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Dietary Factors Modulate *Helicobacter*-Associated Gastric Cancer in Rodent Models

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Abstract

Since its discovery in 1982, the global importance of *H. pylori*-induced disease, particularly in developing countries, remains high. The use of rodent models particularly mice, and the unanticipated usefulness of the gerbil to study *H. pylori* pathogenesis have been used extensively to study the interactions of the host, the pathogen and the environmental conditions influencing the outcome of persistent *H. pylori* infection. Dietary factors in humans are increasingly recognized as being important factors in modulating progression and severity of *H. pylori*-induced gastric cancer. Studies using rodent models to verify and help explain mechanisms whereby various dietary ingredients impact disease outcome should continue to be extremely productive.

Keywords

Helicobacter pylori; *Helicobacter felis*; mice; gerbils; diet

Introduction

Helicobacter pylori, first isolated from inflamed gastric tissues of humans in 1982, is now firmly established as an important human pathogen. Prior to its discovery, despite earlier observations of gastric spiral organisms being present in human and animal gastric tissues (Freedberg and Barron, 1940, Doenges, 1938), an often stated principle, championed particularly by gastroenterologists, was the stomach for all practical purposes was considered to be sterile because of the stomach's low pH. It is now known that *H. pylori* infection is persistent and is always associated with inflammation consisting of mononuclear cells and polymorphonuclear cells. When the infected individual is treated with antibiotics known to eradicate *H. pylori*, the gastritis regresses over time. However, if there is recrudescence of the organism due to ineffective antibiotic therapy or the patient becomes reinfected with another *H. pylori* strain, the gastritis reappears. It has been determined through rigorous clinical and epidemiologic studies that *H. pylori* is directly linked to peptic ulcer disease and importantly, the World Health Organization has listed *H. pylori* as a class I

carcinogen (IARC, 1994). *H. pylori* is also considered to initiate and sustain low grade gastric mucosal-associated lymphoma (Muller, 2009). Prior to the discovery of *H. pylori*, peptic ulcer disease was linked to excessive gastric acid production and/or stress, whereas gastric cancer was linked to a variety of dietary factors documented by extensive epidemiologic surveys.

Although 50% of the world population is infected with *H. pylori*, only a small percentage (1–3%) of individuals have the gastric disease progress to gastric adenocarcinoma. Gastric adenocarcinoma (GAC) represents the most commonly diagnosed (~95%) of gastric cancers, and is the fourth and fifth most common cancer in males and females, respectively (Jemal *et al.*, 2011). Worldwide, there were an estimated 989,000 cases of gastric cancer and 738,000 gastric cancer associated deaths in 2008, making gastric cancer the fourth most common cancer and the second leading cause of cancer death, after that recorded for lung cancer (Ferlay *et al.*, 2010).

A strong genetic predisposition to development of *H. pylori*-associated gastric cancer has been established. Gastric cancer risk is increased up to threefold in individuals with a first-degree relative with gastric cancer, and 10% of gastric cancer cases exhibit familial clustering. While it's well established that *H. pylori* can infect multiple family members, family history remains a risk factor even after controlling for *H. pylori* infection. However, only a small fraction of the familial clustering of gastric cancer is attributable to known family cancer syndromes. It was observed that *H. pylori* infected patients who developed gastric cancer secreted lower levels of gastric acid compared to *H. pylori* infected patients with duodenal ulcers. Thus, in a landmark study, the authors chose to study IL β , a known proinflammatory cytokine with marked acid inhibitory properties in families with precancerous lesions attributable to *H. pylori* (El-Omar *et al.*, 2000). They demonstrated that individuals with specific genetic polymorphisms in IL1 β and IL1RN had a 2 to 3 fold increased risk of developing gastric atrophy and gastric cancer (El-Omar *et al.*, 2000). These findings have been confirmed in some studies, but not others. In a meta-analysis of IL β and ILR antagonist gene polymorphism and risk of gastric cancer, IL1 β -511T and IL1RN*2 were associated with gastric cancer risk in Caucasians, but not in Asians. For IL1 β -511T, the association in Caucasians was stronger when intestinal-subtypes and noncardia gastric cancer cases were examined (Camargo *et al.*, 2006). Further genetic polymorphisms in TNF α and IL10, when combined with IL1B polymorphisms, result in higher risk genotype, conferring a 27-fold or greater risk of developing gastric cancer (El-Omar *et al.*, 2003).

The mechanisms involved in the pathogenesis of GAC remains incompletely characterized but clearly the interactions of host, environmental variables, and *H. pylori* virulence factors, particularly the *cag* pathogenicity island (PAI) and vacuolating cytotoxin, play a role in cancer initiation and progression (Jones *et al.*, 2010). These virulence factors are polymorphic and affect a multitude of host cellular pathways and thereby contribute to differences in disease severity given that various CagA and VacA alleles differentially target selected pathways (Jones *et al.*, 2010). As reviewed by Jones *et al* in 2010, cytotoxin-associated Gene A is encoded on the *cag* PAI, which is a horizontally acquired 40kb DNA segment that encodes for a type IV secretion system (Jones *et al.*, 2010). *cagA* is the terminal gene on the *cag* PAI, and encodes for the 120–145kDa immunodominant CagA

protein (Covacci *et al.*, 1993, Tummuru *et al.*, 1993). Once injected into host cells, CagA acts directly in an unphosphorylated state to influence cellular tight junction (Oliveira *et al.*, 2009), cellular polarity (Zeaiter *et al.*, 2008), cell proliferation and differentiation (Lee *et al.*, 2010), cell scattering (Mimuro *et al.*, 2002), and induction of the inflammatory response (Brandt *et al.*, 2005). Upon entering the gastric epithelia, CagA localizes to the plasma membrane and can be phosphorylated by either Abl kinase or Src family kinases (Poppe *et al.*, 2007, Tammer *et al.*, 2007). These kinase phosphorylate tyrosine residues are located within the carboxy-terminus of CagA. These repeats are based on the amino acid sequences found within the regions flanking the EPIYA sequence and are classified as four distinct EPIYA motifs: EPIYA-A, -B, -C, and -D (Jones *et al.*, 2010). Once phosphorylated, CagA can form cellular interactions that influence cellular shape and motility (Tammer *et al.*, 2007, Jones *et al.*, 2010, Brandt *et al.*, 2007). The presence of CagA is associated with more severe disease. Cancer patients are at least twice as likely to be infected with an *H. pylori* strain that is *cagA* positive than one that is *cagA* negative (Gwack *et al.*, 2006). The VacA cytotoxin is produced and secreted by the majority of *H. pylori* strains. Vacuolating cytotoxin A activity was first noted when *H. pylori* filtrates induced large host cell vacuoles (Leunk *et al.*, 1988). VacA remains on the bacterial surface or is secreted as an approximately 88 kDa toxin (Cover and Blaser, 1992). Exposure to alkaline or acidic conditions amplifies that activity of VacA, where the toxin induces large cytoplasmic vacuoles in host cells that contain the markers for late endosomes and lysosomes. VacA also causes apoptosis, which is dependent on interaction with the mitochondria (Foo *et al.*, 2010). VacA also deregulates multiple cellular pathways as well as inducing inflammation. VacA intoxication of host cells induces production of a variety of inflammatory cytokines that include TNF α , IL-1 β , IL-6, IL-10, and IL-13 (Supajatura *et al.*, 2002).

Animal models, particularly rodents, continue to play an invaluable role in dissecting interconnected mechanisms that determine gastric disease outcome.

Rodent Models for *Helicobacter*-Associated Gastric Cancer

Mice

***H. felis* infection**—Most of the major advances in our understanding of gastric cancer pathogenesis have been derived from studies with mice. While a number of other animal models have been developed for both gastric cancer (e.g., MNNG-induced cancer in rats) and for *Helicobacter*-mediated gastric infection (ferret, gerbil, gnotobiotic pig, primate, etc.), the mouse model has clear advantages with respect to their small size, cost, ease of infection, reproducibility, and especially the power of genetic manipulation. The first study to ascertain whether gastric helicobacters would colonize the stomach of rodents utilized germ-free Swiss Webster mice or Sprague Dawley rats infected with *H. felis* (Fox *et al.*, 1991, Lee *et al.*, 1990). Although the rat is the preferred model for gastric carcinogenesis studies, long-term *H. felis* and *H. pylori* colonization studies in this species have not been performed. This model therefore requires further development before it can be used in carcinogenesis studies. In the first description of *H. felis* gastritis in the germ-free mouse, a moderate degree of fundic glandular epithelial cell hyperplasia was evident at 4 weeks in portions of the gastric mucosa (Lee *et al.*, 1990). This was characterized by an overall

increase in thickness of the mucosa in areas of inflamed tissue. The changes included lack of epithelial cell maturation and differentiation, increased basophilia and increased nuclear to cytoplasmic ratio. Also of interest was the enlargement of individual glands and the presence of columnar type epithelium vs. cuboidal epithelium. Occasional branching of glands was noted as well as luminal epithelium forming small pseudovillus projections. These lesions persisted for the duration of the 8 week study (Lee *et al.*, 1990).

