INTERPLAY BETWEEN *Helicobacter pylori,* DIET, AND DISEASE

By

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CHAPTER 1

INTRODUCTION

***Helicobacter pylori* and disease**

Helicobacter pylori is a gram-negative, microaerophilic rod shaped bacteria that colonizes approximately 50% of the global population (1, 2). Infection with this bacterium is a strong risk factor for the development of disorders such as peptic ulcer disease or gastric adenocarcinoma (3).

Gastric cancer is a major public health concern and a leading cause of cancer mortality, resulting in over half a million deaths annually (4). Infection with *H. pylori* is the strongest risk factor for this disease, which led the World Health Organization to formally designate *H. pylori* as a type 1 carcinogen in 1994 (5). One of the reasons for the high mortality associated with gastric cancer is the fact that it is frequently asymptomatic until late stages, at which point treatment options are limited and offer lower rates of success (6).

While disorders such as gastric cancer are one potential result of infection, only a small proportion of infected individuals develop adverse clinical outcomes attributable to *H. pylori* (3). Additionally, it has been reported that colonization can carry some benefits such as protection from asthma, a disorder afflicting increasing numbers of children in developed countries such as the United States (7).

The exact route of transmission for *H. pylori* infection is unknown. While some evidence suggests infection is spread via the fecal oral or gastric oral route (8, 9), other studies provide evidence that *H. pylori* is transmitted via contaminated water sources, such as well water (10). Upon entry to the gastric environment, *H. pylori* likely uses chemotaxis to migrate to the gastric

mucosa both by navigating away from the strong acidic environment in the lumen, and toward urea produced by gastric epithelial cells (11, 12). The urea also acts as a substrate for the *H. pylori* urease enzyme, the activity of which increases the pH of the microenvironment surrounding the bacterium (12). In early stages of *H. pylori* infection the bacterium is mostly found in the antrum of the stomach, as the acid secreting parietal cells in the corpus protect this site from initial colonization (13). As *H. pylori* infection persists, it triggers an inflammatory response characterized by cytokine production that limits secretion of gastric acid (13). This serves to increase the overall gastric pH and expand the gastric region that *H. pylori* can colonize. Persistent infections can result in gastric atrophy and subsequent loss of parietal cells which allows for *H. pylori* growth in regions such as the corpus that were previously too acidic to colonize (13). Among patients with markedly reduced gastric acid secretion who are also colonized with *H. pylori*, a shift in gastritis is observed, switching from predominantly antrum gastritis to predominantly corpus gastritis (14). These individuals are at increased risk of developing gastric cancer (14). Interestingly, *H. pylori* population density decreases as disease severity increases, with patients experiencing gastric cancer often showing few signs of colonization (13).

Given the wide range of potential outcomes of infection, it is paramount that we elucidate factors that influence infection outcome, in order to improve public health. Three main categories of infection outcome determinants exist: strain-specific bacterial genetic variants, host factors, and environmental factors.

Several strain-specific virulence factors are determinants of clinical outcome. One such virulence factor is Cytotoxin-associated antigen A (CagA). CagA is a 120- to 145-KDa protein that

is encoded on the *cag* pathogenicity island (PAI) (15). The *cag* PAI also encodes a Type IV Secretion System (T4SS) that serves to deliver bacteria proteins (including CagA) into host cells (Figure 1) (15). Not all strains of *H. pylori* possess the *cag* PAI, but those that do are associated with increased gastric inflammation, risk of gastric cancer and peptic ulcer disease (16, 17). One mechanism by which CagA contributes to gastric carcinogenesis is through its ability to bind and activate SHP2, a phosphatase that relays positive signals for cell growth and motility (15). The increased rate of DNA replication necessary to produce new cells increases the likelihood that important genes, such as those involved in tumor suppression, will accumulate mutations. CagA has been designated as a bacterial oncoprotein (15).

Another bacterial virulence factor produced by *H. pylori* is Vacuolating Cytotoxin A (VacA). VacA has several roles in *H. pylori* infection and gastric disease. VacA promotes chronic infection with *H. pylori* by compromising the ability of the immune system to detect and phagocytose bacterial cells. One mechanism by which this occurs is through VacA targeting professional phagocytes and compromising their endocytic pathway signaling to prevent phagocytosis of *H. pylori* (18). VacA has also been shown to utilize the β2 integrin receptor to gain entry to T-cells, where it interferes with cellular proliferation and cytokine production, hobbling the immune response (18). VacA is thought to induce gastric carcinogenesis in a variety of ways (Figure 1), such as by inducing cell death and increasing the rate of cellular proliferation (19, 20). Though most *H. pylori* strains possess the *vacA* gene, they do not all possess the same form. Several different families of *vacA* alleles have been identified, with certain forms possessing more toxigenic activity and associated with worsened outcomes of disease (20).

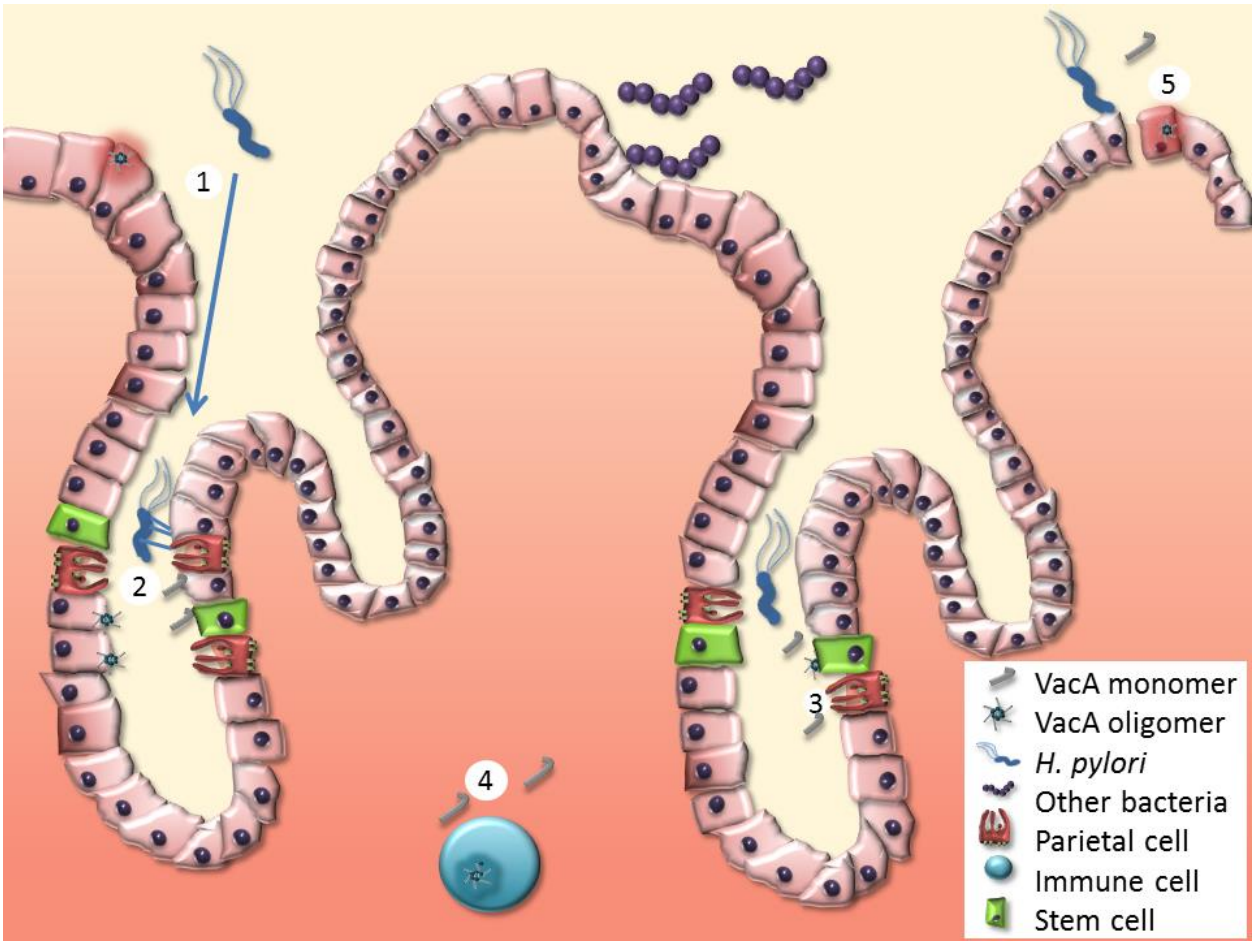

Figure 1. Overview of *H. pylori* pathogenesis. *H. pylori* colonizes the mucus layer overlying foveolar/surface mucous epithelium and also enters gastric glands (1) through preferential chemotaxis to these sites. (2) Targeting of gastric stem cells by VacA may be a critical step in the pathogenesis of gastric cancer. The T4SS is used to translocate the oncoprotein CagA into host cells, increasing risk of gastric cancer development. (3) VacA inhibits acid secretion by parietal cells and CagA may contribute to the loss of parietal cells over the course of infection, raising the pH in the stomach. Increased gastric pH allows other bacterial species to colonize the stomach. (4) VacA interferes with the function of multiple types of immune cells, potentially compromising their ability to function effectively in surveillance for malignant cells, and allowing *H. pylori* to persist chronically. (5) As a consequence of VacA targeting epithelial cells, tight junctions between gastric epithelial cells are disrupted. This potentially allows carcinogenic molecules to enter the gastric mucosa.

Host factors that shape *H. pylori*-infection outcome include genetic polymorphisms, such as in the region coding for IL-1β. IL-1β is a cytokine produced in response to many microbes and various forms of cellular injury (13). IL-1β is produced by several different types of cells, particularly those in the innate immune system such as macrophages, in response to pathogen associated molecular patterns (PAMPS) that can activate pattern recognition receptors and control gene expression pathways (21). Once produced, IL-1β is a proinflammatory cytokine that affects many cell types and serves to increase production of other proinflammatory cytokines such as TNF-α and other mediators of inflammation such as COX-2 (13). Interestingly, IL-1β interacts with gastric epithelial cells to limit secretion of gastric acid (13, 22). This likely aids in *H. pylori* chronic colonization, and allows the bacteria to expand to distal regions of the stomach (13). Certain polymorphisms of the IL-1β gene have been correlated to higher levels of IL-1β production (13). In turn, these polymorphisms have also been associated with worsened gastric disease among *H. pylori* infected individuals (13, 23, 24).

Finally, environmental factors including host diet have also been shown to play a role in infection outcome. High salt or low iron diets increase the risk of gastric inflammation and cancer (25–31). Diet is a major focus of my research, and represents an important disease determinant in *H. pylori*-infected individuals.

Role of diet in *H. pylori* mediated disease

Both increased dietary salt intake and decreased dietary iron intake are associated with a heightened risk of gastric cancer incidence in *H. pylori*-infected humans and in experimentally infected gerbils (32–41). Gerbils maintained on a high salt diet and infected with *H. pylori*

exhibited significantly greater gastric inflammation scores when compared to gerbils infected with *H. pylori* but consuming a normal salt diet (32). This is attributed at least in part to enhanced virulence of *H. pylori* under high salt or low iron conditions (32, 34, 42). High salt conditions have been shown to cause alterations in *H. pylori* gene transcription or protein production *in vitro* and *in vivo*, including augmented production of the bacterial oncoprotein CagA (32, 43–45). In gerbils infected with a CagA null mutant of *H. pylori*, the high salt diet induces no more gastric inflammation than that observed in gerbils infected with the same *cagA* deficient strain that are consuming a normal diet (32). This indicates the importance of *cagA* in mediating the increased inflammation observed under high salt conditions (32).

Similar to the effects of high salt, low iron conditions have been shown to cause alterations in *H. pylori* gene transcription and stimulate increased activity of the *cag* type IV secretion system (T4SS) (34, 46, 47). *H. pylori* strains exposed to low iron conditions produce more T4SS pili (an important structure critical for *H. pylori* virulence) and translocate more CagA into host cells (34, 42). In Mongolian gerbils, low iron diets were shown to accelerate the development of gastric carcinogenesis, and worsen gastric inflammation in a CagA dependent manner (34). In human studies, individuals who exhibited signs of iron deficiency (lower serum ferritin levels) and who were infected with *H. pylori* exhibited more severe gastric pathology when compared to *H. pylori* infected individuals who had normal serum ferritin levels (34). Strains collected from patients with iron deficiency also induced more IL-8 (an important cytokine in the immune response to *H. pylori*) expression when co-cultured with human AGS cells (34).

Of the factors that influence disease presence and severity, host diet is of particular interest, as it serves as a potential point of intervention if individuals consuming diets that promote gastric carcinogenesis were to switch to healthier alternatives.

Extra-gastric effects of *H. pylori* colonization

Although *H. pylori* exclusively colonizes the human stomach, the consequences of infection status extend beyond the stomach lining. As *H. pylori* colonization levels plummet in certain populations (some studies estimate a 10% infection rate in developed countries), potential benefits of colonization are becoming increasingly clear (48). Gastroesophageal reflux disease (GERD) and esophageal cancer are disorders with a reported inverse relationship with *H. pylori* colonization (48, 49). Infected individuals experience less acidic gastric environments over the course of typically life-long *H. pylori* infection, likely conferring protection from esophageal cancer as exposure to gastric acid serves to drive this form of disease (50, 51).

Another reported benefit of colonization with *H. pylori* includes protection from asthma. Studies have noted inverse associations for asthma and colonization with *H. pylori*, particularly cagA+ strains (51). Importantly, the decline in *H. pylori* colonization levels predates the dramatic increase in asthma incidence (52). Murine models have shed light on potential mechanisms by which *H. pylori* could be exerting protective effects. These studies have identified T regulatory cells as important mediators of protection, with T regulatory cell depletion studies abrogating the protective effect of *H. pylori* infection, while transferring the T regulatory cell population of infected donors to uninfected mice confers protection (53).

Changes to systemic levels of circulating blood cells have also been reported. Idiopathic thrombocytopenic purpura (ITP) is a disorder in which autoantibodies target the host's own platelet cells, leading to a reduction in circulating platelets (54). *H. pylori* infection is thought to contribute to ITP development, and eradication studies have suggested that clearing *H. pylori* from the gastric environment leads to increased circulating platelet levels in patients with ITP (54).

***H. pylori* as a causative agent of anemia**

While there is abundant evidence supporting the role of *H. pylori* in gastric carcinogenesis and peptic ulcer disease, there is conflicting data regarding anemia as a potential ramification of colonization with *H. pylori* (3, 35, 48–53). Anemia is characterized by a decrease in the number of healthy circulating red blood cells, or in hemoglobin levels (56). This disorder can be serious, resulting in insufficient oxygen transport throughout the body (56). Anemia is associated with increased risk of organ damage due to anoxic conditions, as well as with mortality among afflicted individuals (56).

Anemia affects up to 1.6 billion people worldwide, resulting in a large segment of the population potentially suffering from fatigue, decreased productivity, increased susceptibility to infection, and death (57–61). There are many causes of anemia, but anemia due to iron deficiency is the most common (IDA, i.e. anemia due to iron deficiency) (62). Iron is an essential component of hemoglobin, required for the trafficking and proper release of oxygen throughout the body. Iron deficiency anemia can be due to inadequate intake of dietary iron,

improper absorption of dietary iron, or excess loss of bodily iron stores (frequently in the form of blood loss, as much of the body's iron stores are found in the blood) (59).

A wide variety of infectious processes can be primary causes of anemia or can exacerbate anemia arising from non-infectious etiologies. For example, intestinal parasitic infections can cause chronic blood loss and subsequent development of iron deficiency anemia (59, 63). Anemia arises in patients with malaria because the parasites invade erythrocytes (58), and bacterial toxins can also cause hemolysis (64). In addition, chronic infections, chronic immune activation, and cancer can cause a form of anemia known as "anemia of chronic disease" (65).

In one of the largest studies to date examining the relationship between *H. pylori* and anemia, serum ferritin and hemoglobin levels were analyzed in 2,794 Dutch adults to determine if *H. pylori* infection was associated with IDA. In men and post-menopausal women, *H. pylori* infection was linked to iron deficiency (as determined by serum ferritin levels) (66). Among pre-menopausal women, no such association was observed. Hemoglobin levels were not affected by *H. pylori* colonization status in any of the cohorts (66). A subsequent meta-analysis of existing studies detected an association between *H. pylori* infection and IDA, based on analyses of hemoglobin and serum ferritin (67). Conversely, several other studies have not detected any association between *H. pylori* and anemia (19).

Animal studies examining a potential link between *H. pylori* infection and anemia have also yielded conflicting results (69–72). In one study, *H. pylori*-infected male INS-GAS mice (which overexpress gastrin) exhibited reductions in both hemoglobin and serum ferritin levels compared to uninfected animals; however the mean corpuscular volume (MCV, a measure of

the average size of erythrocytes) was elevated in the infected cohort (71), a finding that differs from the reduced MCV typically observed in patients with IDA. Infection of INS-GAS mice with the related organism *H. felis* also resulted in anemia (69). A potential limitation of the mouse model for studying *H. pylori*-associated IDA is that *H. pylori* strains often undergo inactivating mutations in the *cag* pathogenicity island during the course of mouse stomach colonization (73, 74). In a study of Mongolian gerbils infected with a *cagA*-producing *H. pylori* strain (ATCC43504), anemia was not detected in *H. pylori*-infected gerbils, although a trend towards decreased MCV in infected animals was observed (72).

H. pylori is present in about half of the human population worldwide, most of whom are asymptomatic. If *H. pylori* contributes to anemia in asymptomatic people, this bacterium could potentially have a substantial impact on the incidence or severity of anemia worldwide (75). The pathogenic mechanisms by which *H. pylori* might contribute to anemia in asymptomatic people are not well understood. Anemia could potentially occur due to blood loss from asymptomatic gastric erosions, impaired absorption of iron due to increased gastric pH, reduced vitamin B12 levels due to atrophic gastritis and parietal cell loss, or anemia of chronic disease. Thus far, there have been few studies designed to discriminate among these possibilities. My study examined each potential cause of anemia in the Mongolian gerbil model (Chapter 1).

Genetic adaptations of *H. pylori* in the gastric environment

Humans and *H. pylori* have co-evolved for tens of thousands of years, a relationship predating the migration of humans from Africa (76). Among different *H. pylori* strains a great

deal of genetic diversity exists, largely driven by the extremely high recombination and mutation rate (77). Individuals colonized by *H. pylori* tend to harbor unique strains that are genetically distinct from other *H. pylori* strains (77). As *H. pylori* infection is typically acquired early in life yet persists chronically, ample opportunities to diversify in the gastric niche exist (77).

Several studies thus far have examined how *H. pylori* evolves over the course of infection. Studies focusing on output strains from humans, primate, and rodent models have identified a wide range of genetic changes that occur, and the term "quasispecies" has been used to describe the degree of genetic diversity present (14, 17–19, 24, 49, 51, 52). Changes to the region encoding CagY (found on the *cag* PAI and essential for functional T4SS formation) have been reported in mice, with these changes leading to the loss of the strain's ability to translocate CagA into host epithelial cells and induce IL-8 (80). Similar studies using rhesus macaques confirmed these changes also occur using this model system (74). Interestingly, most of the mutations present in *cagY* don't lead to a lack of CagY production, but some prevent the formation of the T4SS pilus (74). These mutations are thought to be one mechanism by which *H. pylori* can evade the host immune system by modulating the inflammatory response triggered during infection (74).

While many mutations have been identified, the number of animal adapted strains examined thus far via whole genome sequencing is relatively small, indicating the potential presence of many more unidentified putative genetic changes remaining to be observed. My study examines output strains evolution in 5 gerbils, to better understand mutations that occur *in vivo* (Chapter 3).

Animal models of *H. pylori* infection

The use of animal models in *H. pylori* research has led to many insights that translate to human infections with this species. Animal models are particularly helpful to address the shortfalls of human studies in examining the effect of diet on disease. Animal experiments do not rely on self-reported data, and researchers can more precisely control the diets of the subjects.

Rodent models are popular tools, owing to the large numbers available and ease of use of these systems. Mice can be used to study *H. pylori* infection, and the ease of acquisition and genetic manipulation available in mice make them ideal for studying host factors that influence infection outcome, but there are several drawbacks. Mice that are not genetically manipulated generally only develop mild inflammation as a result of *H. pylori* infection, and do not get gastric cancer (81). Additionally, the *cag* PAI tends to be lost by the colonizing *H. pylori* over the course of infection, making it difficult to study this major virulence factor using a mouse model (81).

Another rodent model system commonly used is the Mongolian gerbil. Gerbils are not outbred, which can be advantageous in that they more closely mimic the natural genetic diversity found in humans, but it also makes it difficult to conduct studies designed to assess the importance of host factors (81).

Primate studies are also done using *H. pylori,* and while cost and the low number of available subjects are limitations to this system, primates are an otherwise excellent model for *H. pylori* infection owing to their close genetic similarity to humans (81).

Goals of study

I wanted to address several gaps in knowledge that were present in the field. Determining if *H. pylori* was a causative agent of IDA and other systemic changes, and gaining a more thorough understanding of the genetic adaptations of *H. pylori* in the gastric niche were my top priorities. Thus, the goals of my study were threefold: 1.) To understand the influence of low iron and high salt diets on gastric pathology and systemic changes in *H. pylori*-infected gerbils. 2.) To provide experimental evidence to address my hypothesis that *H. pylori* is a causative agent of iron deficiency anemia in animal models. 3.) To identify genetic adaptations of *H. pylori* that arise during chronic infection of the gastric environment. I elected to use the Mongolian gerbil model as this rodent model system more closely mirrors the range of gastric pathology displayed by humans in response to *H. pylori* infection, without requiring additional genetic manipulations. Additionally, Mongolian gerbils are outbred, which mimics the genetic variation present in humans and could increase the diversity of bacterial genetic adaptations detected.

CHAPTER 2

H. pylori AND ANEMIA

Introduction

As there was a potential link between *H. pylori* and anemia, we sought to investigate this phenotype using the Mongolian gerbil model. Previous work conducted by former lab members suggested a role for diet in mediating IDA among infected gerbils. In one prior study, gastric pH was significantly increased among gerbils infected with *H. pylori* and consuming a diet with elevated salt content when compared to infected gerbils consuming a normal diet or uninfected gerbils (32). As dietary iron becomes less bioavailable at an elevated pH, we hypothesized that the high salt infected cohort could be especially susceptible to anemia. Additional data from this study revealed a significant reduction in hepcidin transcripts among gerbils infected with *H. pylori*, and consuming a diet high in salt when compared to both infected gerbils on a normal diet, and uninfected gerbils (32). As increased hepcidin levels are a signal that internal iron stores are sufficient and additional iron uptake is halted, reduced hepcidin levels indicate a potential iron deficiency.

