Chapter

Helicobacter pylori Infection and Endothelial Dysfunction

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Abstract

Endothelial cells play a critical role in maintaining the integrity of vascular structure and function. Endothelial dysfunction is closely associated with the development and progression of cardiovascular diseases (CVDs) like hypertension (HTN) and atherosclerosis. Gut microorganisms significantly contribute to atherosclerosis and related CVDs. *Helicobacter pylori* (*H. pylori*) colonizes in human gastric epithelium in a significant portion of general population in the world. Patients with *H. pylori* infection have significantly increased risk for CVDs including atherosclerosis, HTN, coronary heart disease, and cerebrovascular disease especially in younger patients (< 65 years old). *H. pylori* infection significantly impairs vascular endothelial function through multiple mechanisms including increased reactive oxygen species production and oxidative stress, inflammation, decreased nitric oxide formation, modification of the expression of cytokines and microRNAs, abnormalities of lipid and glucose metabolisms, and exosomes-mediated pathways. Endothelial dysfunction associated with *H. pylori* infection is reversible in both animal model and human subjects. Accumulating data suggests that *H. pylori* infection is an important risk factor for endothelial dysfunction and CVDs especially in young patients. Screening young male population for *H. pylori* infection and treating accordingly could be an effective approach for early prevention of CVDs especially premature atherosclerosis associated with *H. pylori* infection.

Keywords: *Helicobacter pylori*, atherosclerosis, endothelial dysfunction, cardiovascular disease, exosomes

1. Introduction

Atherosclerosis is among the principal contributors to cardiovascular diseases (CVDs) especially coronary artery diseases (CAD) and stroke [1]. Despite in-depth understanding of the traditional cardiovascular risk factors including diabetes mellitus (DM), hypertension (HTN), hyperlipidemia, smoking, and obesity, and effective control of these known risk factors, CVDs remain the leading cause of mortality and morbidity in developed countries including the US [2, 3]. It is worrisome that the decline of all cardiovascular mortality rate has been slowing down since 2011 [4]. It is very problematic that patients presenting with ST elevation myocardial infarction over the past 20 years are getting younger [5], and the total number of death from CAD and stroke is projected to increase by

about 18% by 2030 [6]. Clearly, there are other risk factors that have not been defined, and yet contribute significantly to the development and progression of atherosclerosis and related CAD and stroke.

Gut microorganisms significantly contribute to the development of atherosclerosis and related CVDs [7–9]. The microaerophilic Gram-negative bacterium *Helicobacter pylori* (*H. pylori*) colonizes in the epithelium of human stomach in a significant portion of general population in the world with the infection rate ranging from 30% - 50% in developed countries up to 80% in developing countries especially in Asia [10, 11]. Most patients with *H. pylori* infection have no symptoms clinically [12]. However, *H. pylori* infection could cause progressive damage to gastric mucosa, and is closely associated with a number of important diseases including (but not limited to) chronic gastritis, peptic ulcers, and gastric cancer [13]. Recent data indicate that *H. pylori* infection could also contribute to some important extra gastrointestinal diseases such as hematological diseases (especially idiopathic thrombocytopenia), neurological abnormalities, dermatological pathologies, and autoimmune disorders like inflammatory bowel diseases, chronic liver disease, and DM [14–22]. Thus, *H. pylori* infection is a significant cause of morbidity and mortality in humans. In 2005, Dr. Barry Marshall and Dr. Robin Warren were awarded the Nobel Prize in Physiology for their pioneering work on *H. pylori*.

Growing evidences indicate that *H. pylori* infection could also contribute to CVDs. A recent meta-analysis with a large population showed that *H. pylori* infection increased the risk of adverse cardiovascular events by 51%, mostly due to myocardial infarction and cerebrovascular disease [23]. Data also suggests that *H. pylori* infection increases the risk of coronary heart diseases (CHD) and related events predominantly in a patient's early life [24], and is positively associated with HTN [25, 26]. In this chapter, efforts will be focused on: 1) brief overview on the association of *H. pylori* infection and CVDs; 2) relationship between *H. pylori* infection and atherosclerosis; 3) *H. pylori* infection and endothelial dysfunction; 4) role of exosomes in mediating the effect of *H. pylori* infection on endothelial function, and 5) significance and clinical implications.

2. Brief overview on *H. pylori* infection and cardiovascular diseases

The role of *H. pylori* infection in the development and progression of CVDs has been established for the past two decades. Early epidemiology studies have suggested an association between *H. pylori* infection and increased prevalence of atherosclerosis [27] An early study that included 96 patients with CAD and 96 patients without CAD has revealed the followings: 1) there is a significant link between CAD and infection with *H. pylori*, especially the one expressing the virulence factor cytotoxin-associated gene A (CagA) proteins, 2) patients infected with CagA-positive *H. pylori* show significantly greater coronary artery lumen loss and arterial re-stenosis after percutaneous transluminal coronary angioplasty (PTCA) with stent implantation, 3) *H. pylori* eradication significantly attenuates the reduction in coronary artery lumen in CAD patients after PTCA [28]. Diabetic subjects with *H. pylori* infection have more severe peripheral arterial stiffness compared with those without *H. pylori* infection, and a higher cardiovascular risk score and 10-year cardiovascular risk stratification [29, 30]. After adjusting for traditional CVD risk factors, *H. pylori* infection is found to be the only independent predictor of incident carotid plaque with the multivariate odds ratio (OR) of 2.3, and incident acute stroke (with multivariate OR of 3.6) [31]. *H. pylori* infection was positively associated with the prevalence of HTN among Chinese adults [25, 26].

Recently, a study using cardio-ankle vascular index reported that subjects with positive *H. pylori* serology were significantly associated with increased arterial stiffness [32].