These studies have been extended by documentation of persistent active, chronic gastritis in germ-free mice and SPF mice, infected with *H. felis* for 1 year (Fox *et al.*, 1993). Studies of 72–76-week colonization of conventional and SPF mice have provided additional clues on progression of the lesions in the stomach of infected mice. Studying the role of genetic factors in the pathogenesis of *Helicobacter*-associated gastritis by taking advantage of the wide range of inbred and other types of genetically defined mice was a logical next step in further exploration of *Helicobacter* pathogenesis using this model. Two different laboratories inoculated three inbred strains of mice with *H. felis* and tabulated the intensity of inflammation; in BALB/c mice inflammation was minimal, in C3H/He moderate and was most severe in C57BL/6 when examined 2–11 weeks post-infection (Mohammadi *et al.*, 1996, Sakagami *et al.*, 1994). The authors also stated that C57BL/6 mice had mucous cell hyperplasia and parietal cell loss in gastric fundic glands (Mohammadi *et al.*, 1996). Furthermore, the study demonstrated that by using congenic strains on the BALB/c and C57BL background, both MHC and non-MHC genes contributed to *H. felis*-associated gastritis (Mohammadi *et al.*, 1996). The *H. felis* infected mouse model continues to be used frequently with considerable success in studying environmental and host factors centered on *Helicobacter* spp. pathogenesis (See Table 1 and below) (Wang *et al.*, 2000).

In the early investigations by a number of groups, it became clear from studies with inbred mice that progression to preneoplasia was largely determined by the host response. For example, the C57BL/6 inbred mouse strain responded to *H. felis* infection with a robust Th1 immune response, in contrast to the BALB/c inbred strain which showed a predominant Th2 response (Mohammadi *et al.*, 1996, Sakagami *et al.*, 1994, Wang and Fox, 1998). Consequently, C57BL/6 mice showed rapid premalignant progression to atrophy and metaplasia with declining colonization levels, while BALB/c mice maintained high numbers of organisms with minimal epithelial injury or atrophy. Additional studies employing RAG and SCID mice and T-cell-deficient mice pointed to the importance of T-cell responses (Roth *et al.*, 1999), while the use of cytokine knockout mice indicated that *H. pylori*-induced mucosal inflammation is Th1-mediated and exacerbated in IL-4 but not IFN- γ gene-deficient mice (Smythies *et al.*, 2000).

However, while genetic manipulation of the various cytokine genes clarified many aspects of the immunopathogenesis of the disease, the unmanipulated C57BL/6 mouse has proved surprisingly robust as a model for cancer development. Interestingly, many of the early *H. felis* infection studies included observation periods that extended up to one year but not beyond, and described changes of atrophy and metaplasia, but not more advanced pathology. The presence of dysplasia and invasive carcinoma in the *H. felis*-infected C57BL/6 mouse was first described by Fox and Wang (Fox *et al.*, 2002), when the observation period was

extended to 14 months. More recently, progression to antral carcinomas has been reported in mice in which the model has been extended out to 22 months (Cai *et al.*, 2005).

The first *Helicobacter*-dependent mouse model of gastric cancer was reported by Wang *et al.*, who described *H. felis* infection of insulin-gastrin (INS-GAS) transgenic mice in an FVB/N inbred background (Wang *et al.*, 2000). The model has proved ideal as an accelerated model of carcinogenesis. A role for host and dietary factors also has been demonstrated using *Helicobacter*-infected INS/GAS mice (Table 1).

The gastric hormone gastrin stimulates gastric acid secretion and growth of the acid-secreting part of the stomach. *H. pylori* infection in humans results in a mild hypergastrinemia (1.5–2-fold elevation compared with uninfected subjects) that occurs early in the course of infection (Mulholland *et al.*, 1993, Levi *et al.*, 1989), precedes the development of atrophic gastritis, and often resolves after eradication of the infection. Chronic atrophic gastritis, a pathological condition characterized by decreased numbers of parietal cells and low levels of acid secretion and occurs as part of progression of *Helicobacter* spp. associated gastric lesions in the carcinogen cascade. The insulin-gastrin (INS/GAS) transgenic mice have a chimeric transgene in which the human gastrin gene is transcribed from the rat insulin I promoter (Wang *et al.*, 1993). These mice express human heptadecapeptide gastrin (G-17) in the pancreatic *beta* cells, which is then secreted into the circulation. Changes in gastrin secretion are divided into 2 distinct phases: an early phase (1–4 months) and a later phase (5 months and older). Gastrin levels increased modestly during the early phase, and approximately 2-fold compared with wild-type FVB/N mice (Wang *et al.*, 1996). Slightly more than 50% of the serum gastrin (and all of the increase) in young INS-GAS mice is human gastrin resulting from pancreatic secretion. In contrast to the elevated plasma amidated gastrin levels, glycine-extended gastrin levels were all <20 pmol/L. Serum amidated gastrin levels are increased gradually beginning at 6 months, with levels peaking at 550 pmol/L by 20 months of age. Most of the increase in serum gastrin resulted from increased secretion of endogenous mouse amidated gastrin, with lesser changes in the level of human G-17, which increases 3-fold from 3 months to 20 months. During the late gastrin phase (5 months and older), INS/GAS mice have a gradual change in gastric function with a marked decline in the level of stimulated acid secretion. These decreases in acid production are mirrored by decreases in parietal and ECL cell numbers (Wang *et al.*, 2000).

In the INS/GAS mouse model there is an increased expression of growth factors, heparin-binding epidermal growth factor and TNF α . At 20 months of age, INS/GAS mice don't have increased enterochromaffin-like cell number, but rather exhibited gastric metaplasia, dysplasia, carcinoma in situ, and gastric cancer with vascular invasion. Experimental *H. felis* infection of INS-GAS mice led to accelerated (8 mo) development of intramucosal carcinoma (85%), with submucosal invasion (54%) and intravascular invasion (46%; P 0.05) (Wang *et al.*, 2000). These findings support the conclusion that chronic hypergastrinemia in mice synergizes with *H. felis* and *H. pylori* infections and contributes to eventual parietal cell loss and progression to proximal gastric cancer. The model leads to accelerated gastric cancer in part due to the effect of elevated circulating levels of amidated gastrin which bind to CCK-B receptors in the gastric corpus to activate a histamine pathway,

leading to altered growth and immune responses (Takaishi *et al.*, 2005), and to relatively low levels of glycine-extended gastrin which may inhibit progression to preneoplasia (Cui *et al.*, 2003). However, while hypergastrinemia clearly accelerates proximal gastric cancer, it also appears to protect against the more distal, antral gastric cancers (Takaishi *et al.*, 2009, Tomita *et al.*, 2011).

The INS-GAS mouse model has been used to demonstrate that male gender is important in *Helicobacter*-associated cancer progression (Fox *et al.*, 2003a, Fox *et al.*, 2003b). Further studies demonstrated the protective effects of estrogen (Sheh *et al.*, 2011, Ohtani *et al.*, 2007). Thus, the INS/GAS model mimics the 2:1 ratio of male:female in gastric cancer development of humans. The INS/GAS model infected with either *H. felis* or *H. pylori*, consistently reproduces the progression of gastric diseases that is recognized in humans infected with *H. pylori*, known as Correa's Model of Gastric Carcinogenesis (Figures 1 and 2) (Fox and Wang, 2007). However, the hypergastrinemic phenotype to date appears most prominent in INS-GAS mice in an FVB/N background, and is less prominent in a C57BL/6 background (Takaishi *et al.*, 2009).