In addition to IDA, reports have also linked *H. pylori* to pernicious anemia, a type of anemia that arises from chronic vitamin B12 deficiency. This is postulated to be due to parietal cell loss, a phenomenon that frequently accompanies chronic *H. pylori* infection. Parietal cells produce intrinsic factor, an essential component that allows dietary B12 to be absorbed in the gastrointestinal tract. Insufficient intrinsic factor production as a result of parietal cell loss is a potential driver of pernicious anemia. We sought to investigate the potential of *H. pylori* to mediate each type of anemia, and to also examine the role diet played in these diseases.

To interrogate the link between *H. pylori* infection and subsequent anemia development, we placed cohorts of gerbils on one of 4 diets: a normal diet (AIN-93M, a maintained chow commonly fed to gerbils) a high salt diet (AIN-93M engineered to contain 8.25% salt as opposed to 0.25% salt in the normal chow), a low iron diet (AIN-93M engineered to contain 0 ppm iron compared to 39 ppm iron in the normal chow) and a combination diet (AIN-93M engineered to contain 8.25% salt and 0 ppm iron). We chose to include the low iron diets as this was a known risk factor for iron deficiency anemia development.

Materials and methods

Mongolian gerbils

A single cohort of 96 male gerbils between the ages of 3-5 weeks (up to 40 grams in weight) was obtained from Charles River Laboratories. The animals were divided into 4 groups (24 animals per group), each of which received a different diet. One group received TestDiet AIN-93M (Purina Mills) and the other groups received modified versions of AIN-93M: low iron (AIN-93M manufactured to contain no added iron compared to 39 ppm iron in the normal chow), high salt (AIN-93M modified to contain an additional 8% sodium chloride, for a total concentration of 8.25% sodium chloride compared to 0.25% in the normal chow), or a combination high salt/ low iron (AIN-93M manufactured to contain no added iron and 8.25% sodium chloride). Within each group, 16 animals were experimentally infected with *H. pylori* and 8 remained uninfected. The defined diets were fed to each gerbil cohort for 3 weeks prior to *H. pylori* infection and throughout the remainder of the experiment. Uninfected control gerbils were fed the corresponding diets for the same length of time. Gerbils were euthanized 11 or 16 weeks after infection. Experiments were designed with the intent to euthanize 2

uninfected animals and 6 infected animals per diet at 11 weeks post-infection, and intent to euthanize 6 uninfected and 10 infected animals per diet at 16 weeks post-infection. The actual numbers of data points reported for individual assays vary slightly from these intended numbers due to several issues involving individual animals, including clotting of blood samples at the time of harvest, suboptimal quality of gastric tissue collected for histologic analysis, or unexpected death of animals prior to the 11 or 16 week time points. All experimental procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Inoculation with *H. pylori*

Gerbils were experimentally infected with *H. pylori* strain 7.13. Strain 7.13 is a CagA-positive strain with a functional *cag* type IV secretion system, that exhibits increased CagA production under conditions of high salt (82). Additionally, 71.3 has a mutation in *vacA* that abrogates VacA production (83, 84). The parental strain (B128) was originally isolated from a human with a gastric ulcer and was experimentally introduced into a Mongolian a gerbil. An isolate from the gerbil was designated strain 7.13. *H. pylori* strain 7.13 was grown at 37°C in room air supplemented with 5% CO_2 in sterile Brucella broth supplemented with 10% fetal bovine serum (FBS) to mid-log phase (approximate OD_{600} of 0.6) (84). Gerbils were fasted overnight, and then were infected via oral gavage with $1x10^9$ CFU. Gerbils were gavaged twice, on the first and third day of the experiment, each time fasting overnight prior to gastric challenge with *H. pylori* (32, 34).

Hematological, serum ferritin, and serum B12 analysis

At the experimental endpoints, blood was collected from each gerbil by cardiac puncture and placed into tubes containing the anti-coagulant EDTA. Complete blood counts (CBCs) were obtained by the Translational Pathology Shared Research core at Vanderbilt. CBC results included hemoglobin values, mean corpuscular volume measurements, white blood cell counts and platelet counts. Serum was collected at the experimental endpoint and stored at -80°C. Serum ferritin was analyzed using a ferritin ELISA designed for use with mouse blood, following manufacturer's instructions (Kamiya Biomedical Co.) (34). Serum B12 was analyzed using an ELISA designed for use with mouse blood, following manufacturer's (Kamiya Biomedical Co.) instructions.

Gastric pH

Gastric pH was measured at the experimental endpoint by gently pressing pHydrion pH paper (Micro Essential Laboratory) into the freshly incised glandular stomach at the antrum (32).

Bacterial density

To quantify *H. pylori* density in the gastric tissue, a portion of the stomach collected at the time of harvest was weighed, homogenized in sterile Brucella broth containing 10% FBS, and serially diluted onto *H. pylori*-selective tryptic soy agar plates containing 5% sheep blood (Hemostat Laboratories), vancomycin (Sigma-Aldrich) (20 g/ml), nalidixic acid (Sigma Aldrich) (10 g/ml), bacitracin (Sigma-Aldrich) (30 g/ml), and amphotericin B (Sigma-Aldrich) (2 g/ml).

Plates were incubated for 5 days at 37°C under microaerobic conditions (BD GasPak EZ Campy container system). After 5 days, CFUs were counted, and the numbers of CFUs per gram of stomach tissue were calculated (32, 34).

Diagnostic criteria for IDA

To diagnose anemia in humans, the World Health Organization analyzes hemoglobin levels in a healthy reference population. Two standard deviations below the mean hemoglobin value of this reference population is set as the lower limit of normal, and individuals with hemoglobin values below this level are considered anemic (61). To develop similar criteria for anemia in the gerbils, we used the uninfected gerbils on a normal diet as the reference population. The uninfected gerbils harvested at 11 week and 16 week time points had similar mean hemoglobin levels (13.75 g/dl and 13.28 g/dl), and therefore, results for uninfected animals from the two time points were pooled for most analyses. Two standard deviations below the mean hemoglobin value of animals analyzed at the 16 week time point was established as the lower limit for normal hemoglobin scores; gerbils with hemoglobin levels lower than this cutoff were considered anemic. Microcytic anemia was diagnosed using a similar approach, with MCV as the determinant. Two standard deviations below the mean MCV of the normal, uninfected cohort at the 16 week time point was established as the lower limit for normal MCV; gerbils with MCV values lower than this cutoff were considered to have microcytosis. To detect iron deficiency, serum ferritin values were analyzed. The normal, uninfected cohort of gerbils harvested at the 16 week time point was again utilized as the reference population. Due to a high level of variation in serum ferritin values, confidence

intervals were used as a tool to establish lower limits for iron deficiency. The lower 95% confidence interval of the mean represented the lower limit of normal, and gerbils with serum ferritin values below this level were considered iron deficient. Any gerbil meeting the criteria for both anemia and iron deficiency was diagnosed as having iron deficiency anemia.

Fecal Occult Blood

Gerbil stool was collected at the time of harvest by squeezing the contents of the large intestine into Eppendorf tubes. To detect occult blood, the Hemoccult SENSA (Beckman Coulter) assay kit was used, following the manufacturer's instructions.

Statistical analysis

All data are presented as mean $\pm$ SEM. Statistical analysis were conducted using GraphPad Prism Software package. To determine the appropriate test to determine significance, data were first plotted as a histogram to assess the distribution. Data with non-normal distributions were analyzed using non-parametric tests.

All associations were assessed using Spearman's Rank-Order Correlation. Multiple experimental groups were compared to a control group using a Kruskal-Wallis test with Dunn's multiple comparison while two cohorts were compared using Mann-Whitney U test. To determine proportional differences, Fisher's exact test was used. Significance levels were set at $p<0.05$.

Results

Anemia in *H. pylori*-infected gerbils

To investigate possible effects of *H. pylori* and diet on development of anemia, we conducted experiments using a Mongolian gerbil model of *H. pylori* infection. Male Mongolian gerbils were maintained on one of four diets: normal, high salt, low iron or a combination high salt/low iron diet. Among gerbils receiving each diet, one subgroup was experimentally infected with *H. pylori* and the other subgroup served as uninfected controls. At two experimental endpoints (11 and 16 weeks post-infection), *H. pylori*-infected gerbils and uninfected control gerbils were euthanized, blood was collected to examine hematological parameters, and gastric tissue was collected to assess changes in gastric pathology.

In an analysis of all *H. pylori*-infected gerbils compared to all uninfected gerbils (independent of diet), we observed significantly decreased mean hemoglobin values among infected gerbils at the later time point (16 weeks post-infection) compared to the uninfected gerbils (11.3 ± 0.3 g/dl and 13.4 ± 0.1 g/dl, respectively; $p<0.05$) (Fig. 2A). Infected gerbils harvested at the early time point (11 weeks post-infection) also had reduced mean hemoglobin values compared to the uninfected cohort, but the difference did not reach statistical significance (Fig. 2A and Fig. 3A). Among infected gerbils harvested at the 16-week time point, the cohort fed a combination high salt/low iron diet exhibited the lowest hemoglobin levels ($p<0.05$)(Fig. 2B). Similar results were observed when analyzing hematocrit values (Fig. 3B and C).

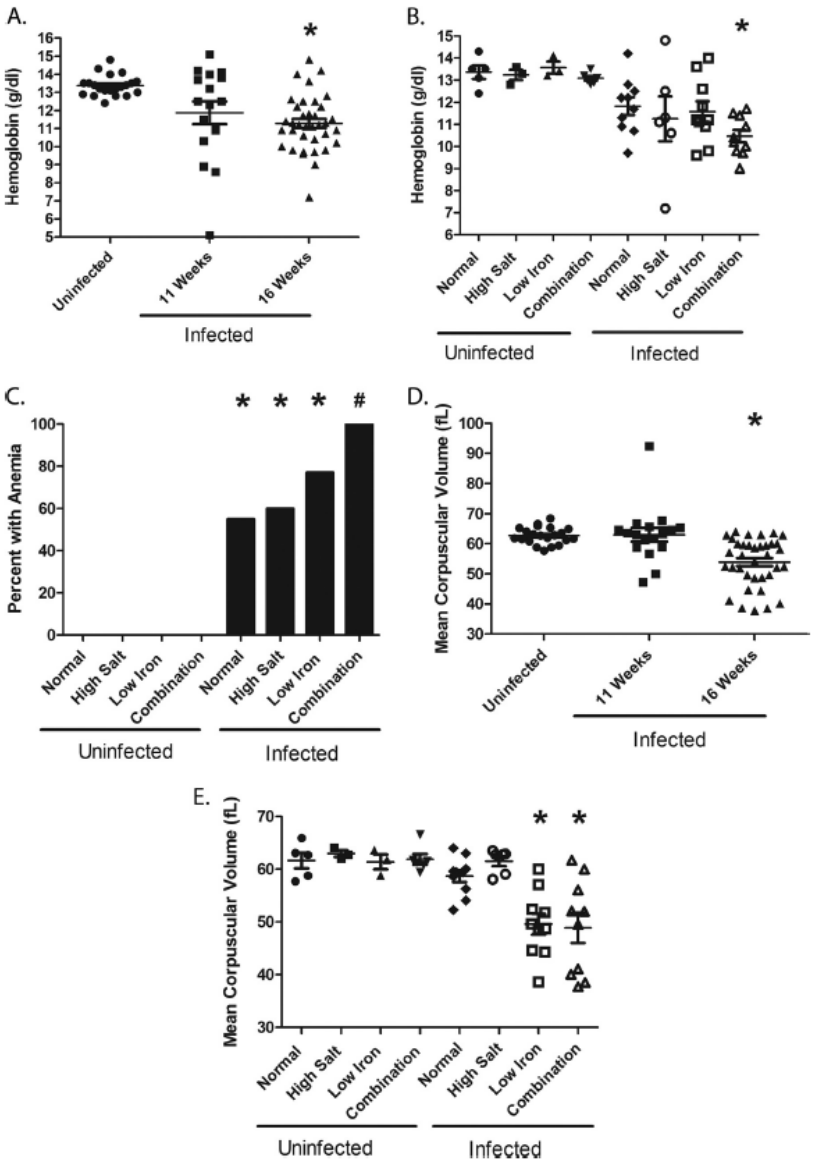

Figure 2: Reduced hemoglobin levels in *H. pylori*-infected gerbils. Horizontal bars represent mean ± standard error of the mean (SEM), and asterisks represent significant differences (p<0.05). A.) Hemoglobin levels are shown for uninfected gerbils (combined results for 11 and 16-week time points), infected gerbils at 11 weeks post-infection and infected gerbils at 16 weeks post-infection. Hemoglobin levels in infected gerbils (16 weeks post-infection) were significantly reduced compared to the uninfected group (p<0.05, Kruskal-Wallis test with Dunn's multiple comparison). B.) Hemoglobin levels at the 16 week time point, by diet. Hemoglobin levels in infected gerbils on a combination high salt/low iron diet differed significantly from the levels in the uninfected cohort on a regular diet (p<0.05, Kruskal-Wallis with Dunn's multiple comparison test) or the combination high salt/low iron diet (p<0.05). C.) Percent of gerbils considered anemic at the 16 week time point, by diet. The incidence of anemia was higher in each of the infected cohorts compared to the entire group of uninfected gerbils (* and #, p<0.005; Fisher's exact test). When comparing various infected cohorts to the

infected cohort on a normal diet, only the infected gerbils on a high salt/low iron diet had a significantly higher incidence of anemia (#, p=0.02). D.) Mean corpuscular volume (MCV) is shown for uninfected gerbils (combined results for two time points), infected gerbils at 11 weeks post-infection and infected gerbils at 16 weeks post-infection. The MCVs of infected gerbils at 16 weeks post-infection were significantly lower than the MCVs of uninfected gerbils or infected gerbils at 11 weeks post-infection (p<0.05, Kruskal-Wallis with Dunn's multiple comparison). E.) MCVs at the 16-week time point, by diet. When comparing each cohort to the reference group of uninfected gerbils on a normal diet, the MCVs of infected gerbils on a low iron diet and MCVs of infected gerbils on a high salt/low iron diet were significantly lower (p<0.05, Kruskal-Wallis with Dunn's multiple comparison test). These two groups also had a significantly reduced MCVs when compared to uninfected groups on the same diets (p<0.05, Kruskal-Wallis with Dunn's multiple comparison).

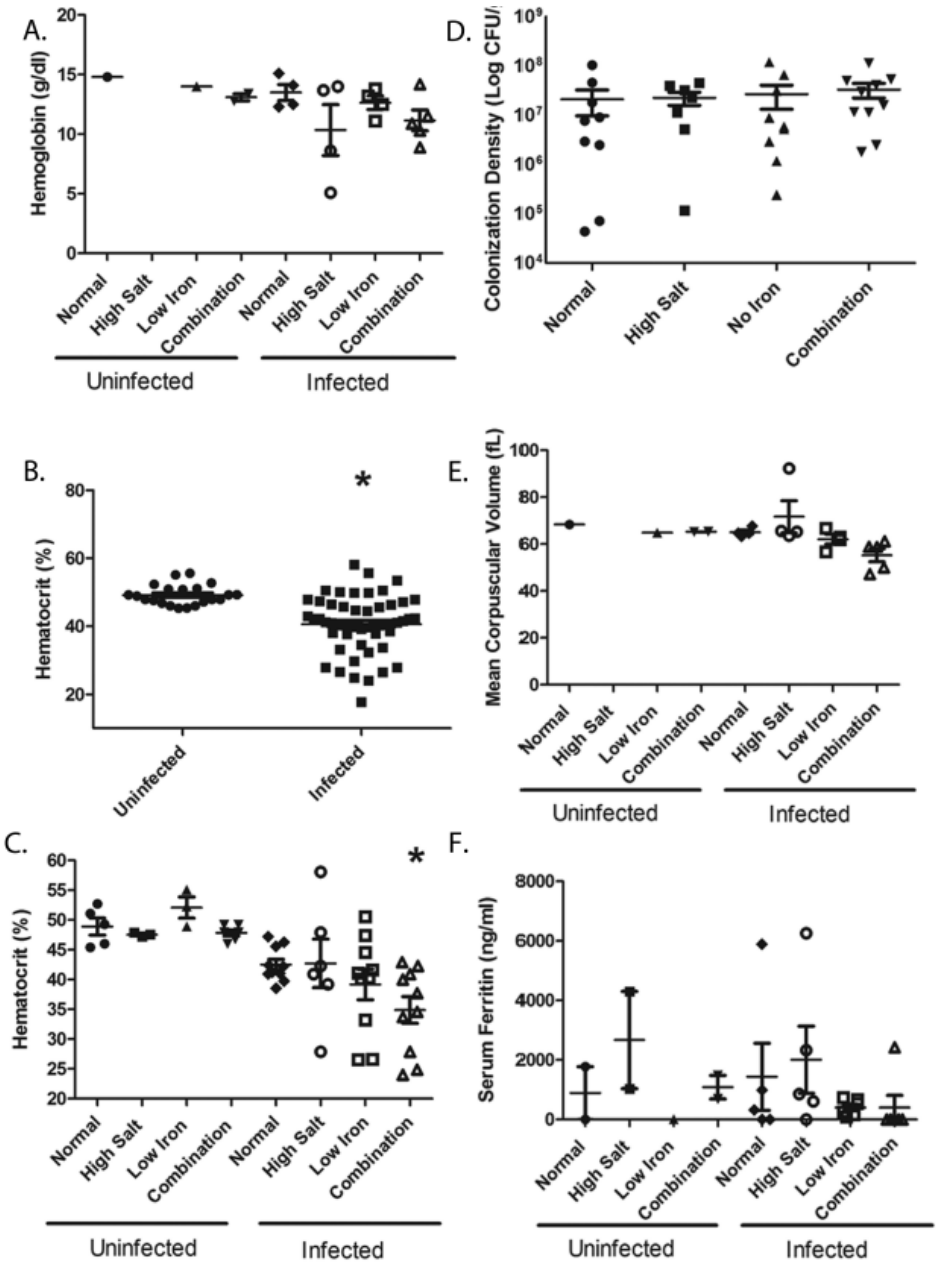

Figure 3: Hematological values of uninfected and *H. pylori*-infected gerbils. Horizontal bars represent mean ± standard error of the mean (SEM), and asterisks represent significant differences (p<0.05). Panels A E and F have fewer samples as these panels contain values from the 11 week time-point only, which was a smaller cohort than the 16-week time point. A). Hemoglobin values among infected and uninfected gerbils at the 11 week time-point. B). Hematocrit values (combined data from both 11 and 16 week time points) were significantly lower in infected gerbils than uninfected gerbils (p<0.0001, Mann-Whitney U test). C). Hematocrit values of uninfected and infected gerbils (16 week time-point), by diet. Hematocrit values were significantly reduced among the infected gerbils (40.6 ± 1.1%) compared to the uninfected gerbils (49.2 ± 0.6%, p<0.0001), with infected gerbils on a combination diet

exhibiting the greatest reduction (34.9 ± 2.3%). In a comparison of all cohorts of gerbils to the uninfected normal diet group, infected gerbils on a combination high salt/low iron diet were the only cohort with a significantly lower hematocrit level ($p<0.05$, Kruskal-Wallis with Dunn's multiple comparison). D.) *H. pylori* colonization density, expressed as log colony forming units per gram stomach tissue. No significant differences were observed between cohorts (Kruskal-Wallis with Dunn's multiple comparison). E.) Mean Corpuscular Volume at the 11-week time-point, by diet. When comparing each cohort to the reference group of uninfected gerbils on a normal diet, no significant differences were observed between cohorts (Kruskal-Wallis with Dunn's multiple comparison). F.) Serum ferritin of gerbils, by diet at the 11-week time-point. No significant differences were observed between cohorts (Kruskal-Wallis with Dunn's multiple comparison).

To determine if anemia was present in individual gerbils, we used criteria described in Materials and methods. By these standards, 72% of all infected gerbils harvested at the later time point were anemic, whereas none of the uninfected animals were considered anemic (Fig. 2C). Anemia rates among groups of infected gerbils differed, depending on the diet that they received. Infected gerbils on a normal diet had the lowest incidence of anemia (55%), and infected gerbils fed a combination high salt/low iron diet had the highest incidence (100%, p=0.02 compared to infected gerbils fed a normal diet) (Fig. 2C). There were no significant differences in *H. pylori* colonization levels among the cohorts. These data indicate that *H. pylori* causes anemia in experimentally infected gerbils and that the composition of the diet influences both the incidence and severity of anemia.

Effect of diet on iron deficiency anemia in *H. pylori*-infected gerbils

As a first step in elucidating the type of anemia present in *H. pylori*-infected gerbils, we analyzed erythrocyte mean corpuscular volume (MCV). The mean MCV was significantly lower among *H. pylori*-infected gerbils at the 16-week time point (53.9 ± 1.3 fL) compared to infected gerbils at the earlier 11-week time point (63.0 ± 2.3 fL, $p<0.05$) or uninfected gerbils (62.7 ± 0.6 fL, based on pooled data from both time points, $p<0.05$) (Fig. 2D and Fig. 3E), indicating the presence of a microcytic anemia (i.e., an anemia characterized by a reduction in MCV, in contrast to a normocytic anemia in which the MCV is not significantly reduced). The lowest MCV values were detected in infected gerbils fed either a low iron diet (49.6 ± 2.0 fL) or combination high salt/low iron diet (48.9 ± 2.9 fL; $p<0.05$ compared to the uninfected cohort on a normal diet, or to uninfected control groups on the same diets) (Fig. 2E).

The most likely causes of microcytic anemia in *H. pylori*-infected gerbils are iron deficiency anemia and anemia of chronic disease (anemia due to persistent infection or disease) (85). A distinguishing feature of IDA is low serum ferritin, whereas anemia of chronic disease is characterized by elevated serum ferritin (86). Therefore, we analyzed serum ferritin levels by Enzyme Linked Immunosorbent Assay (ELISA). Serum ferritin levels were significantly decreased among infected gerbils at both the 11 and 16 week time points (1004.0 ± 379.4 ng/ml and 516.5 ± 123.8 ng/ml, respectively) compared to uninfected gerbils (2036.0 ± 341.4 ng/ml) (p<0.05)(Fig. 4A). Serum ferritin levels among infected gerbils diagnosed with microcytic anemia were significantly decreased when compared to ferritin levels in infected gerbils determined to be non-anemic, infected gerbils with normocytic anemia, and uninfected gerbils (p<0.05) (Fig. 4B). Infected gerbils maintained on a low iron diet or combination high salt/low iron diet exhibited the greatest reductions in ferritin when compared to the uninfected gerbils on a normal diet (p<0.05) (Fig. 4C), and also had significantly reduced serum ferritin values when compared to the uninfected gerbils on the same diets (Fig. 4C).