A recent study, using a large database with a total of 208,196 patients diagnosed with peptic ulcer diseases, compared the cardiovascular outcome for subjects with and without *H. pylori* eradication. A total of 3,713 patients with *H. pylori* eradication treatment within 365 days of the index date were included in the study with randomly selected same number of patients using propensity scores as cohort of non-eradication patients for comparison. The study demonstrated that there was a significant decrease in composite end-points for CHD and death in the early eradication group. The cumulative CHD rate was significantly lower in younger patients (< 65 years old) with *H. pylori* eradication therapy started <1 year of the index date compared to those patients without eradication at all. Interestingly, the study also showed that eradication treatment did not appear to have a significant effect in older patients (≥ 65 years old). Multivariate analysis shows that HTN and renal diseases are risk factors for CHD in patients without eradication, while younger age (< 65 years old) was a protective factor for CHD for the patients with *H. pylori* therapy [33]. Thus, there is little doubt that *H. pylori* infection is indeed associated with significant CVDs including atherosclerosis, HTN, CHD, cerebrovascular disease, and peripheral arterial diseases, as well as their clinical outcomes especially in younger patients (< 65 years old).

3. *Helicobacter pylori* infection and carotid atherosclerosis

The relationship between *H. pylori* infection and atherosclerosis has been inconsistent and sometimes controversial with the findings from a strong positive association, and a mild association, to no association [27, 34–36]. Compared to those without *H. pylori* infection, patients with *H. pylori* infection, especially with CagA+ *H. pylori*, have much higher incidence of atherosclerosis (29% vs. 63%) [37], and acute ischemic stroke (45% vs. 77%) [17]. The prevalence of serologically confirmed *H. pylori* infection was significantly higher in patients with angiographically documented CAD, supporting a positive association between *H. pylori* infection and CAD [38–40]. However, a meta-analysis with inclusion of 18 epidemiological studies and over 10,000 patients showed no positive relationship between *H. pylori* infection and CAD [41]. In contrast, the data supporting a positive relationship between *H. pylori* infection and carotid atherosclerosis with increased carotid intima-media thickness (CIMT) were consistent in most of the studies with patients [17, 42–45]. The reason(s) for the significant difference in consistency on the relationship between *H. pylori* infection and CAD vs. carotid atherosclerosis is unclear. It could be very likely due to the different imaging modalities used for the detections of CAD (using coronary angiogram) and carotid atherosclerosis (using carotid ultrasound). Carotid ultrasound could easily detect early atherosclerotic lesions without significant loss of vascular lumen, while coronary angiogram could not. In a recent study, the investigators used cardiac multidetector computed tomography to identify subclinical coronary atherosclerotic lesions in healthy subjects without clinical CVD, and found that patients with current *H. pylori* infection was 3-fold more likely to have subclinical and yet significant coronary atherosclerosis than the patients without *H. pylori* infection [15]. One of the major features of atherosclerosis is thickening of the intima-media in the arteries that could not be detected with angiogram. Carotid artery is considered an early site of atherosclerosis, and superficially located. Thus, carotid ultrasound examination is an ideal and sensitive non-invasive image modality to diagnose and monitor the

progression of atherosclerosis [46], although it has not been widely used clinically for atherosclerosis screening at this point.

Recently, a large patient database of 17,613 adult patients with carotid ultrasound examination and a ^{13}C-urea breath *H. pylori* test was analyzed [47]. Based on the study designs, the patients were divided into two groups: a cross-sectional study for single measurement group, and a retrospective cohort study for the patients with follow up measurements up to 5 years. Patients were excluded from the study if any of the following conditions was present: 1) history of *H. pylori* eradication, 2) use of any antibiotics, proton pump inhibitors, or H_2-receptor blockers 3 months before the tests, 3) age < 20 or > 70 years, 4) connective tissue diseases or immunological diseases, 5) mental disorders, 6) asthma or COPD, 7) hematological disorders, 8) thyroid diseases, 9) malignancies, 10) recent (within 3 months) or chronic infection (over 3 months) except *H. pylori* infection, 11) congestive heart failure, and 12) abnormal liver or kidney function. Patients with CAD were not excluded from the study since carotid atherosclerosis and CAD share similar risk factors, and it was felt that exclusion of the subjects with CAD could remove the subgroup population who might be at increased risk for carotid atherosclerosis with *H. pylori* infection, leading to potential selection bias. Of note, the patients with CAD accounted only for about 3% of all participating subjects for this study, and there was no stroke in the patients in the database.

The data showed that, after adjusting for age, sex, body mass index, lipid profile, HTN, DM, and smoking, *H. pylori* infection was an independent risk factor for carotid atherosclerosis in male patients ≤50 years, but not in older males or females (OR of 1.229, p = 0.009). The data also demonstrated that *H. pylori* infection was associated with a significant increase in CIMT for males, not females. To further evaluate the relationship between *H. pylori* infection and carotid atherosclerosis, the investigators studied the 5 years follow up data on additional 2,042 subjects with and without *H. pylori* infection for progression on the prevalence of carotid atherosclerosis with annual carotid ultrasound examination and a ^{13}C-urea breath test. The data showed that for males with age of <50 years, there was a 22.5% increase in the prevalence of carotid atherosclerosis in the subjects with *H. pylori* infection compared with the ones without *H. pylori* infection. These data demonstrated that *H. pylori* infection selectively increased the risk for carotid atherosclerosis in young males under 50 years old [47]. However, how *H. pylori* infection could lead to atherosclerosis, and why only in young males is unknown.