***H. pylori* infection**—As described above, the C57BL/6 mouse model of gastric *H. felis* infection has been very useful for the study for manipulation of the Th1-Th2 immune response. However, another factor is likely to be the particular *H. pylori* strain used to infect mice, and this question has been more difficult to address using the wild type C57BL/6 mouse. A preliminary report published in 1991, indicated that fresh clinical isolates of *H. pylori*, but not stock culture isolates colonized the stomach of athymic and euthymic mice for a short time interval (Karita *et al.*, 1991). The mouse model was further developed to test the ability of *H. pylori* strains that expressed vasculating cytotoxin (VacA) and the immunodominant cytotoxin associated antigen (CagA) vs. *H. pylori* strains that did not express the VacA and CagA antigens (Marchetti *et al.*, 1995). The ability of *H. pylori* to colonize was enhanced (over that of clinical isolates) by serial passage of *H. pylori* in mice. Thus 'mouseified' *H. pylori* strains used in the experiments assessing gastric pathology were isolated from mice 2 weeks after inoculation, expanded *in vitro* and used in subsequent studies. The strains expressing the VacA and CagA experimentally produced gastritis for 8 weeks, whereas the strain lacking these antigens did not produce gastric pathology (Marchetti *et al.*, 1995). In addition, the infected mice had a systemic antibody response demonstrated by immunoblot to several dominant antigens. Interestingly, the CagA antigen was not recognized; perhaps because of the early phase of infection in the mice (Marchetti *et al.*, 1995), or expression of the functionality of CagA was lost after colonization (Rieder *et al.*, 2005). Also of considerable importance was the original description of the Sydney strain of *H. pylori* which colonizes mice up to 12 months after inoculation, and importantly induces a significant gastritis, particularly in C57BL mice and B6129 mice (Lee *et al.*, 1997, Rogers *et al.*, 2005). However, most *H. pylori* strains- including the Sydney strain- while showing fairly consistent patterns of colonization, have proven less carcinogenic in C57BL/6 mice (Thompson *et al.*, 2004), although a study published in 2005 demonstrated *in situ* carcinoma in C57BL/6 X 129SvEv (B6129) mice infected for 15 months with *H. pylori* SS1 (Rogers *et al.*, 2005). It is now appreciated that use of *H. pylori* SS1, though successful in generating preneoplastic and neoplastic lesions in specified strains of mice is somewhat

limited due to its inability to express CagA *in vivo*. These results are compatible with *H. felis* mouse models of gastric cancer given that *H. felis* does not have Cag genes located on a pathogenicity island (PAI).

The *cag* PAI encodes an active type IV secretion system (T4SS) and after *H. pylori* epithelial cell adherence, the secretion system translocates the CagA protein into epithelial cells where the CagA protein undergoes tyrosine phosphorylation (CagA^{P-tyr}) (Jones *et al.*, 2010, Muller, 2012). This process is associated with host-cell morphological changes and phosphorylation of host-cell proteins. Several genes within the *cag* PAI, but not *cagA* itself, are required for the induction of proinflammatory cytokines or chemokines, such as IL-8. The production of proinflammatory cytokines by *H. pylori* is dependent on the nuclear factor- κ B and nitrogen-activated protein kinase signal transduction cascades. Increased inflammation in *cag*⁺ *H. pylori* strains is also noted and importantly, human studies indicate that patients infected with CagA⁺ *H. pylori* are at a higher risk of developing gastric cancer than those infected with Cag⁻ strains (Jones *et al.*, 2010, Muller, 2012). Mice appear to be incompatible with *cag*⁺ *H. pylori* strains carrying functionally active T4SS. *H. pylori* strains rapidly switch off the *cag* PAI and down-regulate proinflammatory responses. The widely used *H. pylori* SS1 strain has a complete *cag* PAI, but is unable to induce IL-8 or to deliver CagA into gastric epithelial cells *in vitro*. Using PMSS1 (*H. pylori* SS1 prior to serial mouse passage) with an intact functional CagA elicits more robust inflammation during its initial colonization in C57BL mice, but this feature is lost upon prolonged colonization, presumably due to functional loss of Cag, which recapitulates the *in vivo* behavior of SS1 (Arnold *et al.*, 2011). Other mouse-adapted *H. pylori* strains used in different laboratories carry mutations or gene deletions in the *cag*-PAI and are unable to translocate and tyrosine-phosphorylate CagA.

A number of other very useful murine model of gastric cancer, including genetically engineered mice (e.g. p27 (Kip1^{-/-}); IL1 β), have been developed (Judd *et al.*, 2004, Rogers and Fox, 2004, Zavros *et al.*, 2005, Tu *et al.*, 2008). Indeed, in many of the models of gastric cancer, a long period of achlorhydria, associated with presumed bacterial overgrowth, appears to be the common denominator in mouse models of GAC (Zavros *et al.*, 2005, Lertpiriyapong *et al.*, 2013, Lofgren *et al.*, 2011). It is likely that various mouse models will continue to be developed and used to dissect the role of gastric *Helicobacter* infection and the associated immune response to precisely determine the cancer pathways most relevant to humans.

Gerbils

The Mongolian gerbil has been shown to have particular relevant features that have proven useful in addressing the potential of *H. pylori* to induce gastric cancer and the role cofactors play in the carcinogenic process. Mongolian gerbils were first reported as a model for experimental *H. pylori* infection in 1991 (Yokota *et al.*, 1991). Japanese investigators noted premalignant lesions consisting of intestinal metaplasia, atrophy, and gastric ulcers in gerbils after experimental infection with *H. pylori* (Hirayama *et al.*, 1996, Honda *et al.*, 1998a). In one study, acute gastritis with erosions of the gastric mucosa occurred shortly after infection, whereas gastric ulcers, cystica profunda, and atrophy with intestinal metaplasia were

observed at 3–6 months after *H. pylori* infection (Honda *et al.*, 1998a). Following these findings, others in Japan have noted that gerbils infected with *H. pylori* from periods ranging from 15 to 18 months developed gastric adenocarcinoma (Honda *et al.*, 1998a, Watanabe *et al.*, 1998).

When infected with *H. pylori* for 62 weeks, 37% of the gerbils developed adenocarcinoma in the pyloric region, although vascular invasion and metastases were not observed (Watanabe *et al.*, 1998). In another report, *H. pylori* induced gastric adenocarcinoma at 15 months post inoculation, but again the authors also did not record metastases or vascular invasion (Honda *et al.*, 1998b). Importantly, the histological progression in the gerbil closely resembled that observed in humans, in terms of the early appearance of intestinal metaplasia, well-differentiated histological patterns of the gastric malignancy, and location of the cancer in the antrum. Similar to findings in humans, the gerbils also had gastric ulcer disease in conjunction with gastric cancer (Hansson *et al.*, 1996). The development of metaplasia with production of predominantly acid sialomucins was associated with tumor development. Whether the cancers arose directly from these metaplastic cells is unknown, but the tumors clearly originated deep in the gastric glands, in close proximity to these metaplastic cells (Watanabe *et al.*, 1998).

Although most of the gastric tumors in this *H. pylori* gerbil model originated in the pyloric region of the stomach, significant changes in the oxyntic mucosa consistent with chronic atrophic gastritis were seen (Watanabe *et al.*, 1998). Glandular tissue in the gastric body and fundus were atrophied and replaced by hyperplastic epithelium of the pseudopyloric type. The differences between this lesion and gastric atrophy in humans is that the gerbil corpus is not "thinner" consequent to the pseudopyloric hyperplasia (Wang *et al.*, 1998, Watanabe *et al.*, 1998). The diagnosis of atrophy in *H. pylori*-infected gerbils (as well as in mice) is not involved with the thickness of the mucosa but rather with the loss of oxyntic (parietal and chief) cell populations within the gastric glands. Accumulating data supports that the parietal cell may regulate key differentiation decisions within the gastric glands, and ablation of parietal cells using transgenic technology (Li *et al.*, 1996) or their loss to infection with *H. felis* and *H. pylori* infection leads to altered glandular differentiation and neck cell proliferation; this corresponds to changes in gastric acid and gastrin physiology. The expansion of an aberrant neck cell ("regenerative hyperplasia" or "pseudopyloric hyperplasia") in the gerbils is similar to that observed in the *H. felis* mouse model. This lineage has been shown to be spasmyolytic polypeptide (SP) -positive (Wang *et al.*, 1998), and this SP-positive lineage also develops in *H. pylori*-associated gastric cancers in humans (Schmidt *et al.*, 1998). *H. pylori* induced loss of oxyntic glandular tissue suggests that the gerbil becomes achlorhydric before the development of gastric cancer. Although the precise effects of chronic *H. pylori* infection on gastric acid secretion are not fully elucidated, serum gastrin levels, considered a risk factor in gastric cancer in the *H. pylori* infected gerbil, are increased (Rieder *et al.*, 2005, Franco *et al.*, 2005). Importantly, gerbils infected with *H. pylori* SS1 develop gastritis and dysplasia; however, these experiments published to date do not result in gastric cancer in the absence of carcinogens such as MNU (Martin *et al.*, 2010, Suzuki *et al.*, 2002, Court *et al.*, 2002).