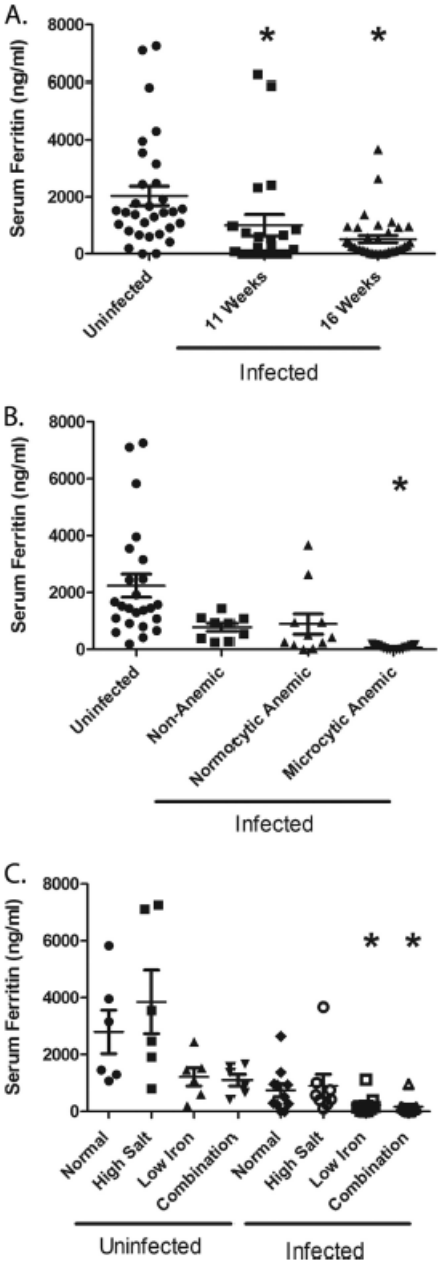

Figure 4: Reduced serum ferritin levels in *H. pylori*-infected gerbils. Horizontal bars represent mean ± standard error of the mean (SEM), and asterisks represent significant differences (p<0.05). A.) Serum ferritin levels are shown for uninfected gerbils (combined results for two time points), infected gerbils at 11 weeks post-infection and infected gerbils at 16 weeks post-infection. Infected gerbils (both time points) had significantly reduced serum ferritin compared to the uninfected gerbils (p<0.05, Kruskal-Wallis with Dunn's multiple comparison). B.) Serum ferritin at the 16-week time point, by anemia status. Serum ferritin levels among infected gerbils diagnosed with microcytic anemia (69.66 ± 17.5 ng/ml) were significantly decreased

when compared to ferritin levels in infected gerbils determined to be non-anemic (780.3 ± 139.5 ng/ml), infected gerbils with normocytic (normal MCV) anemia (894 ± 357.7 ng/ml) and uninfected gerbils (2243 ± 405.5 ng/ml) (p<0.05, Kruskal-Wallis with Dunn's multiple comparison test). C.) Serum ferritin of gerbils, by diet at the 16-week time point. Infected gerbils on a low iron diet and infected gerbils on a combination high salt/low iron diet had significantly lower serum ferritin values compared to the uninfected gerbils on a normal diet (236.1 ± 117.3 ng/ml, 165.2 ± 89.6 ng/ml, and 2796 ± 767.5 ng/ml, respectively, p<0.05 Kruskal-Wallis with Dunn's multiple comparison). The ferritin levels in the former groups also differed significantly from ferritin levels in uninfected cohorts on the same diets (p<0.05).

To determine if IDA was present in individual gerbils, we developed criteria for iron deficiency based on serum ferritin values, as described in Materials and methods. If a gerbil was deemed both anemic and iron deficient, the gerbil was classified as having IDA. By these criteria, IDA occurred in 57% of all infected gerbils and none of the uninfected animals (p<0.001, Fisher's exact test). *H. pylori*-infected gerbils on a combination high salt/low iron diet had a significantly higher rate of IDA (90%), compared to infected gerbils receiving the normal diet (30%) (p=0.0163, Fisher's exact test). Collectively, these data indicated that iron deficiency anemia occurred more commonly in *H. pylori*-infected gerbils than in uninfected gerbils. Moreover, diets containing increased salt content or decreased iron content promoted the development of iron deficiency anemia in *H. pylori*-infected gerbils, but not in uninfected gerbils during the time course of these experiments.

Anemia and gastric pH

Increased gastric pH (hypochlorhydria) can lead to decreased iron absorption, iron deficiency and anemia (87). Therefore, we examined the relationship between gastric pH and hematological alterations. Gastric pH values in *H. pylori*-infected gerbils harvested at both 16 weeks and 11 weeks post-infection (4.4 ± 0.2 and 3.7 ± 0.1, respectively) were significantly higher than in uninfected gerbils (3.0 ± 0.0, p<0.05) (Fig. 5D). There was an inverse association between gastric pH and hemoglobin values (p<0.0001, Fig. 9A). Similarly, there was an inverse association between gastric pH and serum ferritin values (p<0.0001, Fig. 6B). These relationships were not statistically significant if uninfected animals were excluded from the analyses (i.e. if the analyses were limited to only infected gerbils).

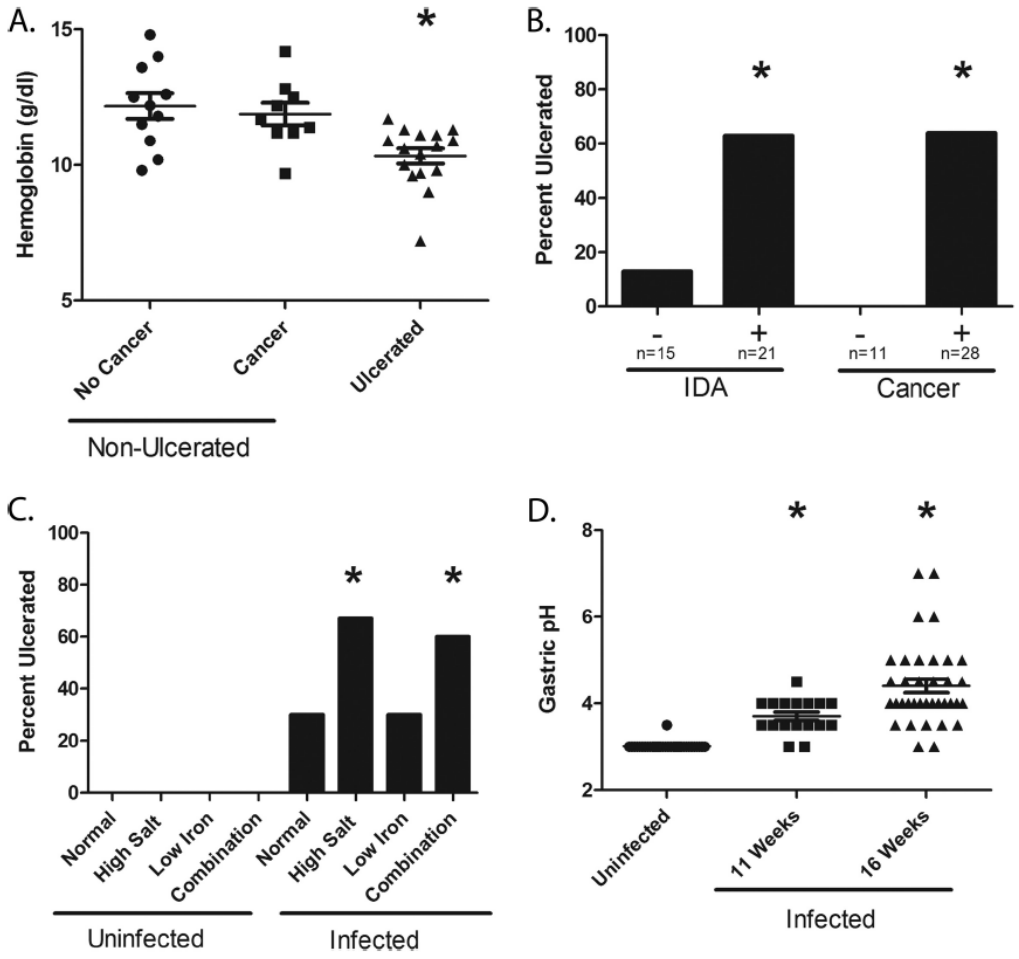

Figure 5: Relationship between anemia and gastric ulceration. Horizontal bars represent mean ± standard error of the mean (SEM), and asterisks represent significant differences (p<0.05). A.) Hemoglobin levels of infected gerbils harvested at the 16 week time point were stratified by cancer and ulceration status. All ulcerated gerbils had gastric cancer. Hemoglobin levels were significantly reduced in infected gerbils with gastric ulcers compared to infected gerbils without gastric ulceration (either with concomitant gastric cancer or without evidence of gastric cancer). (p<0.05, Kruskal-Wallis with Dunn's multiple comparison). B.) Incidence of gastric ulceration at the 16-week time point in infected gerbils with or without IDA, and incidence of gastric ulceration in infected gerbils with or without gastric cancer. Gerbils with IDA or gastric cancer had a significantly higher incidence of gastric ulceration than gerbils without IDA or gerbils without cancer, respectively (p=0.0062 and p=0.0002, Fisher's exact test). C.) Percent of gerbils with a gastric ulcer at the 16-week time point, by diet. Asterisks represent a significant difference (p<0.05) when comparing combined data from cohorts consuming diets high in salt (high salt and combination) to combined data from uninfected animals. D.) Gastric pH of uninfected gerbils (both time points), infected gerbils at the 11-week time point, and infected gerbils at the 16-week time point are presented. Gastric pH was significantly higher in both infected cohorts compared to the gastric pH of the uninfected cohort (p<0.05, Kruskal-Wallis with Dunn's multiple comparison test).

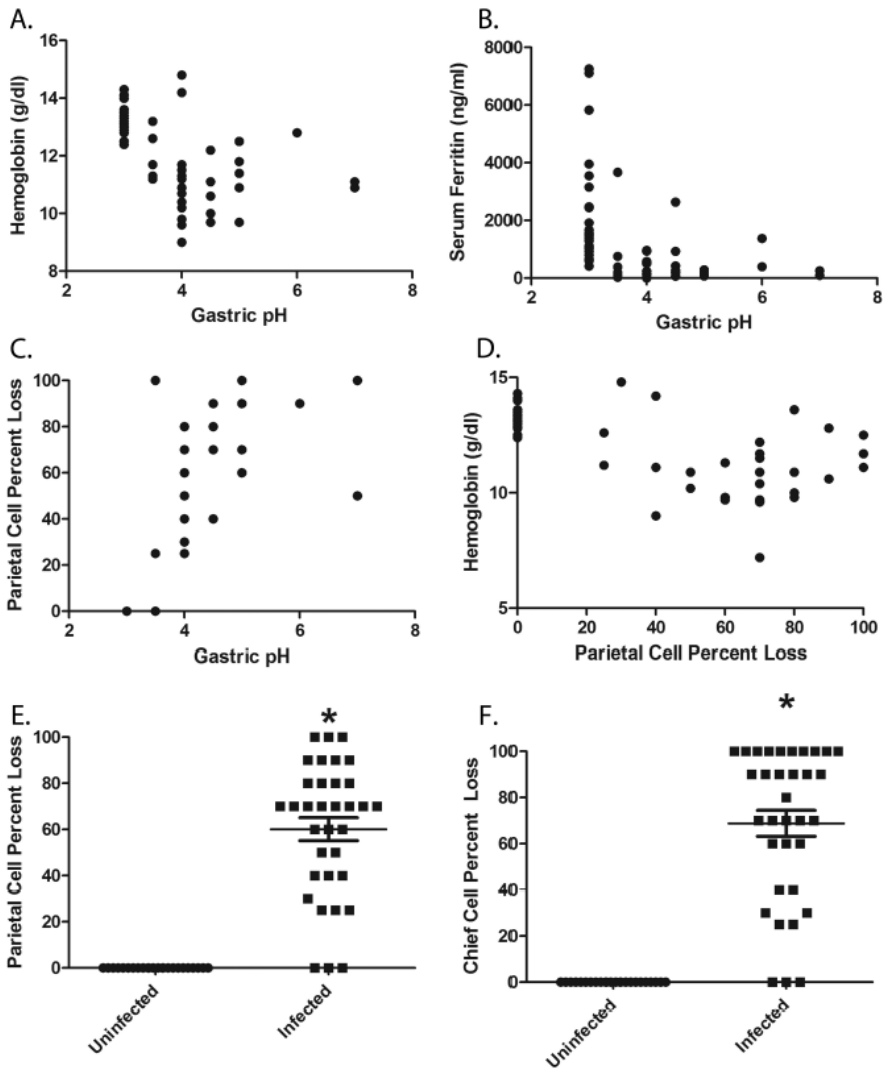

Figure 6: Analysis of hemoglobin, ferritin, gastric pH, parietal cell loss and chief cell loss in *H. pylori*-infected and uninfected gerbils at the 16-week time point. Horizontal bars represent mean ± standard error of the mean (SEM), and asterisks represent significant differences ($p<0.05$). A). Hemoglobin was inversely correlated with gastric pH ($R= -0.64$, $p<0.0001$). B). Serum ferritin and gastric pH were inversely correlated ($R= -0.63$, $p<0.0001$). C). Parietal cell loss was positively correlated with gastric pH ($R= 0.91$, $p<0.0001$). D). Parietal cell loss was inversely correlated with hemoglobin values ($R= -0.63$, $p<0.0001$). E). *H. pylori*-infected gerbils exhibited loss of parietal cells ($p<0.0001$, Mann-Whitney U test). F). *H. pylori*-infected gerbils exhibited loss of chief cells ($p<0.0001$, Mann-Whitney U test). All associations were assessed using Spearman's Rank-Order Correlation.

Consistent with previous reports (32), increased gastric pH was associated with increased gastric inflammation ($p<0.0001$, $R= 0.84$, Spearman's Rank-Order Correlation). This relationship was statistically significant even when uninfected animals were excluded from the analysis. Previous studies have shown that *H. pylori* infection can lead to loss of parietal cells (acid secreting cells found in the stomach) in Mongolian gerbils as well as atrophic gastritis and parietal cell loss in humans (32, 88). Similarly, infected gerbils in the current study exhibited parietal cell loss compared to uninfected gerbils (Fig. 6E and Fig. 7). A reduction in chief cells was also detected in infected gerbils compared to uninfected gerbils ($p<0.0001$, Fig. 6F and Fig. 7). As expected, both parietal cell loss and chief cell loss were positively correlated with gastric pH, suggesting that parietal cell loss was the cause of hypochlorhydria ($p<0.0001$, Fig. 6C). In addition, parietal cell loss was inversely correlated with hemoglobin levels ($p<0.0001$, Fig. 6D). In summary, gastric inflammation and ulceration, parietal cell loss, and increased gastric pH were each correlated with the development of iron deficiency anemia, and likely contributed to the development of this disorder.

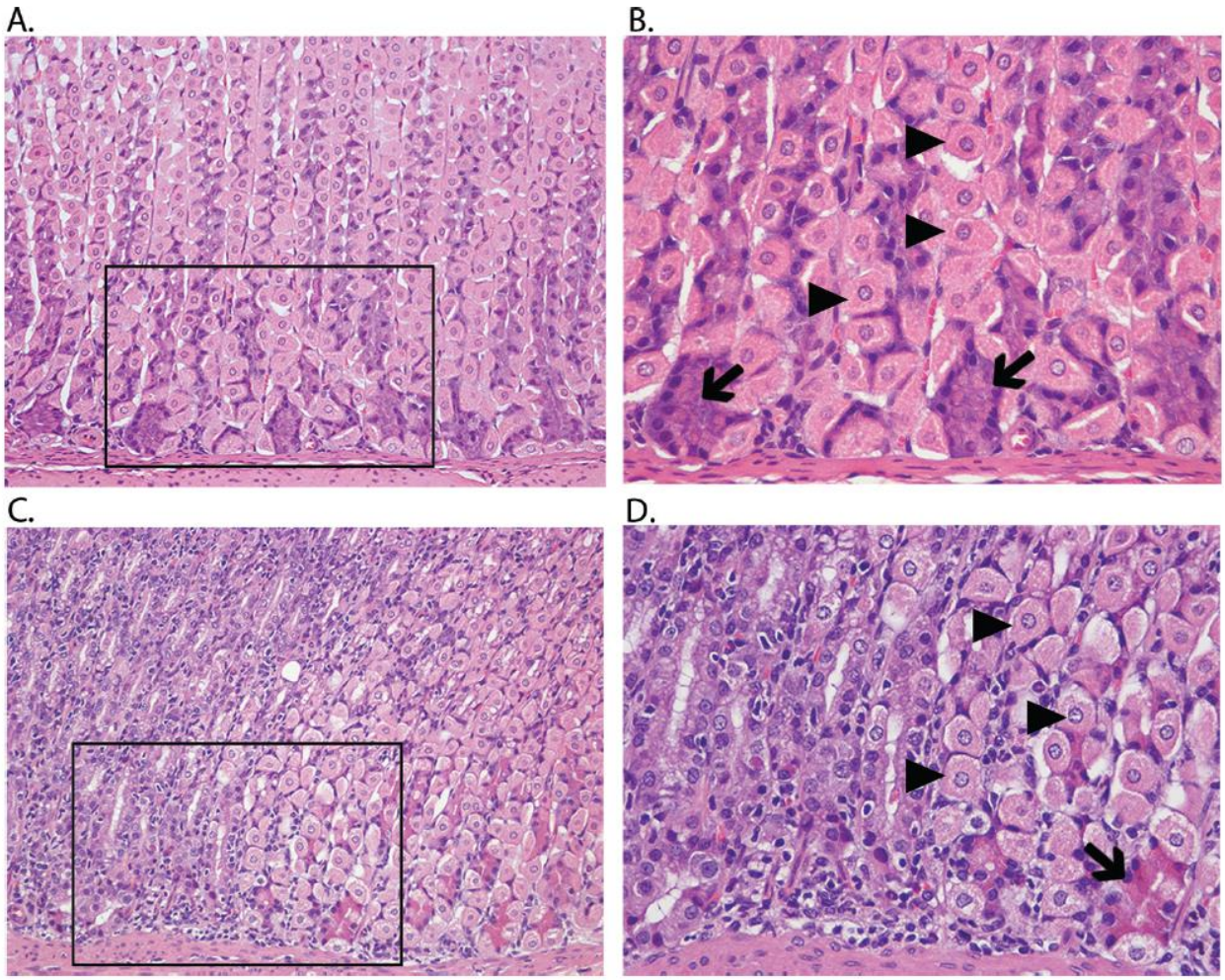

Figure 7. Hematoxylin- and eosin-stained sections of gerbil corpus mucosa. Chief cells (arrows) are basophilic and mainly located at the base of the glands. Parietal cells (arrowheads) are large and eosinophilic and display a triangular or round shape (A and B). Normal mucosa (C and D). An *H. pylori*-induced loss of parietal and chief cells is observed on the left side of the images. These cells have been replaced by antral-type epithelial cells. The boxed areas in panels A and C are magnified in panels B and D, respectively. Magnifications X200 (A and C) and X400 (B and D).

One possible mechanism by which iron deficiency can arise is through chronic blood loss. To detect gastrointestinal blood loss, we tested stool samples collected at the 16-week time point for the presence of fecal occult blood. Fecal occult blood was detected at a significantly higher frequency in the infected gerbils than in the uninfected gerbils (68.57% and 4.76% positive for occult blood respectively, $p<0.0001$, Fisher's exact test). *H. pylori*-induced gastric ulceration represents a potential source of the blood found in the fecal contents.

As expected, infected ulcerated gerbils had significantly lower hemoglobin levels than infected, non-ulcerated gerbils (10.3 ± 0.3 g/dl and 12.0 ± 0.3 g/dl, respectively, $p=0.0002$) (Fig. 5A). Concordantly, gastric ulceration was detected in 62% (13 out of 21) of gerbils diagnosed as having IDA, compared to only 14% (2 out of 14) of infected gerbils that did not have IDA ($p=0.0062$)(Fig. 5B).

Among the infected gerbils, there was no correlation between the presence of gastric ulcers and the detection of fecal occult blood. The detection of fecal occult blood in a high proportion of infected gerbils without gastric ulcers is consistent with a limited sensitivity of the histologic methodology used for detection of gastric ulcers. The methodology used to assess fecal occult blood also has limitations. For example, detection of fecal occult blood may be insensitive if blood loss from gastric ulcers is intermittent.

Another possible cause of blood loss and anemia is the development of gastric adenocarcinoma. To determine if gastric cancer was a cause or also associated with blood loss and anemia independent of gastric ulceration, we classified the infected non-ulcerated gerbils into two subgroups based on presence or absence of gastric cancer (Fig. 5A). No significant difference in hemoglobin levels was detected between these two non-ulcerated subgroups,

suggesting that the anemia was mainly attributable to gastric ulceration instead of gastric cancer. Most of the gastric adenocarcinomas detected in this study were confined to the mucosa and did not penetrate through the gastric epithelium, which probably explains why the presence of gastric cancer was not an independent cause of anemia.

Pernicious anemia

While the anemia observed was a microcytic anemia (consistent with iron deficiency) as opposed to a macrocytic anemia (consistent with B12 deficiency) it was possible that both deficiencies were present. To explore this possibility, we tested the gerbil serum for the presence of vitamin B12 (Fig. 8A). Interestingly, infected gerbils had increased serum B12 when compared to the uninfected cohort. When segregating results by cancer status, the infected gerbils that developed cancer had significantly increased serum B12 when compared to the infected gerbils that did not develop gastric cancer (Fig. 8B).