4. *H. pylori* infection and endothelial dysfunction

4.1 *H. pylori* infection and endothelial dysfunction in patients

Endothelial cells play a critical role in maintaining the integrity of vascular structure and function. Endothelial dysfunction is an important contributing factor to the pathogenesis of CVDs including HTN and atherosclerosis [4]. Early studies with small patient samples suggested that there was no clear association between chronic infections, including infection with Chlamydia pneumoniae, cytomegalovirus, Epstein–Barr virus, and *H. pylori*, and decreased endothelial function [48]. A small study with a total of 53 pediatric patients using Doppler ultrasonography of the brachial artery showed that percent ratio of the change in systolic diameters during hyperemic phase to the basal diameter (endothelium-dependent) was not significantly different between *H. pylori*-negative and -positive groups in pediatric population [49].

However, accumulating data clearly supports the concept that *H. pylori* infection could lead to significant endothelial dysfunction in patients. Using high-frequency ultrasonographic imaging of the brachial artery, it was found that endothelium-dependent flow-mediated vasodilation (FMD) was significantly lower in the subjects with seropositive antibodies to *H. pylori* than in the ones with seronegative antibodies to *H. pylori*, while endothelium-independent nitroglycerin-induced vasodilation was similar in both groups [50]. Similarly, another study with patients with chronic gastritis associated with *H. pylori* infection demonstrated that the level of FMD in patients with positive *H. pylori* infection was significantly lower than those with negative *H. pylori* infection and the healthy control group [51]. Studies also showed that the levels of C-reactive protein and soluble intercellular adhesion molecule-1 (ICAM-1) were significantly higher in subjects with seropositive antibodies to *H. pylori* than in those with seronegative antibodies to *H. pylori* [50]. The levels of endothelial dysfunction biomarkers, including endothelin-1 (ET-1), E-selectin, and ICAM-1, were found to be significantly higher in *H. pylori* (+) patients than in *H. pylori* (−) subjects [52].

One of the important questions is whether endothelial dysfunction associated with *H. pylori* infection is reversible. In a study in 2011, vascular function measurements (ankle brachial index and flow-mediated diameter percent change) were made in patients with *H. pylori* infection at the time of study enrollment and 3 months afterwards with *H. pylori* eradication. Subjects with *H. pylori* infection were treated with standard triple antibiotics therapy. It was found that *H. pylori*-positive subjects had severe endothelial dysfunction that improved significantly after *H. pylori* eradication with triple antibiotics. Subjects without *H. pylori* infection also had endothelial dysfunction, however, that was not improved after treatment with triple antibiotics. These data suggests that endothelial dysfunction in patients with *H. pylori* infection appear to be reversible [53].

In a recent study, the investigators carefully selected 18 young patients (both male and female) with *H. pylori* infection without any known risk factors for endothelial dysfunction to evaluate endothelium-dependent FMD of the brachial artery with ultrasound. A group of 13 age- and sex-matched young healthy volunteers served as the controls. The diagnosis of *H. pylori* infection was confirmed with gastric endoscopic biopsy and ^{13}C urea breath test for each patient. No other confounding variables except the conditions listed in the exclusion criteria were considered for subject selection. Young patients were recruited to minimize the risk factors for endothelial dysfunction. Patients were excluded from the study if any of the following conditions was present: 1) history of *H. pylori* eradication, 2) use of any medications including antibiotics, proton pump inhibitors, or H_2-receptor blockers 3 months before the study, 3) age < 18 or > 35 years, 4) connective tissue diseases or immunological diseases, 5) mental disorders, 6) asthma or COPD, 7) hematological disorders, 8) thyroid diseases, 9) malignancies, 10) recent (within 3 months) or chronic infection except *H. pylori* infection, 11) congestive heart failure, 12) abnormal renal or liver function; 13) congenital heart diseases, 14) hypertension, 15) smoking, 16) diabetes mellitus, 17) lipid abnormalities, 18) stroke, 19) obesity, 20) sedentary life style, 21) alcohol use, 22) any use of energy drinks or coffee or tea within 48 hours, and 23) unresponsive to anti-*H. pylori* therapy. After fasting for 8 to 12 hours, brachial artery FMD was evaluated for patients and control subjects, and presented as percent change in post-ischemia diameter over baseline. The data showed that patients with *H. pylori* infection exhibited a significant reduction in endothelium-dependent vasodilatation compared with the controls (**Figure 1A**). When patients with *H. pylori* infection were treated with BIS-based quadruple oral anti-*H. pylori* therapy (100 mg furazolidone, 100 mg doxycycline, 5 mg ilaprazole, and 220 mg colloidal bismuth tartrate, twice a day for 2 weeks) [54], their endothelium-dependent FMD of the brachial artery was effectively restored (**Figure 1B**) [55]. The effectiveness of *H. pylori* eradication