Unlike mice, gerbils colonize efficiently with *cag*⁺ *H. pylori* strains and the importance in pathogenic potential of *cagA* in *H. pylori* strains has been shown in gerbils. Mongolian gerbils were infected for 32 weeks either with *H. pylori cag*⁺ strain B128 or with the isogenic mutant strain B128 cytoxin-associated gene (*cagY*) or B128 *cagA*, defective in T4SS or in the production of its effector protein CagA, respectively (Rieder *et al.*, 2005). *H. pylori* B128-infected gerbils harbored high numbers of bacteria in the gastric antrum and corpus, whereas B128 *cagY* and B128 *cagA* colonized the antrum more densely than the corpus. All infected animals had robust antral inflammation and epithelial cell proliferation. B128-infected, but not mutant-infected, gerbils had severe transmural inflammation with large lymph aggregates, increased proliferation, gastric atrophy, and mucous gland metaplasia in the corpus. Plasma gastrin levels and gastric pH values were significantly increased only in *H. pylori* WT B128-infected gerbils. In all infected gerbils, proinflammatory cytokines interleukin 1 β , interferon γ , and growth-regulated protein were increased in the antrum, but only in wild type-infected animals was an increase seen in the corpus mucosa (Rieder *et al.*, 2005).

A recent study from Japan provided compelling evidence that the pattern of *H. pylori* colonization depends on the acid output and that this influences the long-term progression to neoplasia (Hagiwara *et al.*, 2011). They examined the potential effect of proton pump inhibitor (PPI) therapy on progression of *H. pylori*-associated disease. The male gerbils were divided into four groups: *H. pylori* (ATCC43504) positives and negatives, with and without administration of a PPI (omeprazole 100 mg/kg body weight/day). At the end point of the 6 month experiment, the results provided support for previously published literature in humans. *H. pylori*-negative male gerbils did not develop gastritis, metaplasia, or cancer irrespective of PPI treatment; whereas all *H. pylori*-positive gerbils developed gastritis and most also developed metaplasia during the later stages of the disease. Treatment with omeprazole in *H. pylori*-positive animals accelerated progression to atrophy and enhanced hypergastrinemia, resulting in a significantly increased incidence of gastric cancer. The authors emphasized that hypergastrinemia might be promoting the development of *H. pylori* gastric cancer (Hagiwara *et al.*, 2011). The findings support previous findings showing the increased risk factor of gastric atrophy in *H. pylori*-infected patients on PPI therapy (Kuipers, 2006, Kuipers *et al.*, 1996, Kuipers *et al.*, 1995, Lundell *et al.*, 2006). In humans, it remains to be shown whether chronic PPI therapy in *H. pylori*-infected individuals also increases the further progression to metaplasia, dysplasia and neoplasia in the proximal stomach, and whether there are protective effects of hypogastrinemia on the gastric antrum (Tomita *et al.*, 2011) are also observed with PPI therapy. These studies will require longer-term follow-up of this subset of patients (Fox and Kuipers, 2011).

The unusual susceptibility of this animal species to gastric adenocarcinoma using standard *cag*⁺ *H. pylori* strains underscores the overriding importance of host factors in determining the outcome from gastric *Helicobacter* spp. infection. It is important to note that many laboratories- particularly those outside of Japan- were unable to document the carcinogenic potential of *H. pylori* in the gerbil model. However, investigators in the U.S. using *H. pylori* strain B128, a *Cag*⁺ strain (7.13) serially passaged in gerbils have now documented rapid

onset gastric adenocarcinoma in gerbils. This study serves to highlight the importance of bacterial factors as well as host susceptibility in cancer induction (Franco *et al.*, 2005).

Dietary Factors and Gastric Cancer

Fat

Epidemiological evidence supports the premise that both *H. pylori* and obesity are independent risk factors for gastric cancer (Ahmed and Sechi, 2005, Chung and Leibel, 2006). Obesity induced inflammation underlies increasingly common diseases, such as type II diabetes and atherosclerosis (Kanneganti and Dixit, 2012, James *et al.*, 2012, Ferrante, 2007, Zuniga *et al.*, 2010). Importantly, several studies employing mouse models and focusing on the direct effect of adipose-derived factors on established tumors, have shown that obesity promotes cancers (e.g. colorectal and pancreatic cancer) and implicate systemic inflammation as an important component of the tumorigenic process (Moon *et al.*, 2013, Zyromski *et al.*, 2009). *Helicobacter*-dependent gastric cancer in mice and humans results from a sustained gastric immune response involving both lymphoid and myeloid derived cells. The population of CD11b/Gr1, immature myeloid cells (IMC), increase in the gastric tissue and circulation during the early stages of gastric helicobacter infection and have been implicated in cancer initiation (Asfaha *et al.*, 2013). IMCs are considered a heterogeneous cell population; myeloid-derived suppressor cells (MDSCs), a component of IMCs, suppress cytotoxic CD8 T cells and effectively prevent their antitumor activity and promote the survival of transformed epithelial cells (Pylayeva-Gupta *et al.*, 2012, Ostrand-Rosenberg and Sinha, 2009, Wang *et al.*, 2013). *Helicobacter*-associated gastric cancer is also correlated with an increased T helper (Th1) and TH-17 immune response (Shi *et al.*, 2010, Fox *et al.*, 2000). Despite its exact role, an elevated Th17 has been positively associated with increased severity of gastritis, whereas its diminution is linked to decreased gastric dysplasia (Wang *et al.*, 2009a). Cytokines and growth factors such as IL6 and granulocyte-macrophage colony-stimulating factors (GM-CSF) also play a role in recruitment and activation of MDSCs (Pylayeva-Gupta *et al.*, 2012, Ostrand-Rosenberg and Sinha, 2009).

A recently completed study utilized *H. felis* infected C57/BL male mice maintained on a high fat diet (HFD), 45% of dietary calories from fat, to study the effect of obesity on immune modulation and the establishment of a protumorigenic gastric microenvironment (Eriksen *et al.*, 2013). Mice on the HFD develop adipose inflammation, hyperglycemia, and insulin resistance (Zuniga *et al.*, 2010, Collins *et al.*, 2004). The authors reported that *H. felis* infected mice in the HFD developed accelerated gastric cancer compared to infected mice fed a lean diet (13% fat). Obese mice had increased bone marrow-derived IMCs in both gastric tissue and blood, as well as increased numbers of CD4 T cells, IL17A, GM-CSF, and pro-survival gene expression in gastric tissue. Phosphorylated STAT3 was increased and likely played a role in promoting cellular transformation as well as regulating inflammatory gene expression. Analysis of adipose tissue in infected obese mice had increased accumulations of macrophages, increased IL6, C-C motif ligand 7 and leptin. Obesity and helicobacter-induced gastric inflammation in concert also increased serum proinflammatory cytokines, including IL6, a cytokine known to promote tumor progression. The authors concluded that obesity accelerated *Helicobacter*-associated gastric cancer via

cytokine mediated cross-talk between inflamed gastric and adipose tissue by augmenting immune responses at both tissue sites and contributing to a protumorigenic gastric microenvironment (Ericksen *et al.*, 2013) (Figure 3).

Folic Acid

Studies have supported the protective role of dietary folic acid against specified cancers. Results of these studies have varied and mechanisms responsible for its protective effective are not defined. Because a global reduction in DNA methylation is an early and consistent observation during carcinogenesis, it has been argued that reversal or protection against DNA hypomethylation provided by folic acid supplementation is a possible mechanism explaining folic acid chemopreventive effect (Kim, 2004, Song *et al.*, 2000b, Song *et al.*, 2000a). *H. pylori* infection and development of precancerous lesions has been associated with a loss of global DNA methylation (Cravo *et al.*, 1996). Lower blood leukocyte (DNA methylation) levels were also observed in gastric cancer patients compared to matched controls (Hou *et al.*, 2010). A recent study also found that high dietary folate increased survival rates in gastric cancer patients compared with low folic acid intake in advanced gastric cancer (Shitara *et al.*, 2010). Interestingly, folic acid supplementation also reduced the incidence of N-ethyl-N-nitroguanidine-induced gastric adenocarcinoma in beagles (Xiao *et al.*, 2002). Though not described in this report, commercially available beagles have a high incidence of gastric helicobacter infection (Lee *et al.*, 1992). Whether Helicobacter promoted ENNG carcinogenesis in the dog is unresolved but should be considered.