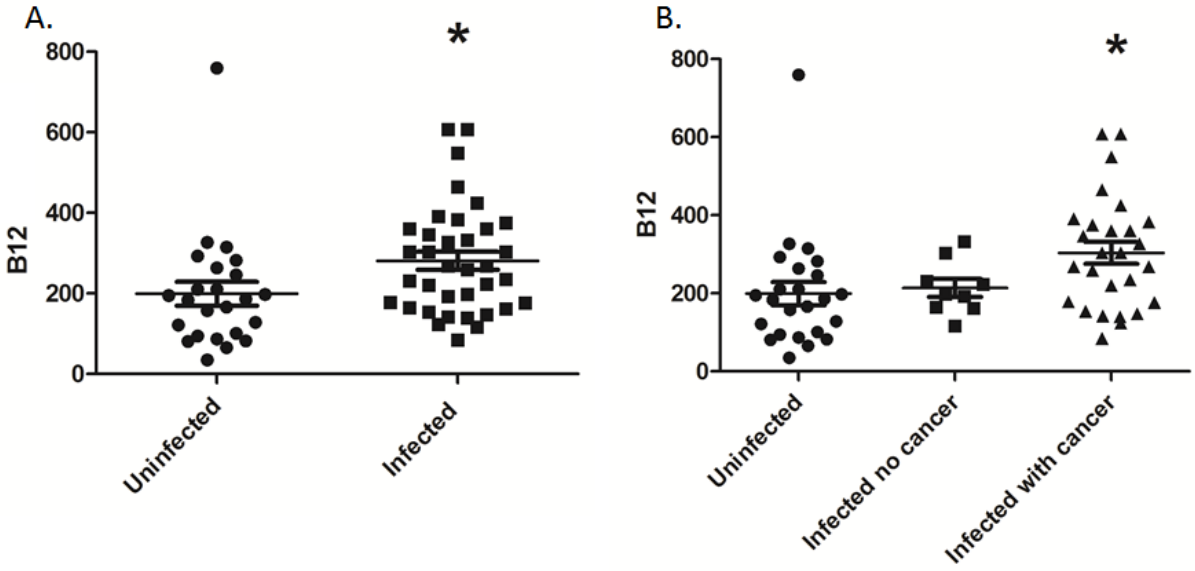

Figure 8: Serum B12 levels (pg/ml) at the 16-week time point. A.) infected versus uninfected gerbils (p=.0074, Mann-Whitney U). B.) Uninfected versus infected, by cancer status (Kruskal-Wallis test with Dunn's multiple comparison, $p<0.05$)

Discussion

In this study, we demonstrate that *H. pylori* infection causes IDA in the Mongolian gerbil model. Previous studies in rodent models have reached varying conclusions about the capacity of *H. pylori* to cause anemia (66–68). The inconsistent conclusions might reflect the use of different *H. pylori* strains, differences in animal models, differences in diets, or different time points selected for analysis. In the current study, we used *H. pylori* strain 7.13, which produces CagA and has a functional *cag* type IV secretion system. A previous study infected Mongolian gerbils with a CagA-positive strain and did not detected anemia in the infected gerbils (72). The reason for the difference in results is not known, but we speculate that the animals in the current study may have had more severe gastric pathology than the animals in the previous study. A direct comparison of gastric histologic features in these studies is not possible because the previous study did not examine gastric tissue for the presence of ulceration, cancer, or inflammation (72).

There are multiple potential mechanisms by which *H. pylori* infection could lead to anemia. Chronic gastrointestinal blood loss is a well-known cause of iron deficiency anemia, and in the current study, we detected gastric ulceration and gastrointestinal blood loss in a high proportion of the *H. pylori*-infected animals. In addition, we detected increased gastric pH and parietal cell loss in *H. pylori*-infected animals. Therefore, both blood loss from gastric ulceration and *H. pylori*-induced hypochlorhydria (with resulting impaired iron absorption) probably contributed to the development of anemia (87).

We also examined the effects of diet on *H. pylori*-induced IDA, and found that *H. pylori*-infected animals fed high salt, low iron, or high salt/low iron diets had a higher incidence and increased severity of IDA compared to *H. pylori*-infected animals receiving a regular diet. Importantly, neither low iron diets nor high salt diets resulted in anemia in uninfected animals. Over a long period of time, a low iron diet would eventually be expected to result in IDA in uninfected animals (57). Presumably the time course of the experiments in the current study was not long enough to permit development of IDA in uninfected animals on a low iron diet. One interpretation is that *H. pylori* infection accelerates or exacerbates the development of IDA in response to a low iron diet (and possibly a high salt diet). In addition, a low iron diet or high salt diet might augment the capacity of *H. pylori* infection to cause IDA.

In support of the latter model, previous studies have shown that administration of diets high in salt or low in iron to *H. pylori*-infected gerbils results in increased severity of gastric inflammation and increased incidence of gastric cancer (32, 34, 36). It has been proposed that these effects are attributable to enhanced *H. pylori* virulence under high salt or low iron conditions (32, 34, 43, 45, 46, 82). For example, a high salt environment stimulates increased production of the *H. pylori* effector protein CagA, and a low iron environment stimulates enhanced activity of the *H. pylori cag* T4SS (which is required for entry of CagA into gastric epithelial cells) (34, 43, 44, 82). Infection of animals with CagA-positive strains that have a functional *cag* type IV secretion system results in increased gastric inflammation and damage compared to infection of animals with *cagA* mutant strains (32, 34) or mutant strains that have defects in *cag* T4SS activity (34, 89, 90). Therefore, increased production of CagA in response to high salt conditions and increased delivery of CagA into host cells in response to low iron

conditions are likely mechanisms by which these dietary alterations influence development of gastric pathology.

Pernicious anemia was not observed in the infected cohort of gerbils. Surprisingly, gerbils infected with *H. pylori* experienced a significant increase in serum B12 levels. Elevated B12 levels have been linked to cancer development in previous studies, including increased risk of gastric cancer development (91). Consistent with these studies, gerbils that were infected and diagnosed with gastric cancer had a significant increase in serum B12 levels when compared to gerbils that were infected, but did not experience gastric cancer. The exact mechanism by which B12 levels are increased in individuals who go on to develop cancer remains poorly understood.

We propose that there may be a positive feedback loop, whereby low iron or high salt conditions lead to increased delivery of secreted *H. pylori* virulence factors such as CagA (32, 34, 43), resulting in gastrointestinal blood loss and impaired iron absorption, and that the consequent iron deficiency drives continued enhanced production and delivery of *H. pylori* virulence factors (Fig. 9). In addition, we speculate that the inflammatory environment generated by a high salt or low iron diet drives selection of *H. pylori* strains that produce multiple virulence factors and are thereby most fit for growth in an inflammatory environment (92).

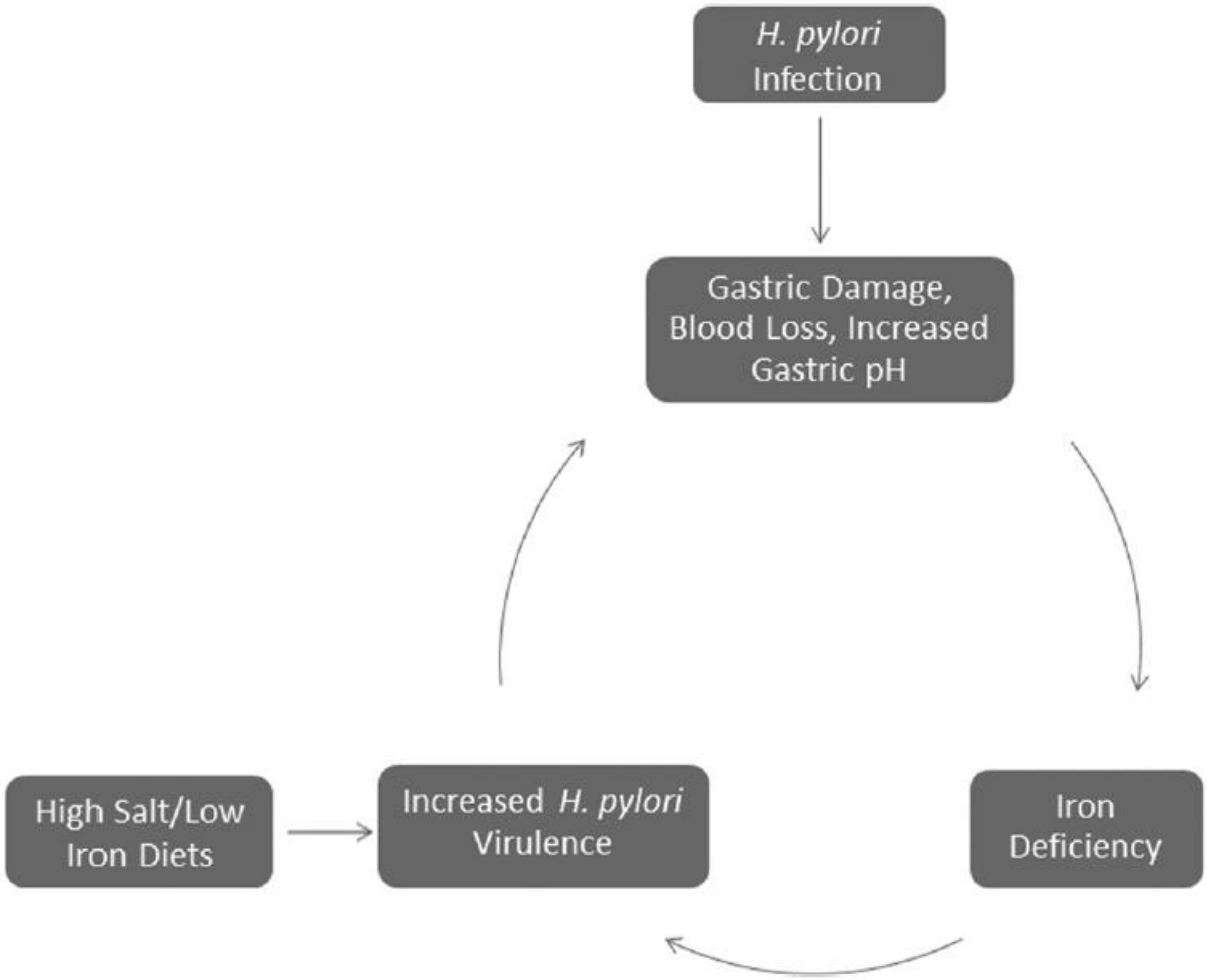

Figure 9: Positive feedback loop for *H. pylori*-induced gastric damage and iron deficiency anemia. *H. pylori* infection causes gastric damage, gastrointestinal blood loss, and increased gastric pH (resulting in impaired iron absorption), all of which can result in iron deficiency. Consumption of a high salt or low iron diet enhances *H. pylori* virulence through multiple mechanisms, including increased production of CagA and increased activity of the *cag* T4SS. Therefore, high salt or low iron diets accelerate or exacerbate the *H. pylori*-induced gastric alterations that lead to iron deficiency. The worsening iron deficiency provides an ongoing stimulus for enhanced *H. pylori* virulence, leading to continued gastric damage and blood loss.

In summary, these data provide strong evidence indicating that *H. pylori* can cause iron deficiency anemia in an animal model. Gastrointestinal blood loss from *H. pylori*-induced gastric ulceration is probably the main cause of the anemia, but reduced iron absorption as a result of increased gastric pH may also be contributory. High salt and low iron diets enhanced the capacity of *H. pylori* to cause gastric ulceration and/or iron deficiency anemia, but had no detectable effect on uninfected animals. These findings highlight the important role of diet as a factor influencing the outcome of *H. pylori* infection.

CHAPTER 3

EFFECT OF DIET AND *H. pylori* INFECTION ON GASTRIC PATHOLOGY AND SYSTEMIC RESPONSES IN THE MONGOLIAN GERBIL MODEL

Introduction

Infections with the gastric bacterium *H. pylori* have diverse outcomes. On one hand, most individuals colonized will experience no ill effects of this colonization, and may, in fact, experience benefits such as protection from allergic asthma. On the other hand, the individuals who do experience adverse clinical outcomes as a result of infection will experience disparate outcomes, ranging from mild gastritis to gastric adenocarcinoma.

While the pathologic outcomes of *H. pylori* infection are fairly well studied, the systemic immune response to chronic *H. pylori* colonization is not. Given the reported link between *H. pylori* and autoimmune disorders, the systemic immune response was of particular interest (93).

Many factors are known to influence the outcome of *H. pylori* infection, and epidemiologic data as well as previous studies from our lab suggest an important role for diet (32, 34, 42). Specifically, high salt and low iron diets increase the likelihood that an individual colonized with *H. pylori* will develop gastric cancer (33, 42). Studies performed in the Mongolian gerbil model have confirmed these findings (32, 34). For these reasons, we decided to focus on diet in our studies. We were curious if high salt, and low iron would demonstrably increase risk of gastric cancer among *H. pylori* infected animals, as well as of the cumulative effects of a diet both poor in iron and rich in salt on gastric cancer rates. Additionally, we were curious if reported associations of *H. pylori* with altered levels of circulating white blood cells and

platelets were present in our gerbil model, and if so what role does diet play on these extra-gastric effects. To examine these possibilities, we designed an experiment utilizing the Mongolian gerbil model of *H. pylori* infection. We placed infected and uninfected cohorts of gerbils on one of 4 diets: normal, low iron, high salt, or a combination of high salt and low iron. We examined the systemic effects of diet, and of disease status (such as anemia). At the experimental endpoint we assessed gastric histology, and systemic immune cell responses via complete blood counts.

Materials and methods

For detailed description of methodologies used in the inoculation with *H. pylori,* hematological, serum ferritin, and serum B12 analysis, gastric pH measurements, bacterial density determination, establishment of diagnostic criteria for IDA, or measurement of fecal occult blood, please see Chapter 1 methods.

Statistical analysis

All data are presented as mean $\pm$ SEM. Statistical analysis were conducted using GraphPad Prism Software package using $p<0.05$ as the upper criteria for establishing significance. All associations were assessed using Spearman's Rank-Order Correlation. Detection of differences between two groups was assessed using Mann-Whitney U, while three or more groups were analyzed using Kruskal-Wallis test with Dunn's multiple comparison, $p<0.05$. Non-parametric tests were used as the data did not fit a normal distribution pattern.

Histology

At the time of harvest, longitudinal strips of the glandular stomach were collected and fixed overnight in 10% neutral buffered formalin (Fisher Scientific). Following fixation, the tissue was embedded in paraffin, sectioned, and subsequently stained with hematoxylin and eosin. Slides were examined by a pathologist who was blinded to the identity of specimens for gastric pathology. Histologic evaluation included evaluation of gastric inflammation, chief and parietal cells, gastric ulceration (characterized by mucosal disruption with inflammatory exudate and cell debris), dysplasia (characterized by irregular glands with budding or branching in the mucosa), and gastric adenocarcinoma (characterized by dysplastic glands penetrating through the muscularis mucosa into the submucosa). These criteria for dysplasia and adenocarcinoma are in agreement with consensus guidelines (94). Acute inflammation (polymorphonuclear neutrophils) and chronic (mononuclear leukocytes) inflammation were graded separately on a 0-3 scale (absent, mild, moderate, marked) in both the antrum and corpus, for a cumulative total score of 0-12. Loss of parietal cells and loss of chief cells were estimated semiquantitatively (as percent cell loss) based on review of hematoxylin and eosin-stained sections containing the entire length of the stomach. Gastric ulceration, dysplasia and adenocarcinoma were recorded as present or absent, but these features were not scored for severity. All gastric cancer cases were independently evaluated by a second pathologist (also blinded to experimental conditions), who confirmed the diagnoses (32, 34, 94, 95).

Results

Analysis of leukocytes and platelets

In addition to analyzing the effects of *H. pylori* infection and diet on development of anemia (Chapter 2), we investigated effects of *H. pylori* and diet on other hematologic parameters. At the 16 week time point, infected gerbils had significantly elevated white blood cell (WBC) counts when compared to uninfected gerbils (14.0 ± 1.1x10^9/L and 9.8 ± 0.7x10^9/L, respectively, p=0.009) (Fig. 10A). Consistent with the elevated total WBC counts, neutrophil counts and monocyte counts were significantly elevated in infected cohorts compared to uninfected gerbils (p=0.001) (Fig. 10B, 10C). When comparing infected anemic gerbils with infected non-anemic gerbils, there was no difference in total WBC counts or neutrophil counts (Fig. 10A, 10B), but infected anemic gerbils had significantly elevated monocyte counts (1.3 ± 0.3x10^9/L) when compared to the infected non-anemic cohort (0.3 ± 0.04x10^9/L, p<0.05) (Fig. 10C).

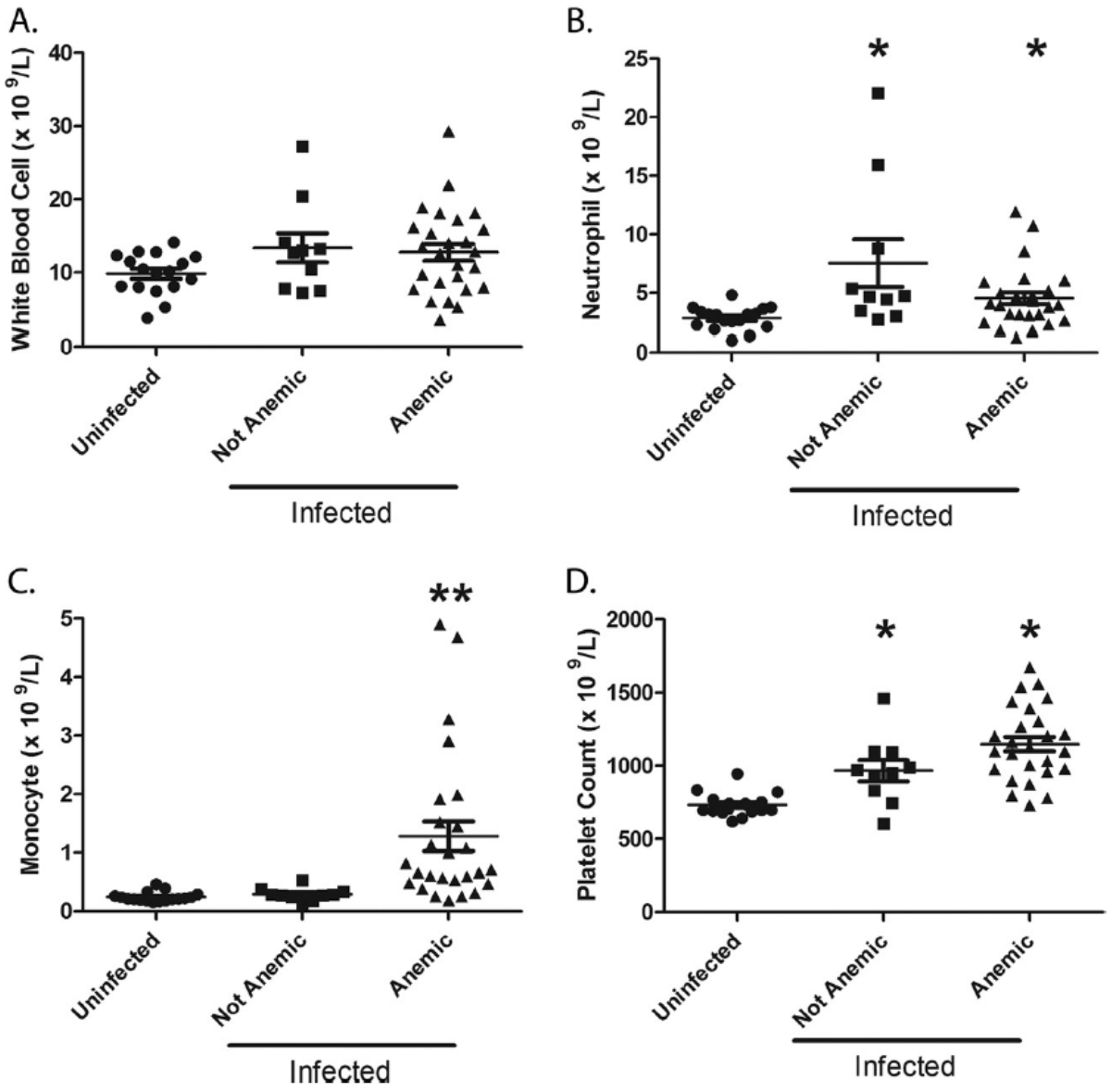

Figure 10: Leukocytosis and thrombocytosis in *H. pylori*-infected gerbils. Horizontal bars represent mean ± standard error of the mean (SEM), and asterisks represent significant differences ($p<0.05$). Total white blood cell counts, neutrophil counts, monocyte counts, and platelet counts were determined at the 16 week time point in uninfected gerbils, infected gerbils without anemia, and infected anemic gerbils. A.) Total WBC counts did not differ among the groups ($p=0.207$, Kruskal-Wallis test with Dunn's multiple comparison). B.) Neutrophil counts were significantly elevated in infected cohorts compared to uninfected gerbils ($p=0.001$ and $p<0.0001$; Kruskal-Wallis with Dunn's multiple comparison). C.) Monocyte counts in the infected anemic (based on hemoglobin values) cohort were significantly higher than monocyte counts in the uninfected cohort or the infected non-anemic cohort ($p<0.05$, Kruskal-Wallis with Dunn's multiple comparison). D.) Platelet counts of gerbils in both infected cohorts (anemic or not anemic) were significantly higher than in the uninfected cohort ($p<0.05$, Kruskal-Wallis with Dunn's multiple comparison).

Infected gerbils had significantly higher platelet counts than uninfected gerbils (1082.0 ± 49.2x10^9/L and 732.5 ± 18.9x10^9/L, respectively, p<0.0001) (Fig. 10D). A positive correlation was observed between platelet count and gastric pH (p<0.0001, Fig. 11A) and between platelet count and gastric ulcers (p=0.013, data not shown). Increased platelet counts were associated with several markers of iron deficiency anemia. Specifically, platelet counts were inversely correlated with hemoglobin (p<0.0001), mean corpuscular volume (p=0.001), and serum ferritin (p=0.013) (Fig. 11B-D). Taken together, these results indicate that leukocytosis and thrombocytosis occur commonly in *H. pylori*-infected gerbils, and monocytosis occurs more commonly in *H. pylori*-infected gerbils with IDA than in infected non-anemic gerbils.

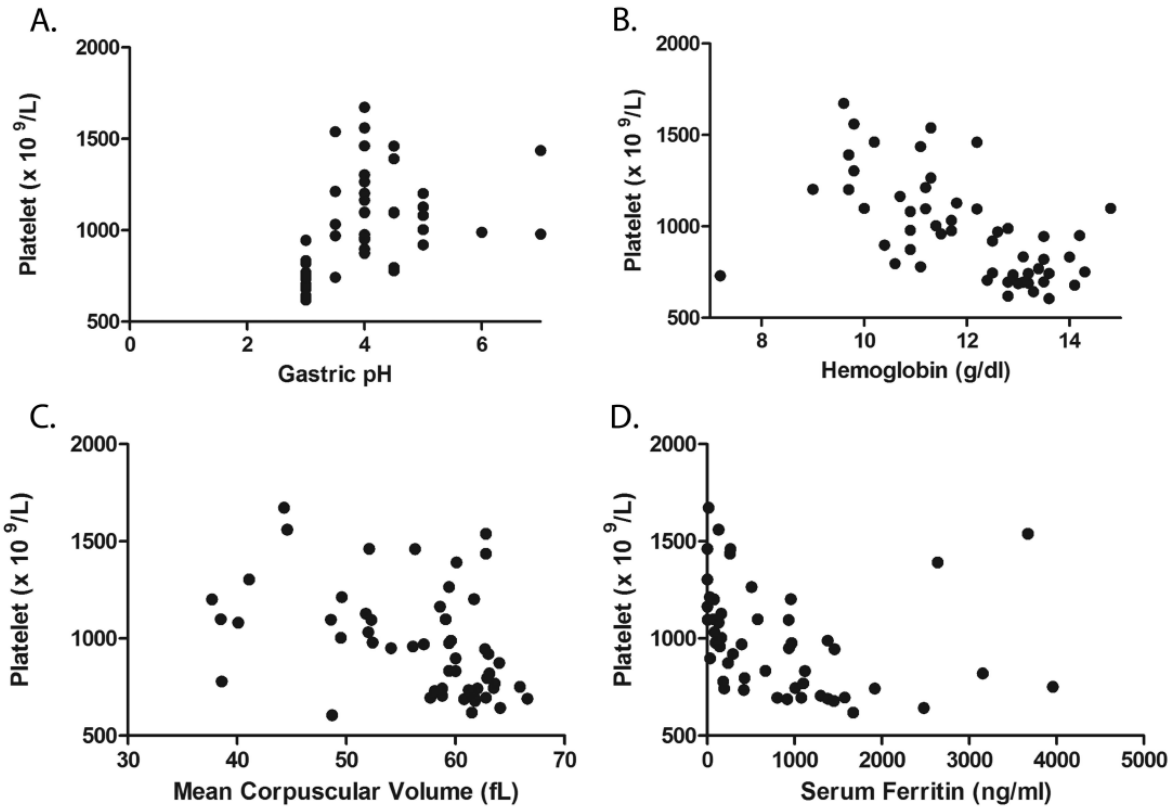

Figure 11: Analysis of platelet counts in *H. pylori*-infected and uninfected gerbils at the 16-week time point. A). Platelet count was positively correlated with gastric pH (R= 0.66, p<0.0001). B). Platelet count was inversely correlated with hemoglobin values (R= -0.62, p<0.0001). C). Platelet count was inversely correlated with mean corpuscular volume (R= -0.46, p<0.0001). D). Platelet count was inversely correlated with serum ferritin (R= -0.53, p<0.0001). All associations were assessed using Spearman's Rank-Order Correlation.