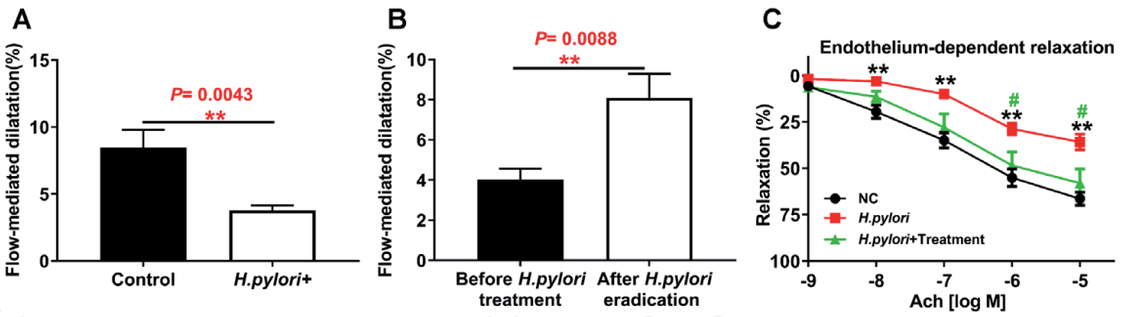

Figure 1.
H. pylori *infection significantly impairs endothelium-dependent flow-mediated dilatation (FMD) in human subjects and endothelium-dependent vascular relaxation in mice. Patients with* H. pylori *infection and healthy control subjects were evaluated for endothelium-dependent flow-mediated dilatation (***FMD***) of the brachial artery with ultrasound. The diagnosis of* H. pylori *infection was confirmed with gastric endoscopic biopsy and* ^{13}C *urea breath test for each patient. Patients with* H. pylori *infection (n = 18 patients) displayed a significant reduction in their endothelium-dependent FMD compared with the controls (n = 13 subjects) (A). Eradication of* H. pylori *infection with anti-*H. pylori *therapy effectively restored the endothelium-dependent FMD in patients with* H. pylori *infection (n = 10 patients with anti-*H. pylori *therapy) (B). Mice infected with CagA*$^+$ H. pylori *for 1 week significantly decreased acetylcholine (***Ach***)-induced endothelium-dependent relaxation of thoracic aorta without change in nitroglycerin (NTG)-induced endothelium-independent vasorelaxation (***data not shown***) after sub-maximal contraction with phenylephrine (***PE***)* (10^{-6} *M). The impaired ach-induced endothelium-dependent vasorelaxation persisted for as long as the infection was present for at least 24 weeks (***C***) without change in NTG-induced endothelium-independent vasorelaxation (***data not shown***). Eradication of* H. pylori *infection with anti-*H. pylori *therapy effectively restored ach-induced endothelium-dependent vasorelaxation in mice with 12 weeks of chronic CagA*$^+$ H. pylori *infection,* $^{\#}p < 0.05$ *(compared to CagA*$^+$ H. pylori *+ treatment mice);* $^{**}p < 0.01$ *(compared to NC mice) (C), n = 8 mice for each group.* **NC***: Normal control;* **Ach***: Acetylcholine;* **NTG***: Nitroglycerin. Data were presented as mean ± SE. [adopted and modified from (55) with permission].*

with antimicrobial therapies was confirmed using ^{13}C urea breath test for the study patients. These data confirms that endothelial dysfunction in patients with *H. pylori* is indeed reversible (very likely within 1 year of infection).

4.2 *H. pylori* infection and endothelial dysfunction in animal models

In the same recent study, the investigators used specific-pathogen-free male C57BL/6 mice to establish a mouse *H. pylori* infection model to determine if impaired endothelial function in human subjects with *H. pylori* infection could be re-produced in animal model using the *H. pylori* bacteria isolated from patients. Since the vast majority (>90%) of *H. pylori* infection patients in East or Southeast Asian countries are infected with CagA$^+$ *H. pylori* [56, 57], and CagA is considered to be involved in the extragastric diseases associated with *H. pylori* infection [44, 58–60], CagA$^+$ *H. pylori* bacteria isolated from gastric ulcer patients were prepared, characterized, and used for the animal experiments with phosphate buffer solution (PBS) as control. After fasting overnight, mice were infected with *H. pylori* inoculum in PBS by intragastric gavage once per day for 3 days to achieve 100% infection rate. Successful infection with CagA$^+$ *H. pylori* in mice was confirmed with both positive Rapid Urease Test (RUT) and Giemsa staining as described [61]. A 100% infection rate was achieved in C57BL/6 mice with this method. Control mice received the same volume of PBS by intragastric gavage.

Thoracic aorta was collected to evaluate endothelium-dependent relaxation to acetylcholine (Ach) and endothelium-independent relaxation to nitroglycerin (NTG) at week 1, 8, 12, and 24 after *H. pylori* infection to determine if there was a significant difference in endothelial dysfunction after acute (1 week) and chronic (24 weeks) *H. pylori* infection. Indeed, Ach-induced endothelium-dependent relaxation was significantly reduced in mice 1 week after *H. pylori* infection without

change in NTG-induced endothelium-independent relaxation. The impaired Ach-induced endothelium-dependent relaxation persisted for as long as the infection was present for at least 24 weeks in the infected mice without change in vascular contraction to either phenylephrine or potassium chloride (**Figure 1C**), while NTG-induced endothelium-independent relaxation remained intact [55]. These data demonstrated that *H. pylori* infection selectively impairs endothelium-dependent relaxation, not endothelium-independent relaxation, of thoracic aorta in mice that are similar to the findings in human subjects with *H. pylori* infection.

Efforts were made to examine if eradication of *H. pylori* infection could improve endothelium-dependent vasodilation to confirm if *H. pylori* infection was indeed the reason for endothelial dysfunction. As expected, elimination of *H. pylori* infection in mice with anti-*H. pylori* therapy (123.3 mg/Kg bismuth potassium citrate, 102.75 mg/kg tinidazole, and 51.38 mg/kg clarithromycin once daily for 2 weeks via intragastric gavage) significantly improved Ach-induced endothelium-dependent vasorelaxation without change in NTG-induced endothelium-independent relaxation (**Figure 1C**). For the control group, *H. pylori* infected mice were given the same volume of normal saline. The effectiveness of *H. pylori* eradication with antimicrobial therapies in mice was confirmed using RUT and Giemsa staining [55, 61]. These findings confirm that impairment of endothelium-dependent vasodilation associated with *H. pylori* infection is reversible in mouse model, similar to the observations in human subjects.