In a study conducted in *H. felis* or control INS/GAS male mice animals were fed a rodent diet containing 2 (control) or 8 (supplemented) mg FA/kg of diet (Research Diets, Newark, NJ). The diets were administered either at weaning or later at 8 and 16 weeks post infection (Gonda *et al.*, 2012). All mice were sacrificed at 28 weeks post infection. The authors measured global DNA methylation in gastric tissue by H-dCTP incorporation assay and expressed data as a percentage of methyl-C content compared with uninfected non-transgenic FVB WT mice. For the first time, the authors found reduced global DNA methylation in the stomach of male *H. felis* infected INS/GAS mice compared to WT uninfected mice; a significant effect was noted at 22 weeks post infection, and because more severe at 28 weeks post infection. There was a significant 18% reduction in folate content in infected INS/GAS mice, reflecting a deficiency of methyl donors in inflamed, dysplastic tissues. Serum homocysteine levels, an inverse measurement of methyl donor status, were also significantly elevated in *H. felis* infected mice at 22 and 28 weeks post infection. Importantly, FA supplementation prevented the loss of global methylation in both dysplastic epithelial and gastric stromal myofibroblasts, measured by 3 independent assays, and markedly reduced gastric dysplasia and mucosal inflammation. FA supplementation also had an anti-inflammatory effect as indicated by gene expression profiling and IHC for lymphocyte markers in gastric tissue (Gonda *et al.*, 2012). However, other studies have demonstrated that treatment with demethylating agents also results in a reduction in preneoplastic lesions in *H. pylori*-infected gerbils (Niwa *et al.*, 2013).

Iron

Nutritionally, iron deficiency remains a common global disorder affecting 500–600 million humans (Zimmermann and Hurrell, 2007, WHO/UNICEF/UNU, 2001), with children being particularly susceptible to iron deficiency anemia (IDA). A variety of clinical syndromes are recognized with IDA, including reduced growth, mental retardation, increased susceptibility to infectious microorganisms, and in extreme cases, mortality (Stoltzfus, 2001). *H. pylori* has been linked to IDA and interestingly, *H. pylori*-infected individuals are refractory to iron supplementation, but IDA can be reversed in these patients with *H. pylori* eradication (Barabino *et al.*, 1999, Kurekci *et al.*, 2005, Choe *et al.*, 2000, Choe *et al.*, 1999, Marignani *et al.*, 1997). Hypochlorhydria, a marker for atrophic gastritis noted in chronic *H. pylori* infections, and sometimes observed with acute *H. pylori* infections, is considered a risk factor for IDA because under non-acidic conditions in the stomach, there is an inability to reduce ferric iron to absorbable ferrous (Fe^{2+}) iron (Windle *et al.*, 2007, Charlton and Bothwell, 1983, Annibale *et al.*, 2003). Also under iron restricted conditions, the virulence factors CagA and VacA in *H. pylori* are increased; iron release due to mucosal injury affords better access of *H. pylori* to the host's iron stores, and allows the organism to bind and utilize iron from hemoglobin, lactoferrin, and transferrin (Senkovich *et al.*, 2010). In a recent report, investigators determined operable mechanisms of iron deficiency in *H. felis* infected INS/GAS male mice over a 9 month period (Thomson *et al.*, 2012). They noted that the infected mice, apparently on a standard mouse diet (although not stated) had parietal cell loss and hypochlorhydria accompanied by decreased serum iron and transferrin saturation, as well as hyperferritinemia, and increased total iron binding capacity (Thomson *et al.*, 2012). The *H. felis* infected INS/GAS mice at 9 months post infection also had decreased gastric expression of iron metabolism regulators hepcidin (expressed in parietal cells and capable of regulatory acid secretion- (Schwarz *et al.*, 2012)), *Bmp4*, and *Bmp6*, but increased expression of the iron efflux protein, ferroportin, iron absorption genes and *Lcn2*, a siderophore-binding protein. The mice had low serum iron concentrations at 6 and 9 months post infection, and at 9 months infected mice also had lowered transferrin, reflecting systemic iron deficiency. Iron storage capacity, measured by serum ferritin, was initially increased at 3 months in infected mice. The authors hypothesized that the *H. felis* infected mice were attempting to sequester iron, making it unavailable for *H. felis* colonizing the stomach. However, by 6 and 9 months, *H. felis* infected mice had significantly reduced serum ferritin, presumably due to overall paucity of the systemic iron (and thereby negating the need for iron storage) (Thomson *et al.*, 2012). Others have observed similar patterns of initial elevation in serum ferritin at 10 weeks post *H. felis* infection followed by a reduction at 30 weeks post infection on WT mice maintained on an iron restricted diet (Keenan *et al.*, 2004). The authors further speculated that the marked changes in gastric iron transporters may also play a role in the rapid development of gastric cancer noted in gastric *Helicobacter* spp. infection in male, INS/GAS hypergastrinemic mice, known to be more susceptible to gastric cancer compared to their female counterparts (Fox *et al.*, 2003a, Fox *et al.*, 2003b). The data generated with the *H. felis* model are Cag-independent given *H. felis* lacks a *cag* PAI. It will be interesting to ascertain if *H. pylori* SS1 or PMSS1 infected male INS/GAS mice will also have iron deficiency.

Importantly, iron deficiency has also been associated with stomach cancer along with other gastrointestinal tumors (Pra *et al.*, 2009, Akiba *et al.*, 1991, Harrison *et al.*, 1997). *H. pylori*-associated gastric cancer has been recently used to explore the role of dietary iron as a cofactor in *cag*⁺ *H. pylori* (strain 7.13) associated gastric cancer in gerbils (Noto *et al.*, 2013). The authors clearly demonstrated that iron depletion accelerated *H. pylori*-induced premalignant and malignant gastric lesions in a *cagA* dependent manner (Noto *et al.*, 2013) (Figure 4) (Table 2). Importantly, irrespective of dietary iron status, infection with a *cagA* isogenic *H. pylori* mutant significantly attenuated inflammation, dysplasia, and gastric adenocarcinoma, highlighting the importance of *cagA* in gastric cancer development. Furthermore, in gerbils maintained on an iron deficient diet, isogenic *cagA* mutants of *H. pylori* have impaired ability to colonize, suggesting that *cagA* plays a role in *H. pylori* iron acquisition (Tan *et al.*, 2011). To determine whether the severity of gastric lesions under conditions of iron depletion were specific for *H. pylori*, gerbils either on iron replete or iron depleted diets were exposed to ethanol-induced inflammation. Proteomic analysis of *in vivo* adapted *H. pylori* harvested from gerbils on replete or deplete iron diets indicated that there was increased abundance of proteins that mediated bacterial-host interactions (*Helicobacter sp.* Q enolase) and *cag* T4SS function (CagA) in strains from the iron depleted group (Noto *et al.*, 2013).

Further, using scanning electron microscopy, the authors indicated that the *H. pylori* isolated from the iron-depleted gerbils and grown on human gastric epithelial cells had significantly more T4SS pili on their surface, compared to *H. pylori* isolated from gerbils on iron-replete diets. This data suggested that the increased assembly of the T4SS pili was attributable to *H. pylori* adaptation under iron depletion (Noto *et al.*, 2013). This observation was tested using *in vitro* iron growth conditions with similar findings; the *H. pylori* grown in iron deficient media had significantly more T4SS pili than the *H. pylori* grown in iron-replete media (Noto *et al.*, 2013).

To compare data collected in the gerbil model, the authors compared *H. pylori* isolated from Colombian patients with the lowest serum ferritin levels to strains isolated with the highest ferritin levels. These *H. pylori* isolates from subjects with low serum ferritin levels induced significantly ($p < 0.05$) higher levels of IL8 compared with *H. pylori* strains isolated from patients with the highest ferritin levels (individuals with gastritis only, or with intestinal metaplasia). The authors argued that the ability of *H. pylori* strains from a low ferritin serum level patient to induce more severe pathology was more likely to have accelerated the diseases early in the carcinogenic process. This hypothesis needs further longitudinal evaluations of *H. pylori*-infected populations on low dietary iron intake (Noto *et al.*, 2013).