Gastric ulceration, gastric cancer and gastric inflammation in *H. pylori*-infected gerbils

To detect gastric ulceration, gastric histologic sections were examined as described in methods. No gastric ulcers were observed in uninfected gerbils (Fig. 12A), whereas gastric ulcers were detected in 46% of infected gerbils at the 16-week time point. Ulcers were predominantly located either in the transitional mucosa between corpus and antrum or in the proximal two thirds of the antrum (Fig. 12B).

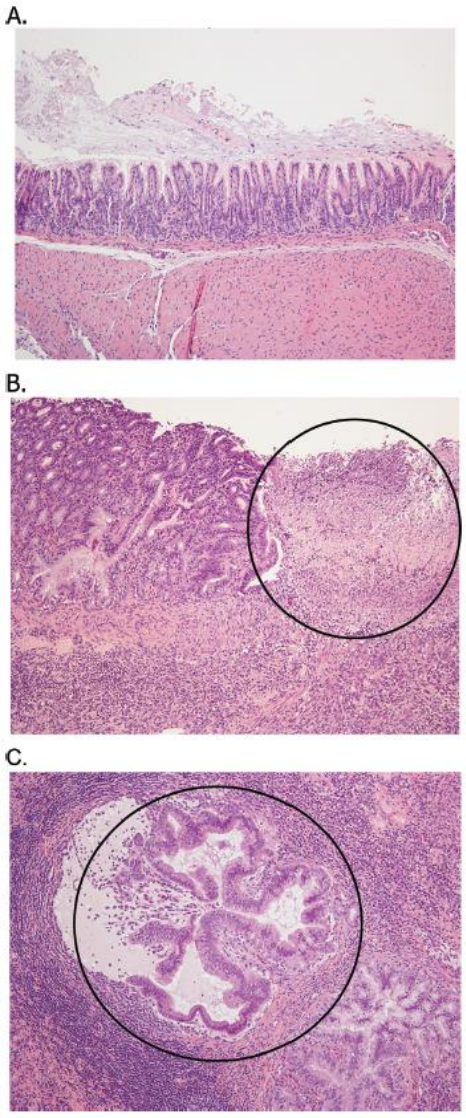

Figure 12: Gastric ulceration and gastric cancer in infected gerbils. A.) Gastric mucosa of an uninfected gerbil on a normal diet. B.) Gastric mucosa of an infected gerbil on a high salt diet. A gastric ulcer in the proximal third of the antrum is visible and circled in upper right portion of the image. C.) Invasive gastric adenocarcinoma (circled) in an infected gerbil on a high salt diet, characterized by irregular glands infiltrating the submucosa (original magnification A-C, x 100).

Among infected gerbils, those on a diet containing increased salt (either the high salt diet or combination high salt/low iron diet) had a significantly higher incidence of gastric ulceration [ulceration in 12 of 19 animals (63%)] compared to gerbils not on a high salt diet [normal or low iron diet, ulceration in 6 of 20 animals (30%), p=0.039]

At the 16-week time point, gastric adenocarcinoma (Fig. 12C) was detected in 72% (28 out of 39) of *H. pylori*-infected gerbils, and none of the uninfected gerbils. Gastric ulcers were detected in 64% of the 28 gerbils with invasive adenocarcinoma, and in none of the 11 *H. pylori*-infected gerbils without adenocarcinoma. Thus, gerbils exhibiting a gastric ulcer were significantly more likely to be diagnosed as having invasive adenocarcinoma (p=0.0002), in comparison to the infected, non-ulcerated cohort of gerbils.

As expected, gastric inflammation was present in *H. pylori*-infected gerbils but not uninfected gerbils (p<0.0001), as determined by gastric histology scores Fig. 13A). The severity of gastric inflammation was associated with markers of IDA (decreased serum ferritin and hemoglobin) as well as gastric pH at the 16-week time point (p<0.0001, Fig.13B-D), but this relationship was not statistically significant if uninfected animals were excluded from the analysis (i.e., if only infected animals were analyzed).

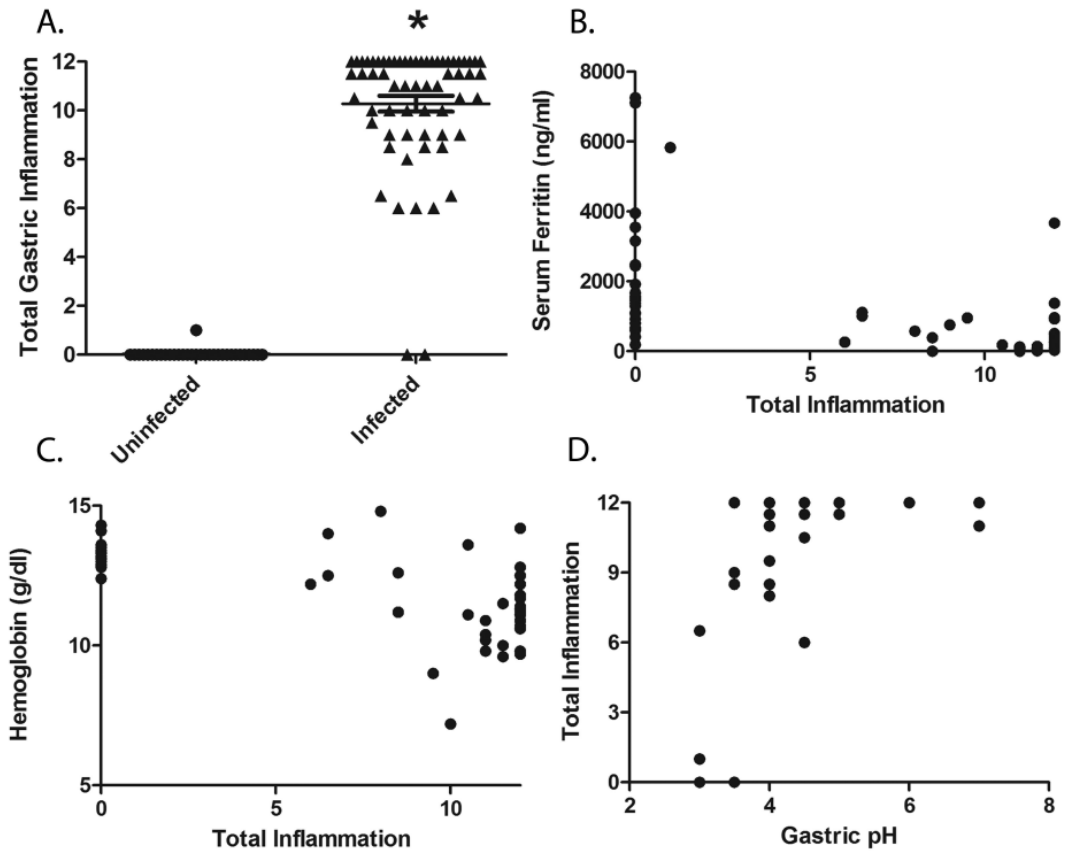

Figure 13: Gastric inflammation at the 16-week time point in *H. pylori*-infected and uninfected gerbils, scored based on histologic analysis (0-12 scale). Horizontal bars represent mean ± standard error of the mean (SEM), and asterisks represent significant differences ($p<0.05$). A). Gastric inflammation was severe in the antrum and corpus of most *H. pylori*-infected gerbils, but no inflammation was detected in uninfected gerbils ($p<0.0001$, Mann-Whitney U test). B). Serum ferritin levels were inversely correlated with total gastric inflammation scores at the 16 week time point ($R= -0.58$, $p<0.0001$ Spearman's Rank-Order Correlation). C). Hemoglobin values were inversely correlated with total gastric inflammation scores ($R= -0.58$, $p<0.0001$ Spearman's Rank-Order Correlation). D). Gastric inflammation scores were positively correlated with gastric pH ($R= 0.84$, $p<0.0001$ Spearman's Rank-Order Correlation).

Discussion

We detected several hematologic abnormalities among our infected gerbils, including leukocytosis and thrombocytosis. These findings indicate that *H. pylori* colonization of the gerbil stomach leads to not only a gastric mucosal inflammatory response, but also a systemic response. Previous studies reported that humans infected with *H. pylori* have significantly higher leukocyte counts than their uninfected counterparts, with the greatest levels of leukocytosis observed among individuals suffering from peptic ulcer disease (96, 97). The leukocytosis observed in *H. pylori*-infected gerbils in the current study was not associated with worsened disease. We speculate that prolonged coevolution of *H. pylori* and humans has favored the selection of *H. pylori*-host interactions characterized by a minimal systemic host response (65, 66). As gerbils are not a natural host for *H. pylori*, this could explain the exaggerated systemic inflammatory response regardless of disease outcome observed in the current study.

In contrast to previous studies that reported elevated rates of gastric cancer in *H. pylori*-infected gerbils receiving high salt or low iron diets (32, 34), the composition of the diet did not influence gastric cancer rates in the current study. Notably, the gastric cancer rates in infected animals on a regular diet were considerably higher in this study than in previous studies (32, 34). When comparing the methods used in the various studies, one difference pertains to the type of diet fed to the control animals. In the current study, the infected control group received AIN-93M chow (which contains 0.26% sodium chloride and 39 ppm iron), and other groups received modified forms of this diet. In contrast, the control groups in previous studies received

either a modified form of AIN-93M chow (containing 250 ppm iron) or a Purina 5001 diet (which contains 0.75% sodium chloride) (32, 34).

The thrombocytosis detected in the current study occurred in a large proportion of animals, presumably as part of a systemic inflammatory response to *H. pylori* infection. This is markedly different from ITP, which occurs in only a very small proportion of *H. pylori*-infected humans through an unrelated immunologic mechanism. Increased platelet counts occurring in association with IDA have been reported in other rodent models of IDA, as well as in humans with IDA (99, 100). This could be due to increased levels of erythropoietin leading to increased platelet production as a result of anoxic conditions resulting from anemia (101).

Unexpectedly, we detected markedly elevated monocyte counts in *H. pylori*-infected gerbils with anemia compared to infected gerbils without anemia. It has previously been suggested that under conditions of iron deficiency, apoptotic signaling pathways in some hematopoietic lineages are rendered non-functional, resulting in a longer lifespan for white blood cells such as monocytes and neutrophils (102). Delayed or absent apoptotic processes could potentially account for the increased circulating monocytes observed in the anemic animals in this study, as this would extend the lifetime of these cells (102).

CHAPTER 4

H. pylori MICROEVOLUTION IN THE GASTRIC NICHE

Introduction

Among individuals colonized with *H. pylori*, the risk of gastric cancer is higher in those who are colonized with *H. pylori* strains secreting proteins that cause alterations in host cells (such as the oncoprotein CagA and s1/i1/m1 forms of the VacA toxin) than in those colonized with strains that do not produce these proteins (23, 103–105). Diets with high salt content, low iron content, or low content of fruits and vegetables are additional risk factors for gastric cancer (106). Several host genetic factors (e.g., certain polymorphisms of the interleukin-1 gene) also influence gastric cancer risk (23, 105).

H. pylori strains isolated from unrelated humans exhibit a high level of genetic diversity (77, 107–109). This diversity is attributable to a high mutation rate and a high rate of intraspecies recombination (77, 108–110). Previous studies have examined *H. pylori* evolution in individual human stomachs over time or during *H. pylori* transmission to human hosts, and have demonstrated that the mutation rate is particularly high during transmission to new hosts (108, 111–114). Microevolution of *H. pylori* has also been detected in experimentally infected animal models, including macaques, mice, and gerbils (74, 115–118).

The Mongolian gerbil model is of particular interest because *H. pylori*-infected gerbils develop severe gastric inflammation, sometimes accompanied by gastric cancer and/or gastric ulceration (32, 119–122). In a previous study, we compared the genome sequences of *H. pylori* isolates cultured from a gerbil that was fed a high salt diet and developed gastric cancer, with the genome sequences of isolates cultured from a gerbil that was fed a regular diet and did not

develop gastric cancer (92). A FurR88H mutation was detected in isolates from the gerbil fed a high salt diet but not isolates from the gerbil fed a regular diet. PCR and sequence analyses of isolates from additional gerbils showed that the FurR88H mutation was consistently detected in isolates from additional animals fed a high salt diet, but was detected less commonly in isolates from animals fed a regular diet. *In vitro* experiments indicated that the FurR88H mutation conferred resistance to high salt conditions and oxidative stress (92). We proposed that a high-salt diet promotes high levels of gastric inflammation and oxidative stress in gerbils infected with *H. pylori*, and that these conditions, along with high levels of intraluminal sodium chloride, lead to selection of *H. pylori* strains that are most fit for growth in this environment (92).

A high salt diet is known to be a risk factor for gastric cancer, and similarly, a low-iron diet is also a risk factor for gastric cancer (106). We hypothesize that these two diets might cause similar changes in the stomach, and *H. pylori* strains containing specific mutations might have a selective advantage in the gastric environments of animals receiving either a high salt or a low iron diet. In support of this hypothesis, the FurR88H mutation (previously detected in strains cultured from animals on a high salt diet) has been detected more commonly in *H. pylori* strains cultured from gerbils on a low-iron diet than in strains cultured from gerbils on an iron-replete diet (123).

In the current study, we sought to sample the diversification of *H. pylori* that occurred during colonization in 5 gerbils from a previously described cohort of Mongolian gerbils (124) under a range of different gastric conditions, including variation in dietary composition and disease state.

Materials and methods

For detailed description of methodologies used in the inoculation with *H. pylori,* hematological, serum ferritin, and serum B12 analysis, gastric pH measurements, bacterial density determination, establishment of diagnostic criteria for IDA, description of histologic analysis, or measurement of fecal occult blood, please see Chapter 1 methods.

Statistical analysis

All data are presented as mean ± SEM. Statistical analysis were conducted using GraphPad Prism Software package using $p<0.05$ as the upper criteria for establishing significance. All associations were assessed using Spearman's Rank-Order Correlation. Detection of differences between two groups was assessed using Mann-Whitney U, while three or more groups were analyzed using Kruskal-Wallis test with Dunn's multiple comparison, $p<0.05$. Non-parametric tests were used as the data did not fit a normal distribution pattern.

Isolation of *H. pylori* chromosomal DNA

H. pylori output strains cultured from gerbils were inoculated onto Trypticase soy agar plates containing 5% sheep blood (Hemostat Laboratories), and then were streaked for single colony isolation. Individual colonies were isolated and expanded by growth on separate plates. Bacteria harvested from one-day-old plates were resuspended in 1 ml of phosphate buffered saline, and genomic DNA was isolated using a Wizard Genomic purification kit (Promega) and eluting the DNA into water.

***H. pylori* genome sequencing**

H. pylori DNA samples were subjected individually to enzymatic fragmentation using the NEBNext™ dsDNA Fragmentase kit (NEB) according to the manufacturer's instructions, with average fragment length of 600bp (Range of 400-1000bp. Libraries of DNA were prepared from purified fragmented DNA samples using the Kapa Hyper Prep library kit with unique indexes as per manufacture's protocol (Kapa Biosystems, Inc. Wilmington, MA USA). Post quantitation of these library preps was performed with the Kapa library quantitation kit (Kapa Biosystems, Inc. Wilmington, MA USA), and sequenced on a MiSeq sequencer using the 600V3 kit (Illumina Inc., San Diego, USA). For the analysis, raw reads were quality trimmed and aligned to the reference sequence (*H. pylori* strain B8) (125) using CLCbio Genomics workbench version 8.5. Alignment parameters were as follows: Mismatch cost = 2, Insertion cost = 3, Deletion cost = 3, Insertion open cost = 6, Insertion extend cost = 1, Deletion open cost = 6, Deletion extend cost = 1, Length fraction = 0.5, Similarity fraction = 0.8. The alignment files exported from the CLCbio workbench in BAM format were then imported into an in-house-developed application (VGAS) for further coverage and SNP analysis. SNP reports were generated using a 10% cut off, and genome-wide comparisons of SNPs in the output strains compared to the input strain were then performed. Sequence data was deposited in NCBI (Bioproject ID: PRJNA414609).

Three single *H. pylori* colonies cultured from each gerbil were isolated, expanded and sequenced individually, and three colonies of the input strain were also sequenced in the same manner. We elected to sequence three colonies of each isolate in order to get an approximate sampling of the mutations present in each output strain. All *H. pylori* sequence data from individual animals (3 single colonies per animal) were analyzed as a group, and all of the

sequence reads of the input strain (3 single colonies) were analyzed as a group. We sought to identify polymorphisms that were detected in ≥75% of sequence reads of output strains from individual animals, and ≤10% of sequence reads from the input strain. This approach allowed identification of polymorphisms that were maximally different when comparing output strain populations with the input strain. Mean annualized SNP rates per site were determined by calculating a ratio of the total number of SNPs in each strain to the genome size (based on the colonization of gerbils for 16 weeks), and then multiplying the values by 3.25 to approximate the number of mutations anticipated to arise over a period of one year.

Preparation of murine PMNs and co-culture with *H. pylori*

Murine PMNs were isolated using a protocol approved by the Vanderbilt University IACUC (V/15/130, Algood). Using a 21-gauge needle, 1 mL of sterile casein solution was injected into the peritoneal cavity of the mouse. An inflammatory response was allowed to develop overnight, and a second dose of casein solution was administered the following morning. The animal was euthanized by carbon dioxide inhalation 3 hours after the second injection. The abdominal skin was sterilized with 70% ethanol and retracted to expose the intact peritoneal wall. The peritoneal cavity was then filled with 5 mL of sterile PBS, using a 25-gauge needle, and the abdomen massaged. The fluid was slowly removed using a 25-gauge needle, placed in a 50 mL conical flask and the procedure repeated a second time. The pooled peritoneal fluid was centrifuged for 10 minutes at 200g, followed by red blood cell lysis using Ammonium-Chloride-Potassium Lysing Buffer (ACK, Gibco). Peritoneal exudates were washed 3 times, resuspended in 1 mL media (F-12 with 5% FBS) and cell numbers were counted. Finally, the cell solution was

brought to the desired concentration ($5x10^5$cells/mL) and 1 mL was distributed to each well of 12-well cell culture plates. Plates were incubated for 1 hour prior to addition of *H. pylori*.

H. pylori strain 7.13 containing wild-type Fur and an isogenic mutant containing FurR88H were tagged with distinct antibiotic resistance markers (chloramphenicol or kanamycin resistance), as described previously (92). Overnight cultures of these strains were inoculated into separate fresh Brucella broth containing 5% FBS and allowed to grow for 6 hours. The *H. pylori* cultures were then used to competitively infect the same culture of PMNs at an MOI of 20:1. In parallel, mock infections were carried out by addition of *H. pylori* to culture plates containing F-12/FBS medium alone. Following a 1 hour infection, the samples were treated with saponin (0.1% final concentration)(126). Dilutions of the saponin-treated samples were plated on Brucella agar plates containing the appropriate antibiotics and then CFUs were counted 5 days after plating. The survival of strains co-cultured with neutrophils was compared to the survival of the same strains in medium alone.

Results

Identification of single nucleotide polymorphisms (SNPs)

To gain a better understanding of how *H. pylori* adapts to different gastric environments, we investigated the diversification of *H. pylori* that occurs during colonization of Mongolian gerbils. We analyzed *H. pylori* strains isolated 16 weeks post-infection from a previously described cohort of gerbils (124). To maximize the number of detectable *H. pylori* genetic adaptions that were positively selected in vivo, we focused the analysis on isolates cultured from five gerbils with substantial variation in gastric environments, including consumption of different diets and different types of gastric pathology (124). One was an

animal on a normal diet with severe disease (defined as high inflammation scores, gastric cancer and ulcer, increased gastric pH and/or anemia). Two gerbils consumed a high salt diet; one had severe disease and one had less severe disease (defined as relatively low inflammation scores, lack of gastric cancer and ulcers, normal pH and not anemic). Two gerbils consumed a low iron diet; one had severe disease and one had less severe disease. Detailed information about these gerbils is provided in Table 1. Three individual *H. pylori* colonies isolated from each gerbil (output isolates), as well as 3 individual colonies of the input strain, were analyzed by whole genome sequencing. We then identified differences in the genomes of the output strains compared to the genome of the input strain, using the stringent criteria described in methods, which were designed to identify genetic changes that occurred in response to strong selective pressure.

Table 1. Characteristics of individual gerbils[a]

Animal	Diet	Hemoglobin	Gastric pH	Gastric Ulcer	Gastric Inflammation Score[b]	Gastric Cancer	Mutation Rate[c]
1	Normal	10.7	4	Yes	12	Yes	1.16E-05
2	High Salt	10.6	4.5	Yes	12	Yes	1.55E-05
3	High Salt	12.5	3	No	6.5	No	7.77E-06
4	Low Iron	14	3	No	6.5	No	9.71E-06
5	Low Iron	10.9	7	Yes	11	Yes	3.11E-05

[a] Gerbils were fed the indicated diets and euthanized 16 weeks after *H. pylori* infection (124).

[b] Gastric inflammation was scored on a scale from 0 to 12 (124).

[c] Mean annualized SNP rate per site.