4.3 Potential mechanisms for the effect of *H. pylori* infection on endothelial function

It is important to know how *H. pylori* infection leads to endothelial dysfunction. *In vitro* study using bovine aortic endothelial cells (BAECs) showed that treatment of BAECs with *H. pylori*-conditioned medium from *H. pylori* 60190 (vacuolating cytotoxin A) significantly decreased the proliferation, tube formation, and migration of the cells (by up to 44%, 65%, and 28%, respectively) through VacA-dependent reduction in the production of endothelial nitric oxide (NO) [62]. Culture of human umbilical vein endothelial cells (HUVECs) with *H. pylori* significantly inhibited the proliferation, migration, and tube formation of HUVECs, and increased the production of the inflammatory factor Chitinase 3 Like 1 (CHI3L1) and phosphorylated p38 in endothelial cells associated with an increased expression of GATA3. Increased levels of GATA3 and CHI3L1 were also found in the arteries of mice with *H. pylori* infection. Knockdown of GATA3 could prevent *H. pylori*-induced dysfunction of HUVECs. These findings suggest that *H. pylori* might impair endothelial function through increased expression of GATA3 and production of CHI3L1 [63].

H. pylori urease (HPU) is considered a key virulence factor that enables bacteria to colonize and survive in the stomach. It has been shown that HPU could trigger the production of reactive oxygen species (ROS) in endothelial cells. Increased intracellular ROS could lead to activation of nuclear factor kappa B (NF-κB) and upregulate expressions of cyclooxygenase-2, hemeoxygenase-1, interleukin (IL)-1β, and ICAM-1, thus increasing oxidative stress and endothelial dysfunction [64]. *H. pylori* infection of primary human endothelial cells is reported to stimulate secretion of important inflammatory cytokines, IL-6 and IL-8 (especially IL-8) in endothelial cells [65]. Treatment of HUVECs with different CagA positive and negative *H. pylori* derived products could enhance the expressions of microRNAs (miRNAs) including miR-21, miR-155, and miR-663 in the cells that are associated with inflammation, apoptosis and necrosis of the cell [66]. Recently, it was reported that *H. pylori* infection could impair endothelial function through exosomes-mediated mechanism [55]. This will be discussed in details below.

5. Role of exosomes in mediating the effect of *H. pylori* infection on endothelial function

H. pylori do not enter the blood circulation themselves because of the gastric tissue barrier and a unique survival and growth environment [67]. However, *H. pylori* virulence factor CagA and *H. pylori* DNA are present in human atherosclerotic lesions and human aorta, carotid and coronary arteries [44, 68–70]. Many cells are known to release extracellular vesicles with unique biophysical and biochemical properties [71, 72], that are referred as exosomes (with diameters from 30 to 200 nm) [73]. Exosomes are found in various body fluids including blood, urine, saliva, and breast milk, and play an important role in cell-to-cell communications through transport of a wide spectrum of bioactive constituents including proteins, lipids, and miRNAs [74, 75]. Recent studies have demonstrated that exosomes are critically involved in the transfer of proteins during infections like prion protein in neurodegenerative disease [76], human immunodeficiency virus-related proteins [77], and human T-cell leukemia virus type-1 proteins [78]. Indeed, it is shown that *H. pylori* infection increases the expression of miR-25 in gastric epithelial cells and is associated with increased levels of exosome-transmitted miR-25 in peripheral blood in human subjects. Further studies demonstrate that Kruppel-like factor 2 (KLF2) is a direct target of exosome-transmitted miR-25 in vascular endothelial cells. MiR-25/KLF2 axis is involved in the regulation of NF-κB signaling pathway, resulting in increased expression of IL-6, monocyte chemoattractant protein-1, vascular cell adhesion molecule-1, and ICAM-1 [79].

To determine how *H. pylori* infection impairs endothelial function, a recent study tested the hypothesis that *H. pylori* could interact with gastric epithelial cells (GES-1), leading to the release of CagA-containing exosomes into the circulation that in turn impair endothelial function [55]. Indeed, Western blotting analysis and immunofluorescence staining demonstrated that the unique *H. pylori* virulence factor CagA entered into human GES-1 after incubation with $CagA^+$ *H. pylori* (**Figure 2A** and **B**). Further studies showed that characteristic exosomes were present in the conditioned media of human GES-1cultured with $CagA^+$ *H. pylori* as defined by specific biomarkers (HSP70 and CD9) using Western blotting, by specific morphologic features using transmission electron microscopy, and by size distribution using a Zetasizer Nano ZS instrument [80]. Western blotting analysis demonstrated that the exosomes from the conditioned media of human GES-1 cultured with $CagA^+$ *H. pylori* contained the unique CagA protein, while exosomes from the control conditioned media of GES-1 (without culture with *H. pylori*) had no CagA protein (**Figure 2C-E**). When the labeled GES-1-derived exosomes with PKH67 were cultured with HUVECs, a detectable amount of PKH67-labeled exosomes was present in HUVECs using 3-D confocal microscopy after 12 hours of culture (**Figure 2F**), confirming the entry of exosomes into HUVECs. Treatment with human GES-1-derived CagA-containing exosomes significantly inhibited the function of HUVECs with decreased proliferation, migration, and tube formation as compared with the control exosomes (**Figure 2G-I**).

Further studies [55], using the serum exosomes from patients with $CagA^+$ *H. pylori* infection and from healthy age-and sex-matched volunteers, revealed that serum exosomes from both patients and healthy subjects exhibited the characteristics similar to the exosomes from human gastric epithelial cells GES-1 cultured with CagA+ *H. pylori* in their morphology using transmission electron microscopy, size distribution using a Zetasizer Nano ZS instrument, and unique biomarkers (HSP70 and CD9) using Western blotting. As expected, CagA protein was detected in the serum exosomes from patients with $CagA^+$ *H. pylori* infection, but not from control

