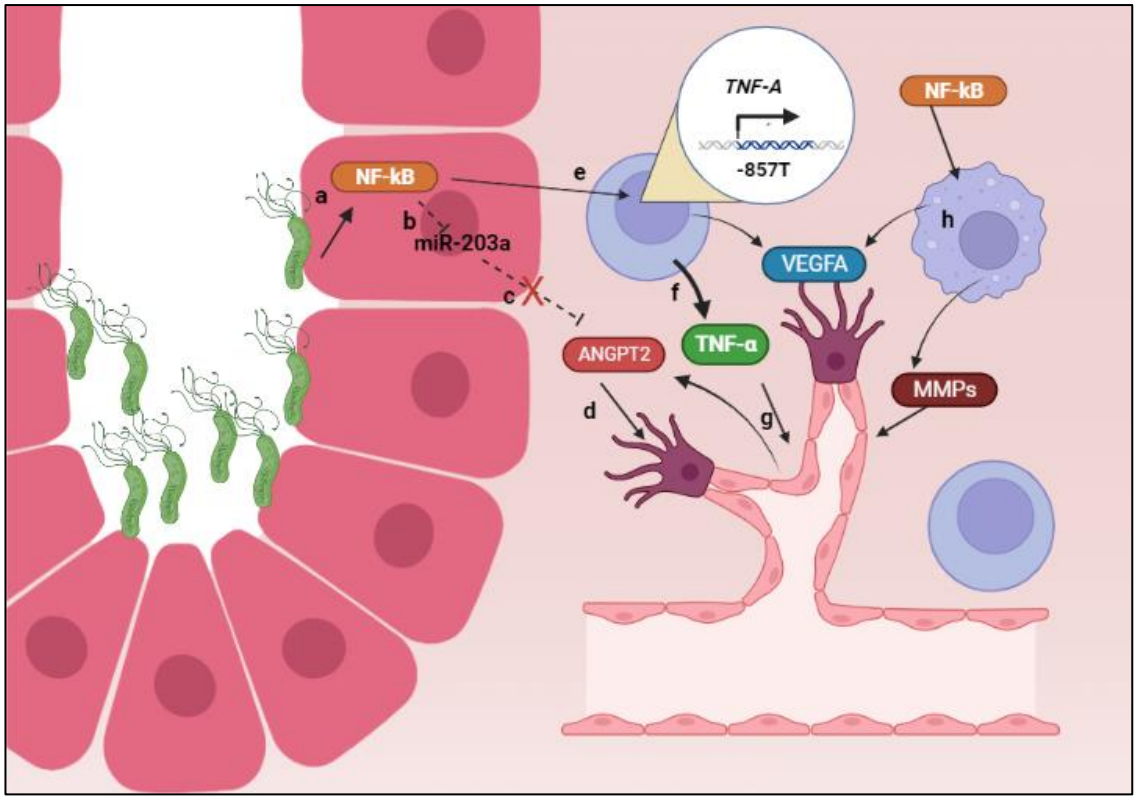

Fig. 6. Model for *H. pylori*-induced angiogenesis in gastric mucosa. In epithelial cells, in a CagA-independent pathway, *H. pylori* infection may trigger the activation of NF-kB (a), which in turn could downregulate the expression of miR-203a (b), losing its capability to repress the translation of ANGPT2 mRNA. (c) Protein ANGPT2 could be secreted from inflamed epithelium, then acting on endothelial cells to induce sprouting phenotype (d). At the same time, NF-κB is a transcription factor for *TNF-A* gene, which in persons with the *TNF-A*-857T polymorphism could have a higher expression in lymphocytes (e), then secreting more protein TNF-α (f). TNF-α activates endothelial cells, which promotes ANGPT2 release from Weibel-Palade bodies (g). NF- κB can also induce the production of VEGFA and MMPs from macrophages (h), which act as mitogen (VEGFA) and in degradation of MEC and basal membrane (MMPs) of endothelial cells, allowing the detachment of pericytes and formation of new vessels. Straight arrow: Induction/action; curved arrow: secretion; discontinuous inhibition line: potential inhibitor. Created with BioRender.com

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