Collectively, the output strains contained twenty-five unique SNPs that were either not detected or detected at very low levels in the input strain (Table 2). Twenty-one were in coding regions and four were in non-coding regions (Table 2). Among the 21 mutations in coding regions, 18 were non-synonymous and 3 were synonymous (Table 2). Four of the non-synonymous SNPs were in *cagY*, which encodes a component of the type IV secretion system that translocates the CagA effector protein into host cells. Two of the non-synonymous changes in *cagY* were instances in which a sense codon was mutated to a stop codon. All four of the SNPs in non-coding regions (Table 2) were localized less than 60 nucleotides upstream of a translational start site (*katA*, *fecA2*, *frpB3*, and *alpA*) (Table 2 and Fig. 14). Two of these were downstream of transcriptional start sites, within 5' untranslated regions of the mRNA (*katA* and *alpA*) (Fig. 14).

Table 2. Single nucleotide polymorphisms detected in *H. pylori* strains cultured from gerbils

Location[a]	Number of Output Strains with SNP[b]	SNP Description	Percent of Output Reads with SNP[c]	Percent of Input Reads with SNP	Nucleotide Change (5'->3')
Non-coding region (Base Position 23384)	1/5	Upstream of *frpB3*	100	<2	C->A
Hypothetical Protein (HPB8_343, Base Position 313249)	1/5	Non-Synonymous Thr to Ile (AA#59)	100	<2	G->A
Non-coding region (Base Position 613051)	1/5	Upstream of *alpA*	100	<5	C->T
cysS (Base Position 641561)	1/5	Non-Synonymous Val to Ile (AA#11)	97	<2	C->T
Non-coding region (Base Position 1001780)	5/5	Upstream of *fecA2*	99,98,99,99,99	<3	G->A
Non-coding region (Base Position 1064871)	5/5	Upstream of *katA*	96,96,96,97,97	<6	G->C
Hypothetical Protein (HPB8_45, Base Position 52225)	1/5	Non-Synonymous Ser to Gly (AA#159)	100	<1	A->G
Hypothetical Protein (HPB8_64, Base Position 71813)	1/5	Non-Synonymous Gly to STOP (AA#50)	100	<4	G->A
Hypothetical Protein (HPB8_593, Base Position 563527)	1/5	Non-Synonymous Ser to Ile (AA#57)	100	<1	G->A
fur (Base Position 1122559)	4/5	Non-Synonymous Arg to His (AA#87)	98,99,99,99	<2	G->A
rpoD (Base Position 1449954)	1/5	Non-Synonymous Arg to His (AA#532)	100	<3	G->A
Hypothetical Protein (HPB8_32, Base Position 38960)	1/5	Non-Synonymous Tyr to His (AA#144)	100	<1	A->G
nadD (Base Position, 135265)	1/5	Non-Synonymous Pro to Leu (AA#154)	100	<2	C->T
folE (Base Position 587541)	1/5	Non-Synonymous Phe to Leu (AA#51)	100	<2	A->G
cagN (Base Position 679850)	1/5	Non-Synonymous Pro to His (AA#122)	100	<2	G->T
cagY (Base Position 693086)	1/5	Synonymous (AA#1005)	88	<3	A->G
cagY (Base Position 693101)	1/5	Synonymous (AA#1010)	100	<10	G->A
cagY (Base Position 693132)	1/5	Non-Synonymous Gln to Glu (AA#1021)	100	<8	C->G
cagY (Base Position 693219)	1/5	Non-Synonymous Lys to STOP (AA#1050)	100	<5	A->T
cagY (Base Position 693226)	1/5	Non-Synonymous Leu to STOP (AA#1052)	100	<9	T->A
cagY (Base Position 693240)	1/5	Non-Synonymous Val to Leu (AA#1057)	86	<6	G->C
thrB (Base Position 1145232)	1/5	Synonymous (AA#101)	100	<3	C->T
hcpE (Base Position 1302755)	1/5	Non-Synonymous Gly to Ser (AA#240)	100	<2	G->A
glmM (Base Position 1462161)	1/5	Non-Synonymous Ala to Thr (AA#148)	100	<2	G->A
cheV7 (Base Position 1572769)	1/5	Non-Synonymous Ala to Val (AA#23)	100	<3	G->A

[a]Base positions in the genome of reference strain B8 are listed.

[b]*H. pylori* output strains cultured from the indicated numbers of animals contained the designated SNPs, based on criteria defined in Methods. See S2 Table for details.

[c]The mean percent of reads containing the designated SNP, based on sequence analysis of 3 individual *H. pylori* colonies cultured from each animal. Multiple values are listed if the SNP was detected in *H. pylori* isolates from multiple animals.

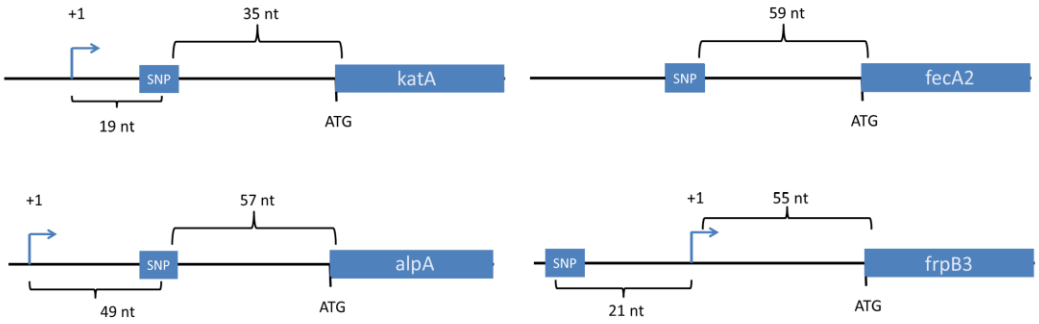

Figure 14: Location of SNPs in non-coding regions. Four SNPs in non-coding regions were mapped in the context of nearby genes. All 4 were within 60 nucleotides of a downstream gene, and 2 were downstream of transcriptional start sites. Transcriptional start sites are labeled as +1. Nt, the number of nucleotides between the depicted genetic elements. We were unable to match the *fecA2* +1 site previously identified in strain 26695 (127) to a corresponding site upstream of *fecA2* in strain B8. Two of the SNPs (upstream of *fecA2* and *katA*) were present in all of the output strains, but not the input strain.

In a previous study, we analyzed the genome sequences of *H. pylori* strains cultured from two gerbils using 454 sequencing methods (92). Five SNPs were detected in 100% of sequence reads of isolates from the animal on a high salt diet, but were not detected (or detected in low abundance) in the input strain or isolates from the animal on a regular diet. Two of these SNPs were also identified in multiple output strains in the current study. Specifically, a mutation upstream of *fecA2* was identified in all 5 output strains in the current study, and a FurR88H mutation was identified in 4 of the 5 output strains (Table 2). The only output strain that did not have the FurR88H mutation was isolated from a gerbil consuming a normal diet (Table 3). We showed previously that the FurR88H mutation confers increased resistance to high concentrations of salt or conditions of oxidative stress (paraquat) (92). Resistance to oxidative stress would presumably provide an important selective advantage in the context of the *H. pylori*-induced gastric mucosal inflammatory response, which is characterized by an infiltration of neutrophils, macrophages, and other immune cell types. To further define how the FurR88H mutation might confer a selective advantage in the gastric environment, studies were conducted in which isogenic *H. pylori* strains containing wild-type Fur or FurR88H (each strain harboring a different antibiotic marker) were co-cultured together (competition study) with neutrophils. A significant survival advantage of the isogenic mutant strain containing the FurR88H mutation was detected, when compared to wild-type strain (mean survival of 74.5% ± 6.8% for the FurR88H mutant compared to 51.8% ± 7.2 for the strain containing wild-type Fur, $p<0.03$)(Fig. 15). These data indicate that the FurR88H mutation confers a survival advantage to *H. pylori* in a neutrophil-containing environment.

Table 3. SNPs by Diet

SNPs Normal Diet				
Location[a]	**Number of output Strains with SNP**	**SNP Description**	**Percent of Reads with SNP**[b]	**Nucleotide Change (5'->3')**
Non-coding region (Base Position 23384)	1/1	Upstream of *frpB3*	100	C->A
Hypothetical Protein (HPB8_343, Base Position 313249)	1/1	Non-synonymous Thr to Ile	100	G->A
Non-coding region (Base Position 613051)	1/1	Upstream of *alpA*	100	C->T
cysS (Base Position 641561)	1/1	Non-synonymous Val to Ile	100	C->T
Non-coding region (Base Position 1001780)	1/1	Upstream of *fecA2*	99	G->A
Non-coding region (Base Position 1064871)	1/1	Upstream of *katA*	97	G->C
SNPs High Salt Diet				
Location[a]	**Number of output Strains with SNP**	**SNP Description**	**Percent of Reads with SNP**[b]	**Nucleotide Change (5'->3')**
Hypothetical Protein (HPB8_45, Base Position 52225)	1/2	Non-synonymous Ser to Gly	100	A->G
Hypothetical Protein (HPB8_64, Base Position 71813)	1/2	Non-synonymous Gly to STOP	100	G->A
Hypothetical Protein (HPB8_593, Base Position 563527)	1/2	Non-synonymous Ser to Ile	100	G->A
Non-coding region (Base Position 1001780)	2/2	Upstream of *fecA2*	99,98	G->A, G->A
Non-coding region (Base Position 1064871)	2/2	Upstream of *katA*	96,96	G->C, G->C
fur (Base Position 1122559)	2/2	Non-synonymous Arg to His	100	G->A, G->A
rpoD (Base Position 1449954)	1/2	Non-synonymous Arg to His	100	G->A
SNPs Low Iron Diet				
Location[a]	**Number of output Strains with SNP**	**SNP Description**	**Percent of Reads with SNP**[b]	**Nucleotide Change (5'->3')**
Hypothetical Protein (HPB8_32, Base Position 38960)	1/2	Non-synonymous Tyr to His	100	A->G
nadD (Base Position, 135265)	1/2	Non-synonymous Pro to Leu	100	C->T
folE (Base Position 587541)	1/2	Non-synonymous Phe to Leu	100	A->G
babA (Base Position 629257)	2/2	Non-synonymous Arg to Gly	99,98	A->G, A->G
cagN (Base Position 679850)	1/2	Non-synonymous Pro to His	100	G->T
cagY (Base Position 693086)	1/2	Synonymous	88	A->G
cagY (Base Position 693101)	1/2	Synonymous	100	G->A
cagY (Base Position 693132)	1/2	Non-synonymous Gln to Glu	100	C->G
cagY (Base Position 693219)	1/2	Non-synonymous Lys to STOP	100	A->T
cagY (Base Position 693226)	1/2	Non-synonymous Leu to STOP	100	T->A
cagY (Base Position 693240)	1/2	Non-synonymous Val to Leu	86	G->C
Non-coding region (Base Position 1001780)	2/2	Upstream of *fecA2*	99,99	G->A, G->A
Non-coding region (Base Position 1064871)	2/2	Upstream of *katA*	97,96	G->C,G->C
fur (Base Position 1122559)	2/2	Non-Synonymous Arg to His	100,100	G->A, G->A
thrB (Base Position 1145232)	1/2	Synonymous	100	C->T
hcpE (Base Position 1302755)	1/2	Non-Synonymous Gly to Ser	100	G->A
glmM (Base Position 1462161)	1/2	Non-Synonymous Ala to Thr	100	G->A
cheV7 (Base Position 1572769)	1/2	Non-Synonymous Ala to Val	100	G->A

[a]Base positions in the genome of reference strain B8 are listed.
[b]The mean percent of reads containing the designated SNP, based on sequence analysis of 3 individual *H. pylori* colonies cultured from each animal. Multiple values are listed if the SNP was detected in *H. pylori* isolates from multiple animals.

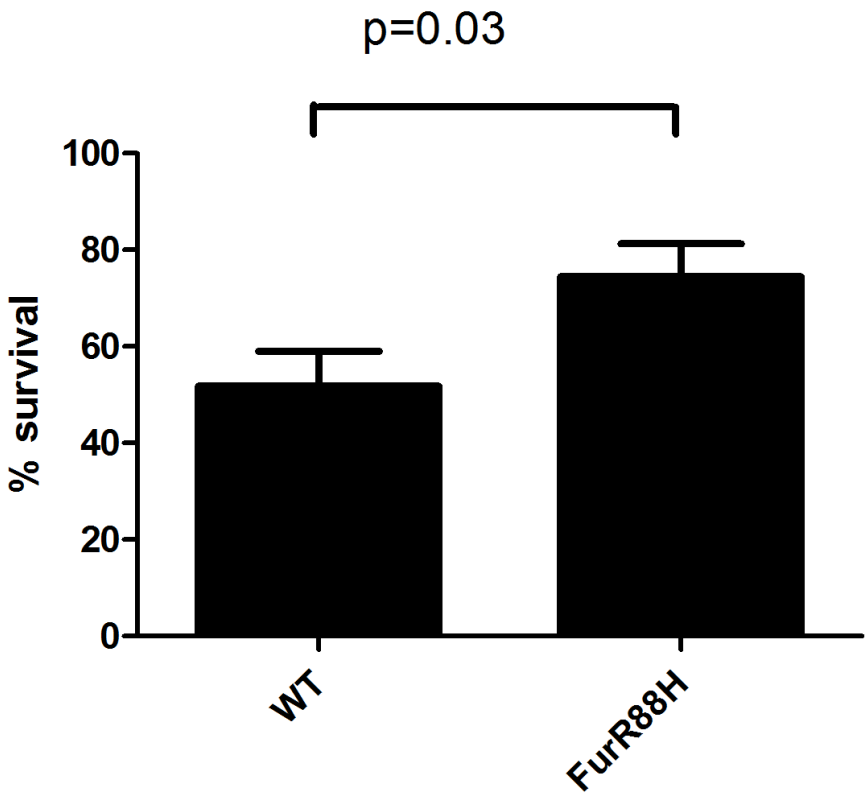

Figure 15: FurR88H confers a survival advantage to *H. pylori* when co-cultured with neutrophils. To determine the effect of the FurR88H mutation on bacterial survival in the presence of neutrophils, we co-cultured a mixture of strain 7.13 containing wild-type Fur and an isogenic mutant containing FurR88H, each labeled with a different antibiotic resistance marker, with freshly isolated murine neutrophils. The survival of *H. pylori* strains in this competition experiment was quantified as described in Methods. The survival of strains co-cultured with neutrophils is compared to the survival of the same strains in medium alone. The strain harboring the FurR88H mutation had a significantly higher percent survival than the wild-type strain ($p<0.03$, Mann-Whitney Test, based on 16 experiments).

Mutation rate

In an effort to quantify the rate of genetic change during the four months in which gerbils were colonized with *H. pylori*, we calculated the annualized SNP rate per site, as described in methods. Overall, the mean annualized SNP rate per site (the number of SNPs that would be expected to occur per site, over the course of one year) among all output strains was $1.5e^{-5}$, with a range of $7.77e^{-6}$ to $3.11e^{-5}$ among individual output strains (Table 1). Interestingly, the mutation rate detected in output strains was positively correlated with the gastric pH in the corresponding gerbils (i.e., higher numbers of SNPs were detected in strains from animals with a high gastric pH) (Pearson correlation coefficient, p=0.0204). An analysis of larger numbers of animals will be required to test the validity of this association.

Deletions and Insertions

We detected 5 unique deletions (ranging from 1 to 4 consecutive nucleotides deleted in individual genes) and 3 unique insertions among the output strains (Table 4). Three of the deletions were in coding regions and 2 were in intergenic regions. The genes containing deletions were *oipA* (also known as *hopH*, encoding an outer membrane protein), *tonB1* (encoding a protein required for activity of outer membrane receptors involved in iron acquisition), and a hypothetical protein (Table 4). The intergenic region deletions were upstream of an LPS 1,2-glucosyltransferase, and a hypothetical protein. Among the deletions in coding regions, all were frameshift mutations. One of the insertions was in a coding region and 2 were in intergenic regions. The insertion in a coding region was in the gene encoding the outer membrane protein FecA, and it was a frameshift mutation. Fifty percent of the deletions

or insertions occurred within polynucleotide tracts (Table 4). All of the insertions or deletions (indels) that occurred in coding regions resulted in protein truncation (Fig. 16).

Table 4. Insertions and deletions detected in *H. pylori* strains cultured from gerbils

Location[a]	Number of Output Strains with Indel[b]	Percent of Input Reads with Indel	Percent of Output Reads with Indel[c]	Indel Type	Polynucleotide Tract?[d]
tonB1 (132930-132931)	3/5	0	76,76,76	Deletion	no
upstream of hypothetical protein (Base Position 217342)	1/5	0	80	Deletion	T(14)
oipA (819621-819624)	1/5	0	90	Deletion	GA(9)
hypothetical protein (HPB8_1200, Base Position 1171489)	2/5	0	100,100	Deletion	T(9)
intergenic region between a membrane protein, and LPS 1,2-glucosyltransferase (Base Position 1332441)	1/5	0	80	Deletion	T(17)
upstream of membrane protein (WP_013195952.1, Base Position 1584449)	1/5	0	75	Insertion (C)	no
fecA3 (Base Position 1661940)	1/5	0	96	Insertion (T)	no
upstream of chemotaxis protein HPB8_1462 (Base Position 1432303)	1/5	0	80	Insertion (G)	no

[a]Base positions in the genome of reference strain B8 are listed.

[b]*H. pylori* output strains cultured from the indicated numbers of animals contained the designated indels.

[c]The mean percent of reads containing the designated indel, based on sequence analysis of 3 individual *H. pylori* colonies cultured from each animal. Multiple values are listed if the indel was detected in *H. pylori* isolates from multiple animals.

[d]The tables shows characteristics of polynucleotide tracts in the input strain.

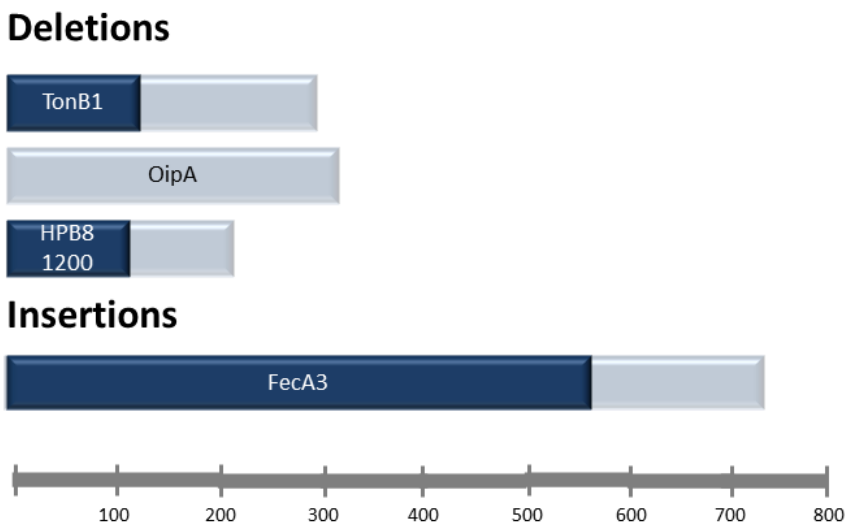

Figure 16: Analysis of insertions and deletions in coding regions. Lengths of the deduced protein products encoded by the corresponding genes containing insertions and deletions were examined. Frameshift mutations (Table 3) were located upstream of the resulting premature stop codons in the ORFs of interest. Pale bars indicate the lengths of the protein products encoded by wild-type (non-mutated) genes, and the darker bars indicate the lengths of the proteins encoded by genes harboring insertions or deletions. For example, *tonB1* encodes a protein 291 amino acids in length in the input strain, whereas in the presence of a frameshift mutation, a protein 111 amino acids in length is encoded.

Detection of genetic changes in multiple animals

Most of the SNPs were detected in *H. pylori* isolates from only one of the gerbils analyzed, but 3 were detected in isolates from multiple animals, ranging from 4 to 5 gerbils (Table 2 and Table 5). The FurR88H mutation discussed earlier was detected in output strains from 4 of the 5 animals. Two SNPs (in non-coding regions upstream of *fecA2* and upstream of *katA*) were found in output strains isolated from all 5 animals (Tables 2 and 5, Fig. 14).

Table 5. Mutations detected in *H. pylori* strains cultured from multiple gerbils[a]

SNPs in Multiple Animals	**Number of Output Strains Containing Mutation[b]**
Non-coding region (Base Position 1001780)	5/5
Non-coding region (Base Position 1064871)	5/5
fur (Base Position 1122559)	4/5
Deletions in Multiple Animals	**Number of Output Strains Containing Mutation**
tonB1 (132930-132931)	3/5
hypothetical protein (HPB8_1200, Base Position 1171489)	2/5

[a]Base positions in the genome of reference strain B8 are listed.

[b]*H. pylori* output strains cultured from the indicated numbers of animals contained the designated SNPs or deletions.

Most of the indels were detected in *H. pylori* isolates from a single animal, but two deletions were detected in isolates from multiple animals. Specifically, a 2-nucleotide in-frame deletion in *tonB1* was detected in isolates from three animals (Tables 4 and 5). Additionally, a one base pair deletion in a hypothetical protein was detected in two animals (Tables 4 and 5). The presence of SNPs or indels in strains cultured from multiple animals suggests that these mutations conferred a selective advantage.

Discussion

In this study, we examined the microevolution of *H. pylori* in the gastric environment of Mongolian gerbils. To maximize the number of detectable genetic alterations that were positively selected in vivo, we analyzed *H. pylori* strains cultured from multiple different gastric environments, including the stomachs of animals fed different diets (normal, high salt or low iron), animals with different gastric pathologies (including gastric ulcer, gastric cancer, and varying severity of gastric inflammation), and animals with different hematologic parameters (either anemia or normal hemoglobin). We used stringent criteria to identify mutations that were detected in a high proportion of sequence reads from output strains, and either not detected or detected in a low proportion of sequence reads from the input strain. Such mutations were likely to have been positively selected *in vivo*. Most of the SNPs detected were non-synonymous mutations, which supports this interpretation. The detection of mutations in *H. pylori* isolates from multiple animals is also consistent with positive selection.

The mutations detected in this study could have arisen *de novo* during colonization of gerbils, or alternatively, these mutations could have been present in the input strain population

but not readily detectable by the sequencing approach used in this study. Recent work suggests that there can be substantial genetic diversity within individual *H. pylori* strains, consistent with the existence of a quasispecies (78, 79). Given the high frequency with which several mutations were detected in output strains, it seems likely that many of the mutations were present in a small subpopulation of organisms in the input strain.