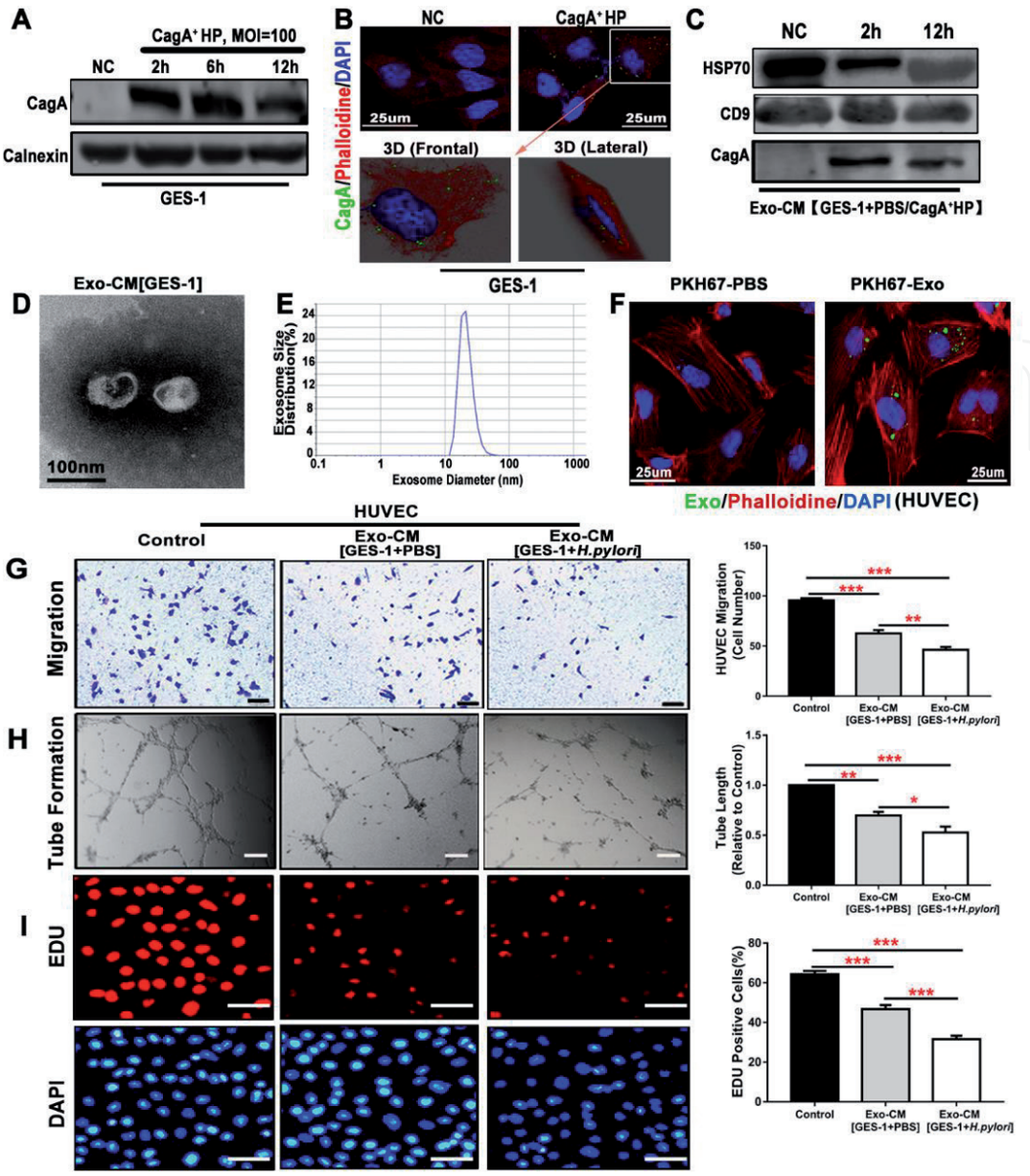

Figure 2.
Exosomes from human gastric epithelial cells GES-1 cultured with CagA$^+$ H. pylori *significantly inhibited endothelial cell function in vitro. Western blotting analysis (A) and immunofluorescence staining (***B***) with 3-D confocal microscope demonstrated that the unique* H. pylori *virulence factor CagA entered into GES-1 after culture with CagA*$^+$ H. pylori*. Exosomes isolated from the conditioned media of GES-1 cultured with CagA*$^+$ H. pylori *displayed typical features for exosomes including characteristic biomarkers HSP70 and CD9 by western blotting (***C***), morphologies on transmission electron microscopy (***D***), and size using a Zetasizer Nano ZS instrument (***E***). Western blotting analysis confirmed the presence of CagA protein in the exosomes from the conditioned media of GES-1 cultured with CagA*$^+$ H. pylori*, but not in the ones from GES-1-conditioned media without CagA*$^+$ H. pylori *(***C***). PKH67-labeled GES-1-derived exosomes (***green***) were incubated with* **HUVECs** *(30 μg protein/5 × 10*^{4} *cells), and a significant amount of PKH67-labeled exosomes were detected inside the HUVECs as visualized using a 3-D confocal microscope (***F***), confirming that the exosomes entered into the cells. Treatment of HUVECs with CagA protein-containing exosomes (50ug/ml) from GES-1-conditioned media for 24 hours significantly inhibited the function of HUVECs with decreased migration (***G,** *scale bars = 200 μm), tube formation (***H,** *scale bars = 200 μm), and proliferation (***I,** *scale bars = 50 μm).* **NC:** *Normal control;* **CagA+ HP:** *CagA*$^+$ H. pylori*;* **GES-1***: Human gastric epithelial cells;* **HUVEC:** *Human umbilical vein endothelial cell;* **Exo-CM***: Exosomes derived from conditioned medium. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data were presented as mean ± SE, n = 3 independent experiments (experiment was repeated 3 times for every measurement). [adopted from (55) with permission].*

subjects using Western blotting analysis. When labeled human serum exosomes with PKH67 were cultured with HUVECs, a significant amount of PKH67-labeled exosomes was present in HUVECs using 3-D confocal microscopy after 12 hours of culture, confirming the entry of serum exosomes into HUVECs. Treatment with