Salt

Historically, the role of salt in gastric cancer has received considerable attention in the literature (Geboers *et al.*, 1985, Tuyns, 1988). The deleterious effect of salt is based on epidemiological studies, biochemical analyses and *in vivo* experimentation (Joossens and Geboers, 1981, Takahashi and Hasegawa, 1986). In a case control Belgium study, consisting of 1087 cases (587 males, 500 females) of gastrointestinal cancer and 2914 controls, the individuals were asked to respond to their taste for salt (Tuyns, 1988). The question asked

was “At the time you eat your food, do you add salt?”. The possible responses were ‘never’, ‘sometimes’, or ‘always’. The relative risks of developing gastric cancer were adjusted for age, sex, and province where the patient lived. The relative risk for development of gastric cancer for individuals with high salt intake was 1.78 (confidence limits 1.19–2.75), which was statistically significant; however the noted increase may be related to another causal factor, e.g. *H. pylori* or other unknown factors of greater importance. The relative risk of 1.78 for gastric cancer in Belgian patients with high salt intake corresponds almost exactly to that noted by Correa studying black Americans in Louisiana (Correa, 1985). A similar increased relative risk for gastric cancer with adding salt to their diet was noted in an Italian multicentre case control study (Buiatti *et al.*, 1990). Other studies have incriminated increased risk of gastric cancer due to ingestion of preserved, often salty food (Tajima and Tominaga, 1985), preference for salty foods (Tuyns, 1988, You *et al.*, 1988), or actual measurement of salt consumption (Correa *et al.*, 1985, La Vecchia *et al.*, 1987, Tsugane *et al.*, 1991). In contrast, salt was not incriminated in a 1992 study (Friedman and Parsonnet, 1992). In this smaller case-control study on 200 individuals residing in the U.S., who subsequently developed gastric adenocarcinoma, and 200 age matched control participants were asked ‘Do you usually salt food before tasting it?’. Analysis of data indicated that there was not statistical evidence that routine salting of food lead to increased risk of gastric cancer (Friedman and Parsonnet, 1992). A joint WHO/FAO report, given the association of dietary salt and increased risk of gastric cancer rarely raised the odds ratio above two, concluded that high salt ingestion “probably increases the risk of stomach cancer” (WHO, 2003). Nevertheless, several more recent reports have analyzed the combinatory effects of high dietary salt and *H. pylori* infections in humans and have consistently shown increased rates of gastric cancer in these populations (Lee *et al.*, 2003, Tsugane and Sasazuki, 2007, Wang *et al.*, 2009b, D’Elia *et al.*, 2012, Brenner *et al.*, 2009, Krejs, 2010).

Given the late onset of gastric cancer in human populations, it is probable that both tumor initiators as well as tumor promoters play an important role in gastric carcinogenesis. In populations with a high risk for gastric cancer, dietary intake of fruits and vegetables is low, and the diet often consists of high starch and low protein. Both low protein and high starch may favor acid-catalyzed nitrosation in the stomach and possible mutagenesis of gastric epithelia (Berretta *et al.*, 2012, Tsugane and Sasazuki, 2007). Because of the purported role of high nitrate diets and subsequent nitrosamine formation in the stomach, numerous studies have been conducted in rodents administering various nitrosamines known to be potent carcinogens. In experiments with rats dosed with MNNG, NaCl was shown to have a promotional effect of induction of gastric adenocarcinoma when salt is given intragastrically weekly or in the diet during 12–20 weeks exposure to MNNG in drinking water (Newberne *et al.*, 1987, Takahashi *et al.*, 1983, Tatematsu *et al.*, 1975). It is also known that salt causes damage to rat gastric surface epithelium followed by proliferation of gastric epithelium (Charnley and Tannenbaum, 1985, Furihata *et al.*, 1984, Ohgaki *et al.*, 1989, Sorbye *et al.*, 1988). In one study, 80% of rats given salt in the diet and MNNG in drinking water developed adenocarcinomas in the antrum, but not in the corpus (Takahashi *et al.*, 1983). Also, ACI rats given a single initiation dose of MNNG [0.25 mL/10 g body weight MNNG in water (5 g/L)] by oral gavage and fed a 10% NaCl diet had significantly increased

tumours in both forestomach and glandular stomach after being on the salt diet for 1 year (Watanabe *et al.*, 1992).

Mice have also been used on a limited basis in studies addressing gastric damage due to salt intake. Mice (strain undesignated) fed a highly salted diet (salted codfish and yakuri sold for food consumption in Japan) developed acute gastric mucosal damage (Sato *et al.*, 1959). Swiss/ICR mice fed salted (10% w/w NaCl) rice diets for 3–12 months were noted to have developed hypertrophy of the forestomach and atrophy (considered a marker of premalignancy in humans) of the glandular stomach (Kodama *et al.*, 1984). Parietal cell loss and statistically increased epithelial proliferation were also noted in B6 129 mice fed a high salt diet (7.5%). A reduction in the parietal cells accounted for the atrophy observed in the fundus of the mice (Kodama *et al.*, 1984). Although parietal cell loss has also been noted in C57BL and INS/GAS mice infected with *H. felis* or *H. pylori*, (Fox *et al.*, 2002, Mohammadi *et al.*, 1996, Wang *et al.*, 2000, Fox *et al.*, 2003a) these studies infer where uninfected mice receive salt in their diet that chronic mucosal damage *per se* may account for parietal cell loss in the fundus of mice.

More recent studies have incorporated NaCl in the diets of rodents infected with gastric helicobacters (Rogers *et al.*, 2005, Fox *et al.*, 1999). C57BL/6 mice infected with *H. pylori* SS1 for 4 months and maintained on a high-salt diet exhibited higher gastric urease activity, serum gastrin, bacterial colonization, and foveolar proliferation levels than did those on a basal diet; however, no meaningful effect of salt on gastric inflammation or oxyntic atrophy scores was observed (Fox *et al.*, 1999). High salt likewise increased foveolar proliferation in uninfected but not *H. pylori* SS1 infected B6129 mice (Rogers *et al.*, 2005). Thus, induction of proliferation in the absence of dysplasia does not seem to increase the risk of carcinogenesis in this model. In agreement with this concept, *H. pylori* SS1 infection was shown to induce DNA damage in Big Blue mice (containing a chromogen-inducible lambda phage transgene for *in vitro* mutation quantitation), but no increase in mutation frequency was evident in infected animals fed a high-salt diet (Touati *et al.*, 2003). B6129 mice fed a high-salt diet showed a shift in anti-*H. pylori* humoral immunity from a Th1 to a Th2 phenotype. This humoral immunity shift is not specific to B6129 mice. Others observed a similar increase in Th2-associated IgG1 relative to Th1-associated IgG2a in *H. pylori*-infected INS-GAS mice on a FVB strain background (Fox *et al.*, 2003a, Rogers *et al.*, 2005). However, the salt-associated shift in humoral immunity to a Th2 pattern seemed to be *H. pylori* specific. The authors found no increase in total IgG1 or IgE consistent with hypersensitivity nor did they detect anti-parietal cell antibodies suggestive of autoimmune gastritis (Rogers *et al.*, 2005).

Gerbils infected with *H. pylori* develop an antral gastritis/duodenitis that mimics the ulcerogenic form of the human disease (Ohkusa *et al.*, 2003). Unlike mice, but similar to humans, the bacterial *cag* PAI seems to be a critical disease determinant in gerbils, particularly gastric cancer (Rieder *et al.*, 2005). The gerbil model has proven highly useful in addressing questions specifically related to *cagA* and its association with high dietary salt and other *H. pylori* virulence determinants *in vivo*. In earlier studies, authors have shown a synergistic promoting effect of *H. pylori* and high salt on N-methyl-N-nitrosurea (MNU)-initiated gastric cancer in the gerbil model (Nozaki *et al.*, 2002). However, chemical

initiation was required to induce cancer, as no tumors developed in gerbils untreated with MNU regardless of *H. pylori* infection status or diet. Gerbils fed a high salt diet (2.5%) for 13 months were noted to have gastric atrophy and foveolar hyperplasia. However, the high salt diet did not promote gastric cancer in gerbils infected with *H. pylori* SS1, which does not express *cagA* (Bergin *et al.*, 2003). However, in a recent study, investigators have demonstrated that a high dietary intake (8.75%) in a Mongolian gerbil model enhances *H. pylori*-induced carcinogenesis (Gaddy *et al.*, 2013). The high-salt diet used approximates the concentration of sodium chloride in dried fish, preserved in 3 to 20% salt, pickled foods containing up to 25% salt, and soy sauce which has 19% salt. The administration of high-salt diet to uninfected gerbils did not result in gastric cancer. The authors concluded that the effects of a high-salt diet on gastric cancer were dependent on the presence of *H. pylori* infection. Importantly, the high salt diet increased incidence of gastric carcinoma in gerbils infected with WT *cagA*⁺ *H. pylori* strain 7.13 but not in animals infected with a *cagA* mutant strain (Gaddy *et al.*, 2013). This observation is consistent with several parts of the world with high rates of gastric cancer, also, a high prevalence of *cagA*⁺ strains; a large percentage of these populations routinely have diets with a high salt concentration.