H. pylori has colonized human hosts for at least 50,000 years and is extremely well adapted to the human gastric niche (1, 3). Experimental introduction of *H. pylori* into animal models, as done in the current study, is invariably associated with disruption of this longstanding bacteria-host relationship. When *H. pylori* enters the stomach of a non-human host, there is strong selective pressure favoring the emergence of mutants that are most fit for growth in the gastric environment of the new host. We presume that some of the mutations detected in the current study reflect the adaptation of *H. pylori* to the gerbil stomach (in contrast to its natural human stomach environment). The insertion and deletion mutations resulting in pseudogenes are particularly relevant in this regard. Presumably the genes that accumulated mutations abrogating protein production were not required or deleterious during chronic *H. pylori* colonization of the gerbil stomach.

Helicobacter acinonychis, isolated from the stomachs of large cats, is one of the species mostly closely related to *H. pylori* (128). It has been proposed that a common ancestor of *H. pylori* and *H. acinonychis* underwent a host jump (from humans to large cats) within the last 200,000 years, leading to the emergence of two separate species (128). Interestingly, many *H. pylori* genes encoding outer membrane proteins correspond to pseudogenes in *H. acinonychis*, suggesting that they were unnecessary or deleterious in stomachs of large cats, or at least not

beneficial (128). The divergence of *H. pylori* and *H. acinonychis* over a very long time period as a consequence of a host jump correlates well with the relatively short-term results of the current study, in which several pseudogenes arose after introducing the natural human colonizer *H. pylori* into the stomach of the Mongolian gerbil.

Previous studies in *E. coli* have reported a higher mutation rate in genes that are highly transcribed, specifically resulting in C to T changes in the DNA sequence (129). Most highly transcribed *H. pylori* genes (such as *ureaA*) were not mutated in the current study. Of the highly transcribed genes that did contain a SNP, none of the mutations detected resulted in a C to T change within the open reading frame (130). Mutations of interest, such as the FurR88H mutation also did not appear to be consistently found in output strains as a result of transcription, due to the fact that *fur* is not a highly expressed gene in *H. pylori*, and the mutation observed is not a C to T change (130). Regardless, future studies should specifically sequence larger numbers of genes that are expressed in relatively high or low amounts in *H. pylori* in order to determine if transcription frequency is an independent driver of genetic mutations in this species.

In total, we detected 25 unique SNPs, 5 deletions, and 3 insertions in output strains from at least one animal. A disproportionately high number of mutations were detected within genes or upstream of genes associated with iron-related functions (*fur*, *tonB1*, *fecA2, fecA3*, and *frpB3)* or genes which encode outer membrane proteins *(cagY, alpA, oipA, fecA2, fecA3* and *frpB3)* (47, 131–133). Moreover, the genes *tonB1* and *cagY* contained multiple mutations.

FecA2, FecA3, and FrpB3 are outer membrane proteins predicted to be involved in iron acquisition (133). TonB1 is a transmembrane protein predicted to be involved in iron

homeostasis as well as nickel import (131, 132). Fur is a master regulator of gene expression, particularly those genes involved in iron homeostasis, central metabolism and energy production (47). The large number of mutations in genes associated with iron-related functions suggests that the availability of iron in the *H. pylori*-infected gerbil stomach might be different from that in the *H. pylori*-infected human stomach. We speculate that iron may be more avidly sequestered in an *H. pylori*-infected gerbil stomach than in an *H. pylori*-infected human stomach.

CagY is a component of the *cag* type IV secretion system localized to the outer membrane, and OipA is an outer membrane protein reported to modulate *H. pylori* interactions with host cells (115, 134–136). The detection of mutations in these proteins suggests that it may be beneficial for *H. pylori* to remodel its surface during colonization of the gerbil stomach, perhaps as a result of immune responses directed against specific outer membrane proteins, or as a consequence of different receptors being available in the gerbil stomach compared to the human stomach (137).

All of the insertion or deletion mutations within open reading frames were frameshift mutations predicted to result in production of truncated proteins or unstable proteins (i.e., generation of pseudogenes). One such mutation occurred in *oipA*. Analyses of *H. pylori* strains cultured from humans has shown that strains possessing a functional, in-frame *oipA* gene are associated with more severe disease outcome, such as gastric cancer or gastric ulceration, compared to strains with an out-of-frame *oipA* gene (135, 138, 139). In the current study, the

one output strain containing an *oipA* frameshift mutation was isolated from a gerbil on a normal diet that exhibited relatively severe gastric disease.

Many of the mutations detected in this study were in intergenic regions (8 of the 33 mutations). The SNPs within intergenic regions were mapped to sites upstream of *katA*, alpA, *fecA2*, and *frpB3*. These mutations could potentially influence transcription or translation rates, or could be in small RNAs that have regulatory functions. In future studies, it will be important to examine the functional significance of these mutations.

In total, 5 mutations (3 SNPs, 2 deletions) were detected in output strains cultured from multiple animals, but not the input strain. The FurR88H mutation and a mutation in *tonB1* were each detected in output strains from at least three animals. The other three mutations detected in output strains from multiple animals were SNPs in intergenic regions. The detection of these mutations in multiple animals suggests that they conferred an important selective advantage.

One of the mutations identified in output strains from multiple animals in the current study was FurR88H. This mutation was detected in output strains isolated from gerbils consuming either a low iron or high salt diet, but not the animal consuming a normal diet. Interestingly, we previously detected the FurR88H mutation in output strains from two different cohort of gerbils experimentally infected with *H. pylori*, and observed that it was detected more commonly in output strains from animals fed a high salt diet than in output strains from animals fed a regular diet (92), and more commonly detected in output strains from animals fed a low iron diet than in output strains from animals fed a regular diet (140). *In vitro* experiments showed that the FurR88H mutation conferred a survival advantage when *H.*

pylori was cultured under conditions of oxidative stress (92). Here, we co-cultured wild-type and FurR88H strains with neutrophils, and noted a significant survival advantage for strains harboring the FurR88H mutation. This result provides further evidence that the FurR88H mutation may enhance the ability of *H. pylori* to evade immune defenses, and may help to explain why strains harboring this mutation are able to out-compete other strains *in vivo*.

Previous studies have analyzed the microevolution of *H. pylori* in individual humans over time, during transmission to new human hosts (108, 111–113), and in animal models of infection (74, 108, 125). These studies have detected mutations in many genes, particularly those encoding outer membrane proteins (including BabA and other members of the Hop family)(74, 112, 115–117, 141, 142). Mutations in *cagY*, which encodes a component of the *cag* T4SS, have also been detected frequently (74, 115). Mutations resulting in loss of CagY production have been detected at a particularly high frequency in the mouse model of *H. pylori* infection (74). It is likely that mutations in genes encoding outer membrane proteins enhance the ability of the bacteria to colonize new hosts, evade the immune response, and establish persistent infection. In the current study, we detected many genetic changes in output strains similar to those reported in previous studies, including mutations in genes encoding outer membrane proteins such as OipA and CagY. Conversely, many of the mutations we detected within or upstream of genes with functions related to iron (such as *tonB1*, *fecA2*, *fecA3,* and *frpB3)* have not been commonly reported to undergo genetic adaptations during the course of chronic infection in humans.

We calculated a mean annualized SNP rate per site (the number of SNPs that would be expected to occur per site, over the course of one year) of $1.5e^{-5}$, with a range of $7.77e^{-6}$ to $3.11e^{-5}$ among individual output strains. One prior study examining the *H. pylori* mutation rate in chronically-infected humans detected an annualized mutation rate per site of $2.5e^{-5}$ (112), and another reported an annualized mutation rate per site of $6.1e^{-4}$ (108). The variation in mutation rates among studies could be due to differences in the criteria for identification of SNPs, strain differences, differences in the selective forces of individual gastric environments, or differences in phase of infection (chronic versus acute). For example, there is evidence that *H. pylori* strains colonizing humans or rhesus macaques undergo a "mutational burst" in the acute phase of infection, and then exhibit a slower mutation rate during chronic infection (108). In general, the current results suggest that *H. pylori* mutation rates in the Mongolian gerbil model are similar to the corresponding mutation rates in humans, and higher than the mutation rates observed in previous studies of *S. aureus* or *P. aeruginosa* infections in humans (108, 143, 144).

A notable limitation of the current study is that we analyzed output strains from a relatively small number of animals. Therefore, it was not possible to determine the specific effects of diet or disease state on the development of mutations. We anticipate that sequence analysis of strains from a larger number of animals will more clearly reveal correlations between specific mutations, diet and disease state. Such studies could potentially lead to the identification of biomarkers for strains associated with severe disease states. It will be important in future studies to analyze the functional consequences of the mutations that are selected for during *H pylori* colonization of the gerbil stomach under varying conditions, and to

analyze the selective advantages associated with these mutations in larger populations of animals.

In summary, these results provide new insights into the microevolution of *H. pylori* under a wide range of gastric environmental conditions. They also reveal a physiologically relevant phenotype of the commonly detected output strain mutation FurR88H in conferring a survival advantage to *H. pylori* when co-cultured with neutrophils. In addition, these studies shed new light on the genetic changes that occur when *H. pylori* is introduced into a new host species.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

Summary

H. pylori infects approximately 50% of the world's population, and can lead to gastric maladies ranging from peptic ulcer disease to gastric cancer (1, 2, 3). Only a minority of infections result in adverse clinical outcomes, and some individuals even derive benefits from *H. pylori* colonization, such as protection from allergic asthma (3, 7, 145). With such a broad range of clinical outcomes of infection, determining which instances of infection are likely to become problematic is paramount. Individuals in developing countries who are treated with antibiotic therapies designed to eradicate *H. pylori* frequently become re-infected, further complicating therapeutic efforts (146).

Given the high prevalence of *H. pylori* colonization and the disparity in infection outcomes, I sought to further study and define factors that determine if infection is beneficial, neutral, or associated with adverse clinical outcomes. Several factors influencing colonization outcome have already been identified, such as host age, blood type, strain of infecting *H. pylori*, and host diet (32, 42, 147–149). Host diet is of particular interest, as this can potentially be altered through intervention strategies. To determine which factors contribute to infection outcome, it is essential to have a thorough understanding of diseases that could be caused by *H. pylori*.

H. pylori **as a causative agent of iron deficiency anemia**

In 2005, Barry Marshall won the Nobel Prize in Medicine for his work uncovering the role of *H. pylori* in gastritis and peptic ulcer disease(150). My studies suggest that in *H. pylori*-infected gerbils maintained on a high salt diet, ulcers occurred at a higher rate when compared to infected gerbils fed a normal diet. Bleeding from gastric ulcers represents a possible mechanism through which *H. pylori* could be causing anemia (151). CagA has also been suggested to play a role in this process, with individuals testing positive for antibodies to CagA positive strains of *H. pylori* experiencing higher rates of ulceration than individuals infected with CagA negative strains (152). As high salt conditions upregulate *cagA* expression, the role of salt in ulceration observed in our study might be dependent on CagA and activity of the T4SS (32, 92).

Anemia is a global problem, affecting up to 1.6 billion people worldwide and an estimated 3 million individuals in the US alone (66, 153). The most common cause of anemia is iron deficiency (154). *H. pylori* has been clinically associated with systemic iron deficiency and iron deficiency anemia (IDA) (66, 67). The literature has been conflicted regarding the role of *H. pylori* in potentiating iron deficiency anemia in humans. *H. pylori*-associated anemia in animal models was an understudied phenomenon, undermining efforts to establish a formal link and disease mechanism. In a previous study, authors attempting to replicate *H. pylori*-associated hematological disease in a Mongolian gerbil model did not detect hematologic disease in infected gerbils (72). Decreased hemoglobin or other markers of anemia were not detected, with the exception of a reduced mean corpuscular volume (MCV) in infected animals (72). I

sought to address this gap in knowledge by studying *H. pylori*-induced iron deficiency anemia using the Mongolian gerbil model.

My data indicated that infected animals on a low iron diet developed a microcytic anemia, characterized by a reduction in red blood cell volume and hemoglobin levels. Uninfected animals on a low iron diet did not develop this anemia, indicating that *H. pylori* was necessary for disease development. Infected animals on a combination high salt and low iron diet developed a microcytic anemia at earlier time points than animals on a low iron diet, indicating a role for salt in this process. Serum ferritin levels, an important marker for iron deficiency anemia, were also significantly lower in the anemic group. I propose a model of IDA development in which multiple factors interact to cause IDA. *H. pylori* alone is both necessary and sufficient for IDA development; however rates increased significantly if a low iron diet is present to help unmask the IDA phenotype. Addition of another risk factor (in this case, a high salt diet) served to increase rates of anemia even more dramatically. While the link between salt and gastric cancer has been explored in previous studies, the possible connection between salt intake and iron deficiency anemia development is less scrutinized. Animals on a high salt diet had an increased rate of gastric ulceration, which might be a source of blood loss contributing to iron deficiency. Interestingly, infection with *H. pylori* was a bigger risk factor for iron deficiency than a low iron diet in our study. My findings have implications for public health in regions at risk for either gastric cancer or iron deficiency. Additionally, my work establishes the Mongolian gerbil model as suitable for study of *H. pylori*-induced iron deficiency anemia.

Our data suggest there is a correlation between decreasing hemoglobin levels and the presence of gastric dysplasia or cancer. This is an important link with implications for

individuals with IDA, and indicates lower hemoglobin values as a potential biomarker for infections likely to lead to altered gastric pathology.

Other *H. pylori* induced systemic alterations

One of the more surprising findings of our studies was the systemic changes observed among infected gerbils. While I expected to see a reduction in circulating platelets (as *H. pylori* has been associated with ITP), I instead observed a significant increase (155). This is likely due to the association of increased platelet counts with IDA, a disorder afflicting many of our gerbils (99, 100). While the exact mechanism driving increased platelet counts in anemia patients is unknown, one study suggested that increased levels of erythropoietin (a cytokine secreted by the kidneys in response to low blood oxygen levels, in order to increase production of red blood cells) could be responsible (156). Future studies should test the gerbils for levels of erythropoietin, in addition to measuring platelet levels, to establish if such a link exists in this model.

Microevolution in the gastric niche

We were also able to positively identify many mutations that occurred over the course of infection. Interestingly, many iron acquisition genes were mutated, including the previously detected FurR88H mutation, present in 4 out of 5 output strains (and was not found at detectable levels in the input strain). Fur (Ferric Uptake Regulator) serves as a global transcriptional regulator, controlling expression of genes in response to changes in iron availability, oxidative stress, salt concentration and pH (47). The Fur protein has an iron binding

pocket used to coordinate iron, and the iron bound state of Fur determines if it can bind to DNA to effect transcription of downstream genes (47). While Fur typically serves only as a transcriptional repressor in other bacterial species, it can serve as a repressor or activator in *H. pylori*, capable of activating and repressing different genes depending on whether Fur is in its apo or iron-bound state (47).

Previous work in our lab and the Algood lab tied the FurR88H mutation to a survival advantage when *H. pylori* stains containing this mutation were co-cultured with neutrophils, when compared to strains lacking this mutation. This could be due to the role of the FurR88H mutation in oxidative stress survival, as this mutation has been shown to provide a competitive advantage when strains possessing it are co-cultured alongside of wildtype strains with paraquot, a mediator of oxidative stress (92). This is an important finding with physiological relevance to *H. pylori* infections in humans.

I also uncovered a significant association between output strain mutation rate and gastric pH of gerbils from which strains were isolated. Gerbils with increased gastric pH tended to yield output strains with increased mutation rates. Since only 5 gerbil output strains were sequenced, this finding should be confirmed in follow-up studies.

Future directions

Mechanism by which *H. pylori* drives IDA

Several interesting questions remain regarding the role of gastric ulcers in *H. pylori*-induced iron deficiency anemia. While I hypothesize that gastric ulceration leading to chronic

blood loss is one mechanism by which *H. pylori* drives the development of iron deficiency anemia, this remains to be conclusively demonstrated experimentally. Future studies should focus on determining the relative importance of ulceration in IDA. An anti-ulcer drug, such as geranylgeranylacetone (successfully used to protect against ulcers in a rodent model) can be given to a cohort of gerbils infected with *H. pylori* and on a combination high salt diet (to promote ulceration) and low iron diet (to promote IDA) (157). The ulceration rate should be lower in these gerbils when compared to infected gerbils on the same diet that are not given the drug. Given the apparent importance of gastric ulcers in IDA, I believe protection from ulceration would result in significant protection from IDA as well.

The other mechanism by which *H. pylori* could be causing IDA is by increasing the gastric pH of its host, thus making dietary iron less bioavailable. To separate out the increase in gastric pH versus the other effects of *H. pylori* colonization on IDA, an uninfected cohort of gerbils could be given proton pump inhibitors, in order to maintain the gastric pH at a level comparable to that which is seen in chronic *H. pylori* infection (a pH of 5-6). I would expect to see anemia development in these gerbils, provided the gastric pH increase is a legitimate cause of IDA in this model.

Effect of CagA on IDA development

The virulence factors responsible for IDA have not been identified experimentally. One *H. pylori* protein of interest is the effector protein CagA, which has been epidemiologically associated with gastric ulceration (152). Additionally, other studies suggest a role for CagA in

the loss of parietal cells that can occur over time in *H. pylori*-infected gastric environments. Increased gastric pH resulting from the loss of these acid-secreting cells makes dietary iron less bioavailable, thus constituting a second mechanism by which CagA could impact IDA risk. Future studies evaluating the capacity of a *cagA* mutant strain to cause IDA in the gerbil model would be key to identifying the role of CagA in *H. pylori* induced-anemia and other hematologic alterations.

I observed a significant increase in gastric ulcers among infected gerbils consuming a high salt diet when compared to infected gerbils consuming a normal diet. I postulate that salt plays a role in anemia development by increasing expression of *cagA* (a phenotype observed in *in vitro* studies), which in turn increases risk of gastric ulceration. I hypothesize that CagA translocation is necessary for this phenotype and that increased salt leads to increased CagA translocation; other T4SS-dependent processes may also play a role in this pathway. To better define the effect of the *cag* pathogenicity island (c*ag* PAI), mutant strains of *H. pylori* should be tested in the gerbil model: one of which is deficient in CagA production, the other lacking a functional T4SS. This will allow us to separate the effects of CagA production from that of the T4SS in IDA, gastric ulceration, and alterations in systemic circulating blood cell levels.

Biomarkers for *H. pylori* colonization associated with adverse outcome

One of the goals with the anemia study was to determine if *H. pylori* caused IDA. As this appeared to be the case in our experiments, I wondered if anemia could serve as a biomarker for severe gastric diseases. IDA was correlated with gastric ulceration and increased gastric inflammation. While I cannot determine if the anemia predates the gastric pathology observed,

given the 16 week time course it could not have predated it by a substantial amount. Since humans tend to not develop gastric cancer until old age, after a presumably life-long infection, anemia could still serve as a red flag for problematic infections (158). Erythropoietin levels could be informative even in the absence of anemia if *H. pylori* infection is present, as a healthy host may be able to overcome chronic blood loss by increasing the rate of red blood cell production. Elevated erythropoietin levels could be an important marker for this process, so it is essential for future studies in the gerbil model to test the validity of this hypothesis by also measuring erythropoietin blood levels at various time points.

Ideally, a simple blood test could be administered to determine if an *H. pylori* infection was likely to result in disorders such as gastric cancer or anemia and thus should be targeted for eradication. Currently, patients with peptic ulcer disease routinely receive antibiotics to treat *H. pylori* infection. The ability to identify individuals at high risk of severe disease would allow *H. pylori* infection to be treated before severe gastric disease develops, resulting in better patient outcomes (159). To that end we have initiated a pilot study examining metabolites in the serum of *H. pylori*-infected gerbils compared to the serum of uninfected gerbils. Preliminary results are encouraging, with nearly 2,000 significant differences in metabolite concentration identified ($p \leq 0.05$, fold change ≥ 1.5) out of over 20,000 features detected using Progenesis software. One of the metabolites found to be upregulated in infected gerbil serum compared to uninfected was trihexosylceramide, a metabolite that is also highly expressed in metastatic colon cancer (160). Next steps should focus on testing larger numbers of serum and time course studies to identify metabolites that change prior to development of gastric cancer or anemia. We anticipate that these studies would result in major advances in knowledge about *H. pylori*-

associated disease and disease prevention, and might provide mechanistic insights into *H. pylori*-associated diseases.

***H. pylori* microevolution**

I were curious if the output strains isolated from gerbils experiencing severe gastric disease would be more likely to cause severe gastric disease in subsequent instances of infection. To test this hypothesis, I set up an experiment using Mongolian gerbils on either a high salt or normal diet, and infected the animals with either a high salt high disease output strain, or high salt low disease output strain (Fig. 17). The experiment was carried out for 12 weeks after infection, and gastric tissue was collected at the time of harvest for histological analysis. I did not observe a significant difference in cancer occurrence or gastric inflammation between gerbils colonized by the high salt high disease strain and those colonized by the high salt low disease output strains (Fisher's exact test, p=0.1)(Fig. 18A and B). I did observe a significant difference in cancer incidence between gerbils consuming a high salt diet and those consuming a normal diet, with the high salt diet cohort experiencing a higher rate of gastric cancer development (Fisher's exact test, p=0.04,).

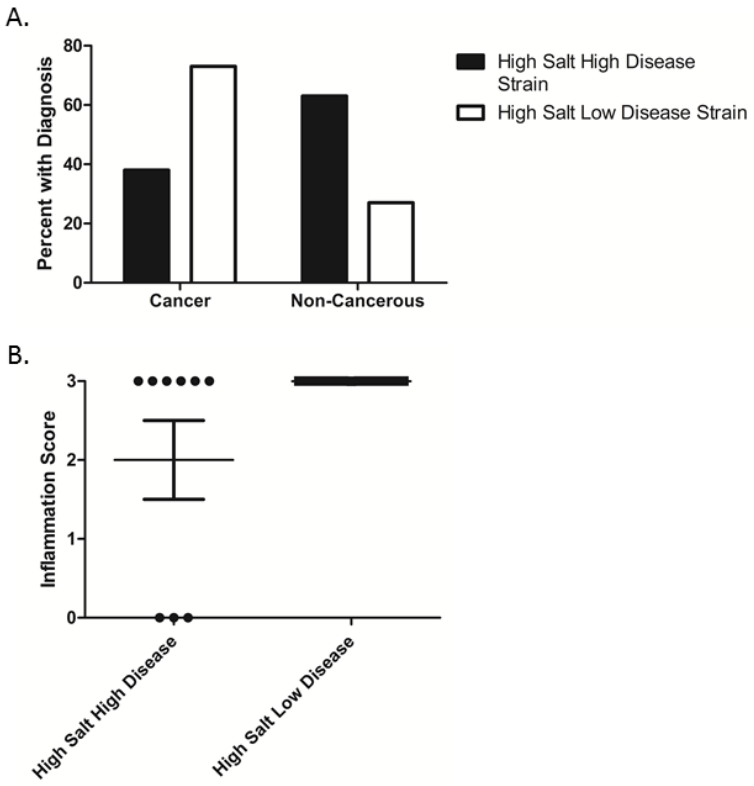

Figure 17. Cancer and inflammation scores by output strain and by diet. A.) Percent of gerbils infected with either the high salt high disease output strain or the high salt low disease output strain that developed gastric cancer. B.) Inflammation scores, by output strain.