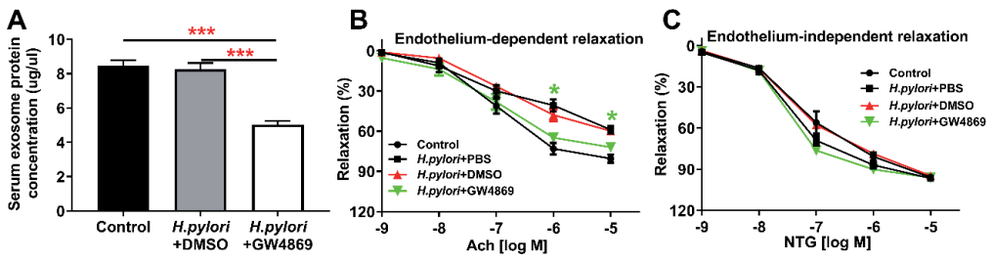

Figure 3.
Inhibition of exosome secretion by GW4869 significantly improved endothelium -dependent vascular relaxation in mice with CagA⁺ H. pylori *infection. Treatment with GW4869 significantly decreased the serum exosome level in the mice with CagA⁺* H. pylori *infection (A) as reflected by the significantly decreased total exosome protein levels (***p < 0.001 by one-way ANOVA with Bonferroni's test), and significantly improved acetylcholine (***ach***)-induced endothelium-dependent relaxation (B) of the aorta in the mice with CagA⁺* H. pylori *infection without change in nitroglycerin (***NTG***)-induced endothelium-independent relaxation (C). *p < 0.05 (when CagA⁺* H. pylori *+ GW4869 group compared with CagA⁺* H. pylori *+ DMSO group).* **Ach***: Acetylcholine;* **NTG***: Nitroglycerin;* **DMSO***: Dimethylsulfoxide (solubilizer of GW4869). Data shown were mean ± SE. n = 8 mice for control group and 10 mice for other groups. [adopted from (55) with permission].*

serum-derived CagA-containing exosomes from patients with CagA⁺ *H. pylori* infection significantly inhibited the function of HUVECs with decreased migration, proliferation, and tube formation. Of note, culture with serum exosomes from healthy control subjects also moderately and yet significantly inhibited endothelial function with decreased migration, tube formation, and proliferation, suggesting that some endogenous substances in the normal serum exosomes could also lead to endothelial dysfunction. However, the serum exosomes from patients with CagA⁺ *H. pylori* infection exhibited significantly greater inhibitory effects on endothelial functions than the ones from healthy subjects.

Studies were also performed to determine if blocking exosomes release with GW4869 *in vivo* could improve endothelial function in mice with CagA⁺ *H. pylori* infection [55]. Indeed, treatment with GW4869 significantly decreased the level of serum exosomes in the mice with *H. pylori* infection (**Figure 3A**), and effectively preserved Ach-induced endothelium-dependent relaxation of the aorta without change in NTG-induced endothelium-independent relaxation (**Figure 3B** and **C**). These findings suggest that *H. pylori* (especially CagA⁺ *H. pylori*) infection could lead to significant endothelial dysfunction in both patients and mice through exosomes-mediated mechanisms.

6. Effect of *H. pylori* infection on other cardiovascular risk factors

It is not surprising that *H. pylori* infection increases the risk for atherosclerosis and other CVDs including HTN and stroke. It has been reported that *H. pylori* infection promotes the release of IL-1, IL-6, TNF-α, and other cytokines, and activates local and systemic inflammatory response, thus leading to endothelial dysfunction and atherosclerosis [81–83]. *H. pylori* infection could also lead to malabsorption of vitamin B12, which could increase serum level of homocysteine, and promote the development and progression of atherosclerosis [84]. In addition, *H. pylori* could enhance the oxidation of low-density lipoproteins (LDL) and increase atherosclerotic plaque formation with decreased plaque stability [85, 86]. We also observed that the levels of LDL-cholesterol in patients with *H. pylori* infection were significantly higher than those without *H. pylori* infection, while the level of high-density lipoprotein cholesterol (HDL-C) were significantly decreased in the patients with *H. pylori* infection than those without *H. pylori* infection [47]. Patients with *H. pylori* seropositivity were shown to have increased brachial-ankle

pulse wave velocity (a marker of atherosclerosis), and impaired glucose metabolism [87]. It is believed that *H. pylori* could interact with gastric epithelial cells to up-regulate the expression of adhesion molecules, and secrete cytokines, which could activate leukocytes, damage the vascular endothelium, aggravate local and systematic inflammatory responses, and thus promote the development and progression of atherosclerosis and related CVDs.

7. Significance and clinical implications

It is very concerning that cardiovascular mortality has been increasing since 2010 especially for males for unknown reasons [6]. It is also reported that the patients with ST elevation myocardial infarction over the past 20 years are getting younger [5]. The reasons for this reverse trend in cardiovascular mortality and mobility have yet to be defined. *H. pylori* infection selectively increased the risk for carotid atherosclerosis in young male patients (≤ 50 years), not in older males or female patients. A recent study [33] that analyzed a large database with a study population of 208,196 showed that the mortality rate was significantly lower in patients with early eradication of *H. pylori* infection. The cumulative CAD rate was significantly decreased in younger patients (<65 years old) with *H. pylori* eradication therapy within 1 year of infection compared to those patients without eradication at all. Interestingly, the treatment of *H. pylori* eradication did not have a benefit in older patients (>65 years old). These data strongly suggested that *H. pylori* infection could be a significant risk factor for endothelial dysfunction, atherosclerosis and CAD in young patients, and could provide a potential explanation for young patients who develop CAD without a clear etiology. It is unclear why *H. pylori* infection does not increase the risk for atherosclerosis for patients older than 50 years. It is possible that other significant risk factors like DM, HTN, and hyperlipidemia play a dominant role that could mask the contribution of *H. pylori* infection to the development and progression of atherosclerosis in this age group of patients. Further studies are needed to investigate the mechanism(s) on the selective effect of *H. pylori* infection on atherosclerosis in young population.