Others have reported that exposure to *H. pylori* to high-salt conditions *in vitro* leads to alterations in the expression of multiple *H. pylori* genes, included *cagA* and neutrophil activation protein (Liao *et al.*, 2013, Loh *et al.*, 2012). The authors also noted that *cagA* gene expression was elevated in the stomachs of WT-infected gerbils maintained on a high-salt diet compared to WT-infected animals maintained on a regular diet (Gaddy *et al.*, 2013). Interestingly, both IL-1 β transcription and iNOS transcription were significantly elevated in WT-infected gerbils maintained on a high-salt diet compared to the regular-diet fed to gerbils infected with WT-*H. pylori*. WT-infected animals maintained on a high-salt diet had markedly increased gastric pH compared to their regular diet WT-infected gerbil counterparts. This is consistent with the finding of atrophic gastritis and hypochlorhydria preceding the development of gastric cancer in humans. Parietal cells are reduced in the hypochlorhydric animals and likely accounts, at least in part, for the observed hypochlorhydria. They also hypothesized that hypochlorhydria could be due to perturbations in parietal cell function. To test this, the authors also measured hepcidin (produced in parietal cells), gastrin, and H,K-ATPase transcripts. The animals infected with WT *H. pylori* and maintained on a high-salt diet had decreased levels of all 3 transcripts compared to animals infected with WT *H. pylori* and maintained on a regular diet. They concluded that increased dietary salt consumption markedly alters the outcome of infected with *cagA*⁺ *H. pylori* strain 7.13 and proposed that high salt concentrations stimulate increased expression of *cagA*, and the actions of CagA lead to inflammation, hypochlorhydria, and enhanced carcinogenesis. They also raised the possibility that high salt concentrations lead to altered expression of multiple *H. pylori* genes (including *cagA*) as well as to the alterations in the host and that this constellation of alterations could enhance progression of carcinogenesis. Regardless of the mechanisms, their data argues that reduction in dietary salt could lower risk of gastric cancer in *H. pylori*-infected patients (Gaddy *et al.*, 2013).

Vitamin C

Antioxidants, particularly vitamin C and b-carotene, have anticarcinogenic properties based on laboratory data as well as epidemiological evidence. Ascorbic acid scavenges nitrous oxide and makes the compound unavailable for nitrosation in the stomach. Furthermore, evidence indicates that many, but not all, patients with *H. pylori* gastritis have lowered ascorbic acid concentrations (Sobala *et al.*, 1991, Sobala *et al.*, 1993). Other investigators have shown that patients with precancerous lesions consisting of intestinal metaplasia and multifocal atrophic gastritis also have lowered ascorbate and elevated N-nitroso compound concentrations (Chen *et al.*, 1990, Bartsch *et al.*, 1988). Supplementation of vitamin C has been associated with reduced gastric cancer risk in some human studies (Su *et al.*, 2000, Correa *et al.*, 1998). Despite initial promising results in a prospective trial in a very high-risk gastric cancer population supplemented with vitamin C at 2 g per day for 6 years (Correa *et al.*, 2000), a follow-up study on this population indicated that vitamin C supplementation over a 12-year period did not provide any lasting protection against gastric cancer (Mera *et al.*, 2005).

To test whether *H. pylori* does indeed impair ascorbic acid secretion into the stomach, animals which need dietary supplementation of vitamin C should ideally be used to mimic vitamin C metabolism in humans. Of the *Helicobacter* sp. infected animal models presently available, non-human primates fulfill this criterion, but to date has not been used in answering this important question. The guinea pig is the only other laboratory animal that has dietary requirements for vitamin C. Published data indicated that *H. pylori* induced gastritis in this model (Shomer *et al.*, 1998). Supplementation with vitamin C in the diet at 10-fold higher levels than a maintenance dose (3,102 mg/kg) for 6 weeks in guinea pigs, which lack L-gulono- γ -lactone oxidase similar to humans and *gulo*^{-/-} mice, did not impact *H. pylori* colonization levels nor severity of gastritis (Taylor, 2007).

In a later study using vitamin C-deficient B6.129P2- *Gulo*^{tm1Umc/mmc} (*gulo*^{-/-}) mice lacking L-gulono- γ -lactone oxidase, authors compared gastric lesions and Th1 immune responses in *H. pylori* SS1-infected *gulo*^{-/-} mice supplemented with low (33 mg/L) or high (3,300 mg/L) vitamin C in drinking water for 16 or 32 weeks (Lee *et al.*, 2008b). Vitamin C levels in plasma and gastric tissue correlated with the dietary vitamin C supplementation levels in *gulo*^{-/-} mice. *H. pylori* SS1 infection resulted in comparable gastritis and premalignant lesions in wildtype C57BL/6 and *gulo*^{-/-} mice supplemented with high vitamin C, but lesions were less severe in *gulo*^{-/-} mice supplemented with low vitamin C at 32 weeks post infection. The reduced gastric lesions in infected *gulo*^{-/-} mice supplemented with low vitamin C correlated with reduced Th1-associated IgG2c, gastric IFN- γ and TNF- α mRNA and higher *H. pylori* colonization levels. These results in the *H. pylori*-infected *gulo*^{-/-} mouse model suggest that although supplementation with a high level of vitamin C achieved physiologically normal vitamin C levels in plasma and gastric tissue, this dose of vitamin C did not protect *gulo*^{-/-} mice from *H. pylori*-induced premalignant gastric lesions. In addition, less severe gastric lesions in *H. pylori* infected *gulo*^{-/-} mice supplemented with low vitamin C correlated with an attenuated Th1 inflammatory response (Lee *et al.*, 2008b).

It is interesting to note that low vitamin C-supplemented *gulo*^{-/-} mice tended to have lower degrees of inflammation, oxyntic atrophy and dysplasia and significantly less severe epithelial defects, pseudopyloric metaplasia and foveolar hyperplasia relative to high vitamin C-supplemented *gulo*^{-/-} and WT mice (Lee *et al.*, 2008). Helicobacter-induced gastric disease in humans and mice is mediated by a Th1-predominant host immune response (Mohammadi *et al.*, 1997, Fox and Wang, 2007, D'Ellos *et al.*, 1997). *Gulo*^{-/-} mice supplemented with high vitamin C had a similar Th1-promoted inflammatory response to *H. pylori* as infected WT mice. Low vitamin C-supplemented, *H. pylori*-infected *gulo*^{-/-} mice had less severe gastric lesions associated with suppressed Th1 responses as evidenced by reduced levels of plasma IgG2c, reduced expression of gastric IFN- γ and TNF- α mRNA, and increased *H. pylori* colonization levels. These data suggest that low dietary vitamin C impairs the host's ability to sustain a proinflammatory response and conversely, that adequate vitamin C levels in the infected host may be important for maintaining a robust Th1 immune response to chronic *H. pylori* infection. Consistent with this report, others have reported attenuated Th1 immune responses in vitamin C-deprived *gulo*^{-/-} mice. This was manifested by less severe pneumonia and suppressed pulmonary expression of proinflammatory IL-1 β and TNF- α mRNA in vitamin C-deprived *gulo*^{-/-} mice during the first few days of acute viral influenza (Li *et al.*, 2006). In hosts with low or no vitamin C intake, attenuated Th1 responses to other types of infection, such as tuberculosis in humans or *Klebsiella pneumoniae* sepsis in *gulo*^{-/-} mice, may also be important in predicting survival (Gaut *et al.*, 2006, Hemila *et al.*, 1999).

Using a mouse model of intestinal parasitic infection that causes a Th2 immune response, the effect of modulating the Th1-associated response to *H. felis* infection has been analyzed (Fox *et al.*, 2000). In C57BL/6 mice coinfecting with intestinal helminths and *H. felis*, reduced systemic Th1 immune responses and lower levels of Th1-mediated gastric cytokines were associated with increased *H. felis* colonization levels (a marker of less severe gastric pathology) and less severe premalignant lesions (Fox *et al.*, 2000). In another series of mouse experiments, the authors demonstrated that lower bowel colonization with enteric helicobacters accentuated the severity of *H. pylori* gastritis. Colonization with these helicobacters led to a lower gastric Th1 immune response, apparently triggered by regulatory T cells sensitized by shared antigens between enteric *Helicobacter* spp. and *H. pylori* (Ge *et al.*, 2011, Lemke *et al.*, 2009). This may have direct relevance to the immune status of the African population where enteric helicobacters are commonly isolated from diarrheic children (Lastovica and le Roux, 2000). These results may in part explain the "African enigma" where helminth and enteric helicobacter infections are common and the incidence of gastric cancer is low, despite a high prevalence of *H. pylori* infection (Holcombe, 1992). The *H. pylori*-infected, low vitamin C-supplemented *gulo*^{-/-} mouse model may offer another possible explanation to the African enigma. In Gambia, due to the impact of drought on the food supply, mean daily intake of vitamin C approaches zero for 7 months of the year, and is accompanied by low plasma vitamin C levels (Bates *et al.*, 1994). It is tempting to speculate based on studies of the *H. pylori* infected *gulo*^{-/-} mice on low vitamin C, that minimal vitamin C intake during *H. pylori* infection may be one of the factors contributing to a lower reported incidence of gastric cancer in some populations in Africa because of attenuated gastric immune responses resulting in less severe premalignant

lesions. Although this hypothesis is consistent with a report of diminished mitogen responses of peripheral blood mononuclear cells from pigs affected by heritable vitamin C deficiency (Schwager and Schulze, 1998), the biological significance of these responses requires further studies. The authors concluded that high vitamin C supplementation in this model, similar to previous epidemiological studies in humans (Mera *et al.*, 2005, Waring *et al.*, 1996, Phull *et al.*, 1999, Bjelakovic *et al.*, 2004), did not prevent progression of *H. pylori*-induced gastritis and development of premalignant gastric lesions. In contrast, low dietary vitamin C resulted in less severe gastric disease by downregulating gastric and systemic Th1 immune responses to *H. pylori* infection.