My prior large scale gerbil study did not show a difference in cancer rates when comparing gerbils on a high salt diet and gerbils on a normal diet. Complicating factors include the fact that the normal diet cohort experienced unusually high rates of cancer (potentially due to the decreased iron content in this diet compared to previous baseline diets, and compared to the iron content of the normal diet in the new study). Additionally, our largest sample size had an experimental endpoint that may have been carried out too far at 16 weeks. This study, although small, reassures us that we can consistently replicate this finding of increased cancer incidence in infected gerbils consuming a high salt diet compared to infected gerbils consuming a normal diet.

My hypothesis that a high disease output strain would go on to cause high disease in future infections was not supported by the evidence. Interestingly, there were differences in the colonization rates of these two strains: 80% of the gerbils infected with the low disease output strain were colonized, and 50% of the gerbils infected with the high disease output strain were colonized. Adaptation to the gastric niche is dynamic and likely changes as environmental conditions in the stomach change (pH, inflammation, ulceration). Strains taken from a gastric environment with high levels of inflammation and increased pH may not be equipped to immediately colonize an uninfected, acidic gastric environment. This hypothesis could be tested with future, larger scale gerbil studies as well as *in vitro* assays designed to test the acid sensitivity of high disease versus low disease output strains. I hypothesize that strains colonizing a gerbil experiencing low disease with a normal, acidic gastric pH would be more acid tolerant in *in vitro* assays than a strain taken from a high disease gerbil experiencing severe hypochlorhydria.

Our study provided insights into the cumulative *H. pylori* genetic changes over the course of infection. Future studies should focus on sequencing individual strains isolated at multiple times over the course of infection. This could be accomplished by collecting small amounts of gastric tissue via endoscopy. These types of studies could reveal how *H. pylori* adapts to the same gastric environment over the course of infection, and could reveal if there is a chronological order to the mutations detected. Ideally, gastric pH and histology of the stomach could also be noted at the time of strain isolation.

I observed several gerbils that were colonized with *H. pylori* that did not exhibit signs of gastric disease. These strains are attractive targets for future sequencing studies, to attempt to find SNPs that are associated with strains that tend to not cause gastric disease. One possibility is that these gerbils cleared the initial infection and then were infected by a cage mate, and thus were not infected over the full 12 weeks. Time course experiments could allow isolation fo strains that don't cause disease, and could provide confirmation that the infection was present over the intended time period.

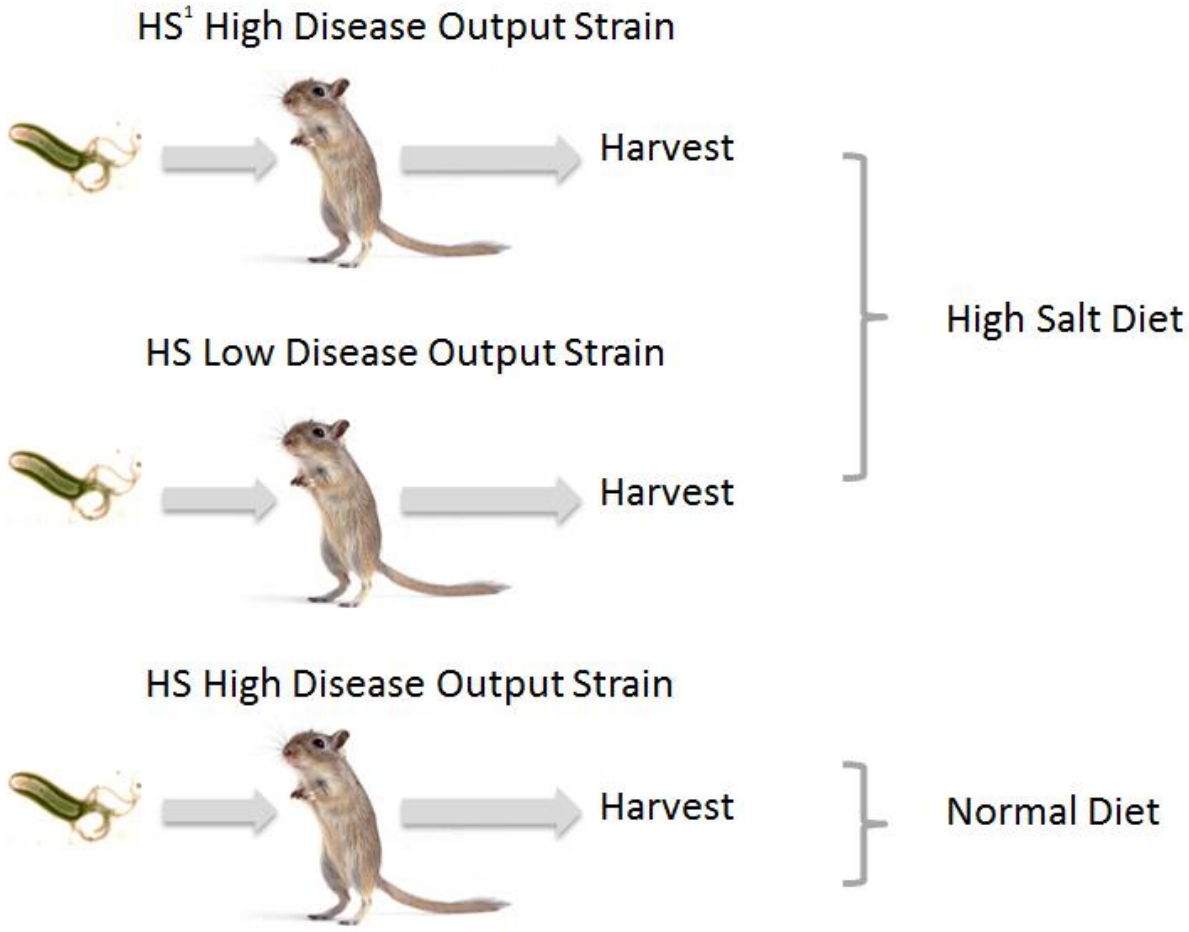

Figure 18: Schematic of experimental set-up. Three cohorts of gerbils were utilized for the study. The first two cohorts were maintained on a high salt diet (an additional 8% salt compared to the normal diet). The first cohort was infected with a high salt[1] (HS) high disease output strain isolated from the first study; the second cohort was infected with a HS low disease output strain. The third cohort was also infected with the high salt high disease output strain; however this cohort was maintained on a normal diet.

Identifying the FurR88H mutation consistently among output strains (as well as some strains recently isolated from human stomachs) makes this mutation a prime target for future study. Presumably FurR88H confers a selective advantage to strains possessing this mutation.

We initially thought that the FurR88H mutation interfered with the ability of Fur to coordinate iron, as the region where it is mutated encodes the region corresponding to the iron binding pocket in the resulting protein (161). Prior experiments suggested that an isogenic FurR88H mutant strain expressed two iron acquisition genes at higher levels than a wild-type strain (92). Additionally, output strains taken from a gerbil on a high salt diet (and which were found to possess the FurR88H mutation) produced higher levels of these same iron acquisition proteins (92). Taken together, these results suggested that the FurR88H mutation resulted in less iron bound fur in the bacterial cell, which led to less repression of iron acquisition genes and more production of the iron acquisition proteins that they encoded. Proteomic experiments did not support this hypothesis however, with the wild-type strains producing levels of FecA2 and FrpB1 that did not significantly differ from that of the FurR88H mutant strains at the 15 hour time-point. Follow-up studies designed to assess if this mutation conferred a growth advantage when cultured in iron restricted media also did not result in significant differences between the FurR88H containing strains compared to the wildtype strains. Finally, manganese chloride assays designed to identify strains that do not possess a form of Fur capable of coordinating metal also did not yield significant differences in survival (Fig. 18).

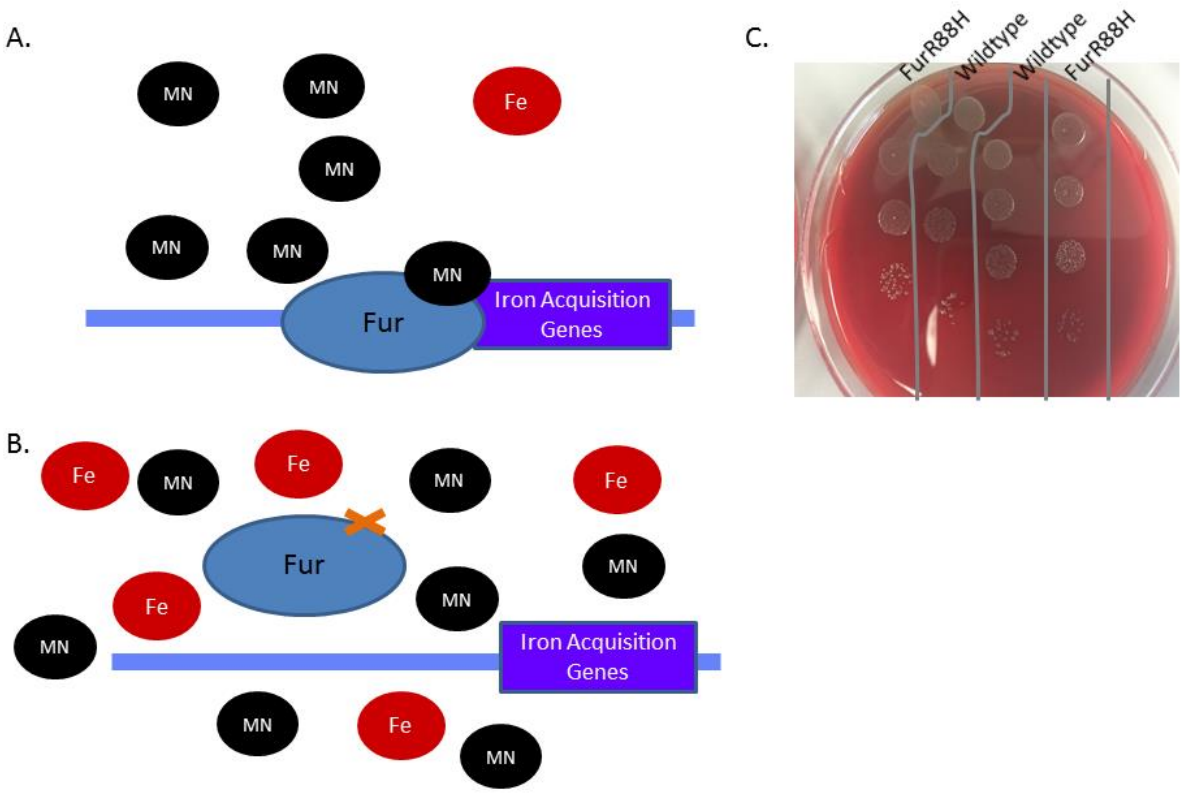

Figure 19: Manganese chloride assay. A. In strains possessing a prototypical Fur, adding excess manganese chloride to the media results in manganese chloride coordinating with Fur in its iron binding pocket, causing Fur to subsequently bind DNA and repress transcription of iron acquisition genes. This causes the bacterium to undergo iron starvation, and subsequent cell death. B. If a mutation in the iron coordination pocket of Fur prevents the binding of manganese chloride, iron acquisition genes are continuously expressed, allowing for cell survival. C. No significant differences in bacterial survival were observed when comparing the FurR88H mutant strains (first and last column) with wildtype strains (middle two columns) after treating the bacteria with excess manganese chloride.

In order to determine if the FurR88H mutation results in less iron bound by Fur, ICP-mass spec studies should be performed on the cell pellets of mutant and wildtype strains, across a spectrum of iron availability. This should allow for an approximate stoichiometry to be established, determining the effect of this mutation on cellular levels of iron. Eventually, purified wildtype Fur and FurR88H could also be tested in a similar manner.

Competition studies using a FurR88H variant and a wild-type strain to confirm the presence of a selective advantage *in vivo* would also be ideal. I generated the necessary FurR88H and wildtype strains; unfortunately, they failed to colonize the gerbil stomach. Since even the wild-type strain failed to colonize, it may be necessary to remake them, with as few laboratory passages as possible in order to minimize the risk of acquiring a mutation leading to lack of colonization. My initial plan was to inoculate both strains (each containing a unique antibiotic marker) into the same cohort of gerbils, and to determine what proportion of the output bacteria contained the FurR88H mutation.

Follow-up studies to determine if the FurR88H mutation conferred immune cell survival advantage in a neutrophil-specific manner, or was a reproducible phenomenon for a variety of immune cell types (macrophages, dendritic cells) would also be an excellent follow-up study. Identifying the mechanism that conferred this advantage would be a primary focus. Adding in excess oxygen detoxification enzymes such as catalase to the co-culture media would help determine if it was an oxidative stress-related phenotype as hypothesized, as we would expect to see the survival advantage of FurR88H abrogated if this were the case. If it is neutrophil specific, it would be interesting to determine if the FurR88H mutation arose in a mouse strain lacking neutrophils.

Relationship of output strain mutation rate and gerbil gastric pH

Another question that arose from the sequencing of output strains involved the relationship between output strain mutation rate and gastric pH of the gerbil from which the strain was isolated. Based on my analysis of 5 animals, there was a statistically significant relationship, with higher mutation rates found in strains isolated from gerbils with increased gastric pH. In order to confirm this finding, future studies should focus on output strains isolated from larger numbers of gerbils. If it remains a significant finding, expanding the analysis to other models and output strains would shed light on its relevance to human infections.

Temporal analysis of CagA translocation in gastric disease

In previous studies CagA has been identified as an oncoprotein and has been shown to play an important role in gastric carcinogenesis, based on both epidemiologic studies and animal experiments (162). Gastric cancer occurs at a higher frequency among the elderly compared to younger individuals, and the presence of *cagA*-positive *H. pylori* strains is a risk factor for gastric carcinogenesis. In gerbil experiments, *cagA* mutant strains do not induce gastric inflammation, even in the presence of a high salt diet (32). The carcinogenic potential of CagA mediated by several mechanisms, including the ability of CagA to deregulate oncogenic pathways such as Wnt (163). Mutations in tumor suppressing genes can likely promote cancer development even after CagA has been removed, whereas the ability of CagA to deregulate oncogenic pathways is likely dependent on its continued presence in the cell. While it has been shown that eradicating *H. pylori* can improve clinical prognosis even after disease progress, it is not known how much of this can be attributed to the removal of CagA, and how much is due to

the removal of other bacterial factors (164). It is also unknown if there is a temporal component to gastric cancer risk from CagA exposure, i.e., does early exposure to CagA result in a higher overall risk for gastric cancer than exposure at later time points?

There are several challenges inherent in working to improve our understanding of the importance of CagA at various time points. Chief among these is the difficulty in halting host exposure to CagA, without clearing *H. pylori* from the gastric tissue. To isolate the effects of CagA at various time points from the effect of confounding variables (such as other *H. pylori* virulence factors), an inducible promoter system such as TetR presents a unique solution to address this problem. This system is naturally found in several species of bacteria. It functions as a modulator of gene expression in response to environmental tetracycline (or doxycycline) concentrations. In the absence of tetracycline, the Tet repressor binds DNA at TetO sites and inhibits transcription of downstream genes. When tetracycline is present, the drug binds to the Tet repressor protein, causing it to change into a conformation that will no longer bind DNA. This allows genes previously repressed by this regulator to be expressed. By applying the Tet repressor system to create an on/off switch in an *in vivo* model, we can explore the temporal component of CagA translocation. This will allow us to determine if early time points of CagA translocation can lead to IDA and gastric carcinogenesis even after CagA translocation has been halted. Halting CagA translocation after IDA development is a useful method through which we hope to address a basic question: does preventing CagA translocation allow for a partial recovery from IDA and recovery from more advanced stages of gastric and systemic disease? If true, this proof of principle could form the foundation of further experiments measuring the efficacy of dietary interventions as a strategy for combating IDA and gastric cancer. Candidate

diets for these interventions would be those in which CagA translocation is not up-regulated, such as a low salt, high iron diet.

Previous work in our lab led to the development of a strain of *H. pylori* capable of regulating gene expression via this system. We built on this strain by inserting the TetO sites in front of the *cagUT* locus (Fig. 19). This site was chosen as CagT is required for activity of the T4SS and CagA secretion.

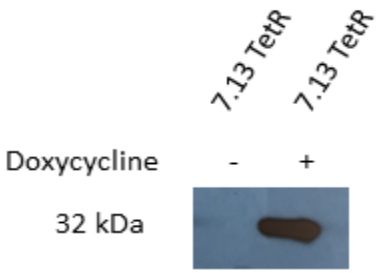

Figure 20. Regulation of CagT production by *H. pylori* strain 7.13 containing TetO sites in front of the *cagUT* locus. By adding doxcycyline to the media, *cagT* is expressed.

I initially tested our TetR strain in the gerbil model immediately after introducing the tet repressor TetR into the genome of strain 7.13. Preliminary results were promising, with our strain colonizing 3/3 gerbils. Immediately after this study, I attempted to colonize a second cohort of gerbils with a strain that in addition to containing the Tet repressor also contained TetO sites in front of the *cagUT* locus. I did not observe consistent colonization with this strain. After several attempts at remaking the strains and testing the strains for colonization, we finally generated a strain that reliably colonized, and upon isolation back out of the gastric tissue we were still able to regulate *cagT* expression using tetracycline analogs.

In order for the TetR system to be a viable method of gene regulation *in vivo,* I needed to be able to administer a dose of doxycycline that is high enough to activate the TetR system, but not so high as to eradicate the colonizing *H. pylori*. After several experiments, I found 200 ppm doxycycline administered in the gerbil chow to be the upper range of what the bacteria will tolerate. Due to difficulties in detecting the *cagT* mRNA isolated from gerbil gastric scrapings, we are still working to establish if we have successfully regulated T4SS expression via this system. A recently concluded study should yield this answer, as we will use gerbil pathology as another endpoint. If CagA is not being translocated (indicating successful repression of the T4SS machinery via TetR), gerbil histology should be markedly attenuated in severity of inflammation and gastric erosion. Gerbils consuming diets containing lower concentrations of doxycycline exhibited higher rates of gastric colonization than gerbils consuming diets containing higher dosages of doxycycline (Fig. 20). Future studies can focus on the effect of

switching off CagA translocation midway through infection on gastric pathology and systemic immune cell circulating levels.

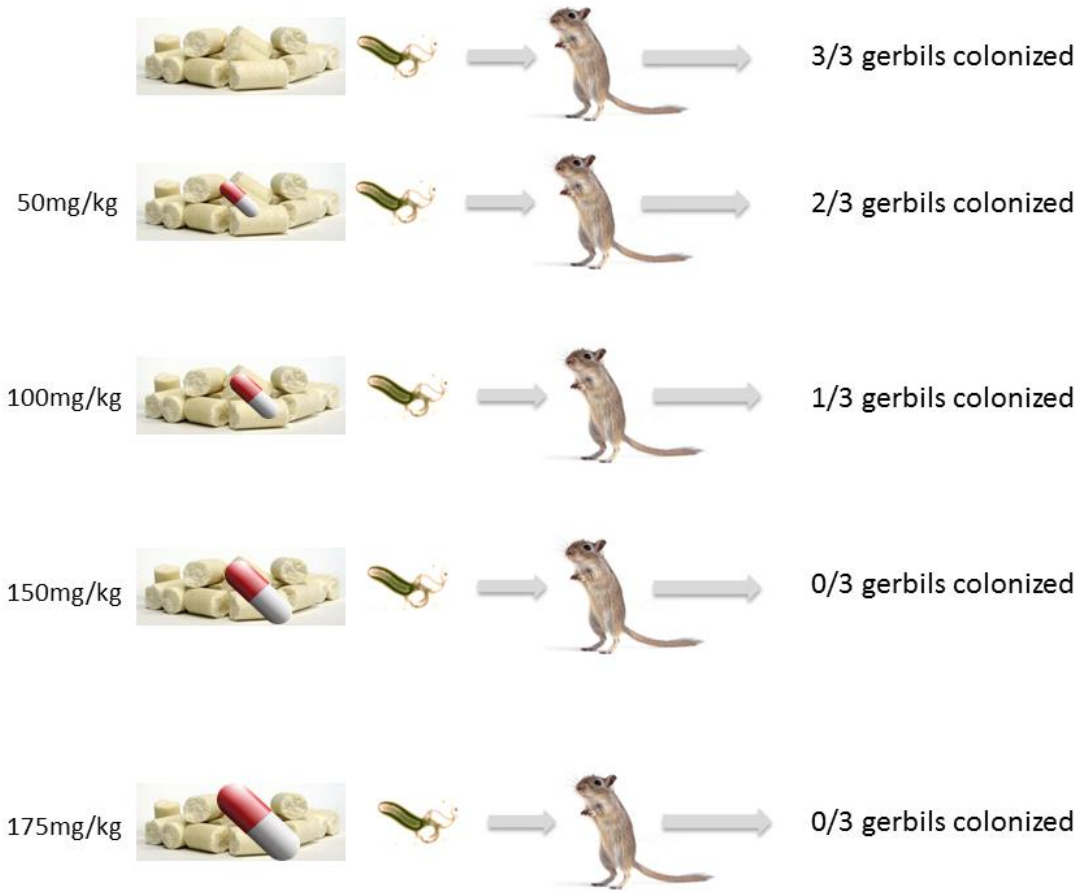

Figure 21. Colonization rates of TetR strain 7.13 on diets containing various concentrations of doxycycline. Colonization rates decreased as doxycycline dosage increased, with gerbils consuming a diet containing 50 mg/kg doxycycycline exhibiting a 2/3 colonization rate.

Future studies can follow-up on these experiments in a variety of ways. Identifying a mechanism by which *H. pylori* causes IDA is a top priority. This should be done by separating out the increased gastric pH and the gastric ulceration phenotypes using the Mongolian gerbil model. Erythropoietin levels should be monitored in uninfected and infected gerbils to determine if this cytokine could be responsible for some of the systemic changes observed over the course of infection, such as the increased platelet counts. Output strains should continue to be isolated and saved after harvest, in order to later identify interesting strains for further sequencing study, such as those that colonized well but did not cause disease. The FurR88H mutation should also be further studied to determine the mechanism by which it confers increased oxidative stress resistance. Additionally, the pilot studies examining serum metabolites in infected versus uninfected gerbils should be continued in greater numbers and at various time points, in order to identify a change in the metabolomic profile that predicts gastric cancer development. Finally, the TetR strains are a continued priority. At this point we have a strain capable of colonizing gerbils, and that maintains the tet regulation phenotype upon isolation back out of the gerbil. Future studies should seek to ensure we can regulate this system *in vivo*, and if so, perform temporal studies to determine the effects of this manipulation on gastric histology.

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