There are substantial sex differences in many CVDs including (but not limited to) myocardial infarctions, heart failure, hypertension, and cardiac hypertrophy [88]. It is well known that premenopausal women are relatively protected from CVDs when compared to men. Typically, women are almost 10 years older than men when they have their first myocardial infarction [89]. It was believed that the decreased cardiovascular morbidity and mortality in young females was due to possible cardio-protective effects of estrogen [90]. However, several large clinical studies, including the HERS trials and the Women's Health Initiative study [91, 92] showed that hormone replacement therapies (HRT) had no cardiovascular benefit in post-menopausal women. In contrast, there might have been an increased risk of CAD during the first year of HRT, and there was an increased risk of nonfatal ventricular arrhythmias among the women on HRT [91]. Thus, the mechanism(s) for decreased CVD risk in premenopausal women is still unclear. The prevalence of *H. pylori* infection was the same in males and females, and yet, *H. pylori* infection only increased the risk for carotid atherosclerosis in male patients≤50 years, not in older males or female patients. It is possible that the significant sex and age difference in the development of atherosclerosis associated with *H. pylori* infection may be one of the reasons for decreased risk for CAD in young females. Further studies are needed to confirm these findings with both patients and experimental animal models.

Currently available data strongly suggest that *H. pylori* infection is an important risk factor for endothelial dysfunction and CVDs especially in young male population. The available data also provide solid evidence to support screening young male population for *H. pylori* infection once a year and treating accordingly for early prevention of CVDs especially premature atherosclerosis associated with *H. pylori* infection.

8. Conclusions

H. pylori infection significantly increases the risk for CVDs including atherosclerosis, HTN, CHD, cerebrovascular disease, and peripheral arterial diseases especially in younger patients (< 65 years old). *H. pylori* infection significantly impairs vascular endothelial function through multiple mechanisms including increased ROS production and oxidative stress, inflammation, decreased NO formation, modification of the expression of cytokines and miRNAs, interruption of lipid and glucose metabolisms, and exosomes-mediated pathways as shown in **Figure 4**. Endothelial dysfunction associated with *H. pylori* infection is reversible in both animal model and human subjects if the infection could be eliminated in a timely fashion (within one year of infection for human subjects and 6 months for mice). Accumulating data suggests that *H. pylori* infection is an additional risk factor for endothelial dysfunction and CVDs. Screening young male population for *H. pylori* infection once a year and treating accordingly could be an effective approach for early prevention of CVDs especially premature atherosclerosis associated with *H. pylori* infection.

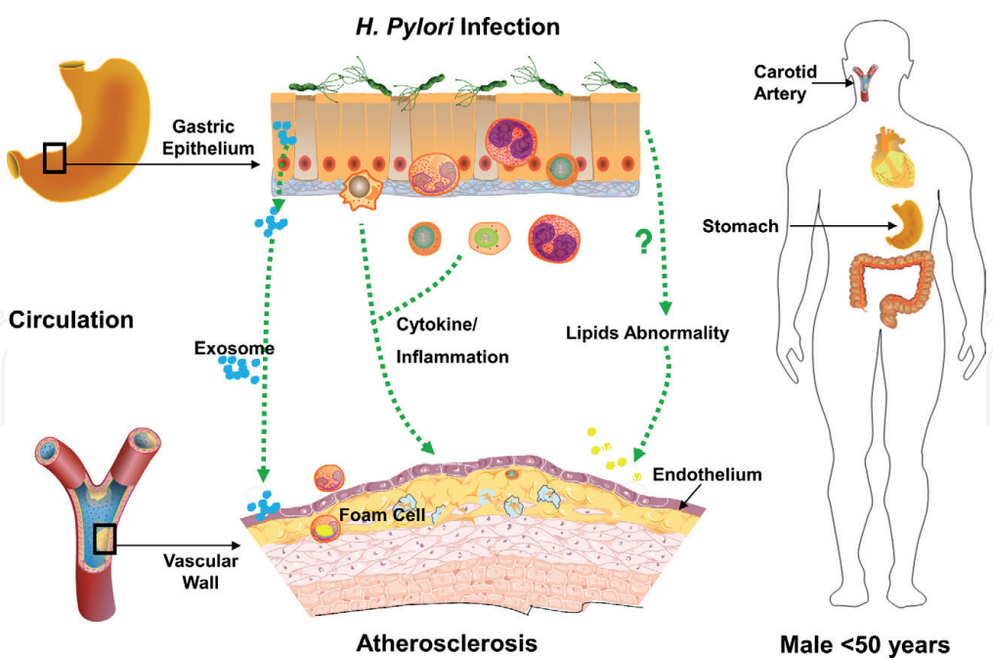

Figure 4.
Schematic illustration of the mechanism on endothelial dysfunction and atherosclerosis associated with H. pylori *infection. It is proposed that* H. pylori *infection could impair endothelial function through exosome-mediated mechanisms. CagA protein is only from CagA*$^{+}$ H. pylori*, and could serve as an ideal tracking molecule for exosome trafficking in vivo. CagA*$^{+}$ H. pylori *translocate CagA protein into gastric epithelial cells (GES-1). CagA-containing exosomes are released into circulation from GES-1, then enter into endothelial cells, leading to endothelial dysfunction.* H. pylori *Infection could also decrease endothelial function through increased production of reactive oxygen species, oxidative stress, and inflammation, decreased cellular nitric oxide formation, modification of the expression of cytokines and miRNAs, and interruption of lipid and glucose metabolisms. [adopted and modified from (47) with permission].*

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Conflict of interest

None.

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