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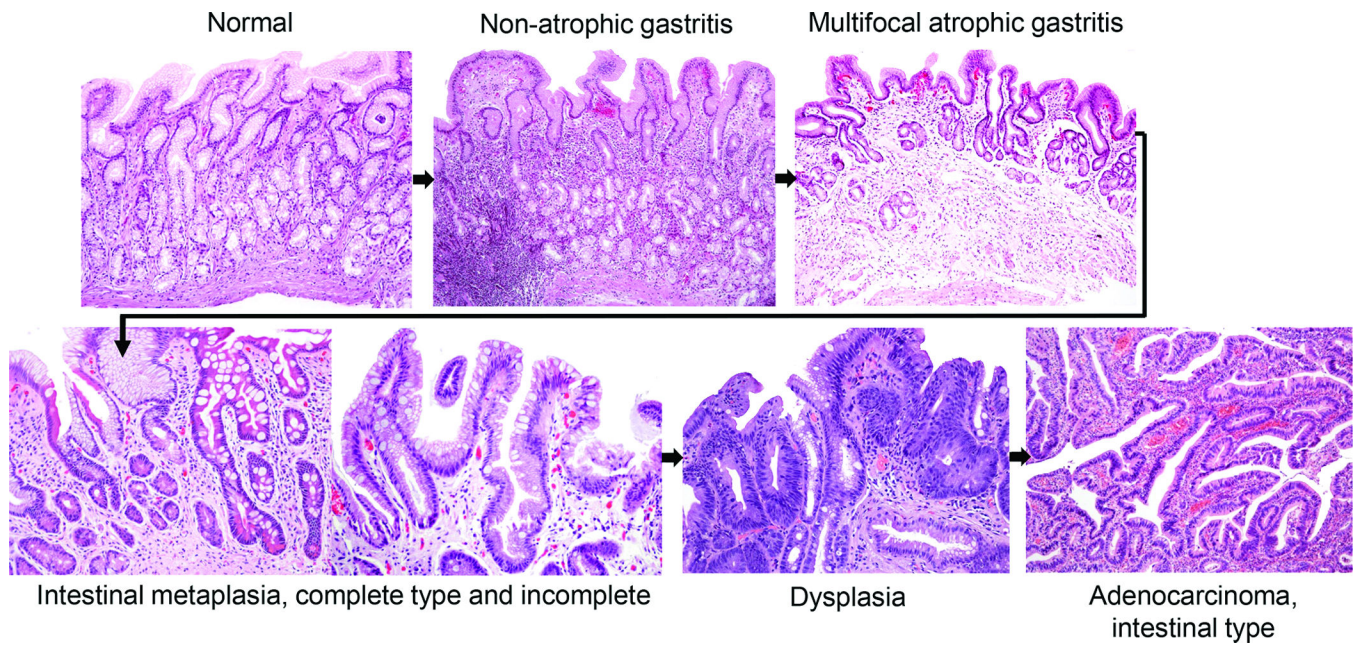


Figure 1.
Correa's Cascade of Gastric Carcinogenesis. Courtesy of Pelayo Correa

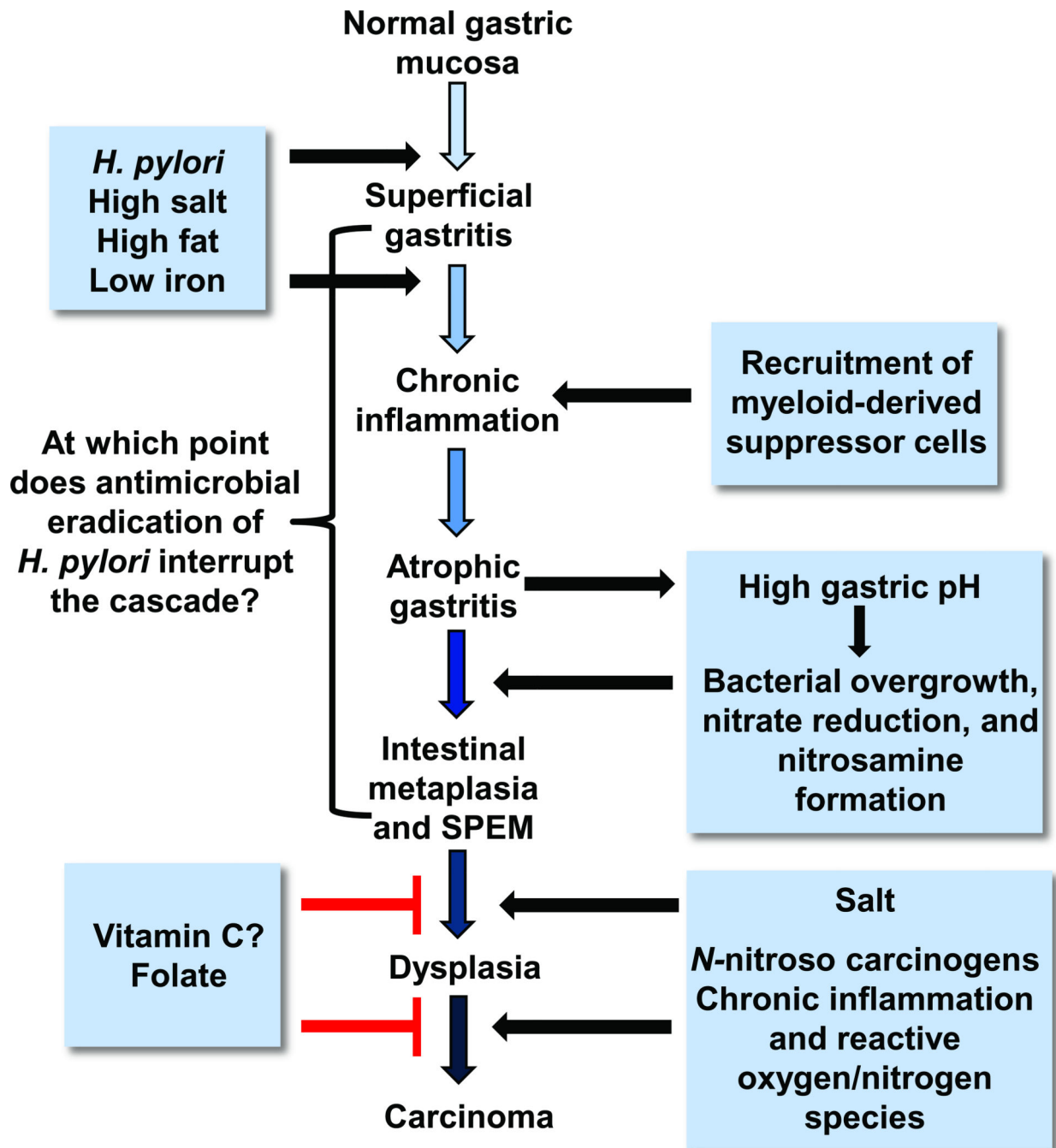


Figure 2. Proposed Correa pathway of pathological events and dietary influence on in gastric cancer progression. In well-differentiated, intestinal-type gastric cancer, histopathological studies indicated that chronic *H. pylori* infection progresses over decades through stages of chronic gastritis, atrophy, intestinal metaplasia, dysplasia, and cancer (Correa, 1991). The development of cancer has been attributed to alterations in DNA caused by chronic inflammation, recruitment and engraftment of bone marrow-derived cells, an imbalance between epithelial cell proliferation and apoptosis, and, in a milieu of atrophy and

achlorhydria, gastric colonization by enteric bacteria with nitrate reductase activity, which facilitates the formation of carcinogenic nitrosamines. Corpus-predominant atrophy, or the loss of specialized glandular cell types such as parietal and chief cells, appears to be the critical initiating step in the progression toward cancer. (Modified from (Fox and Wang, 2007))

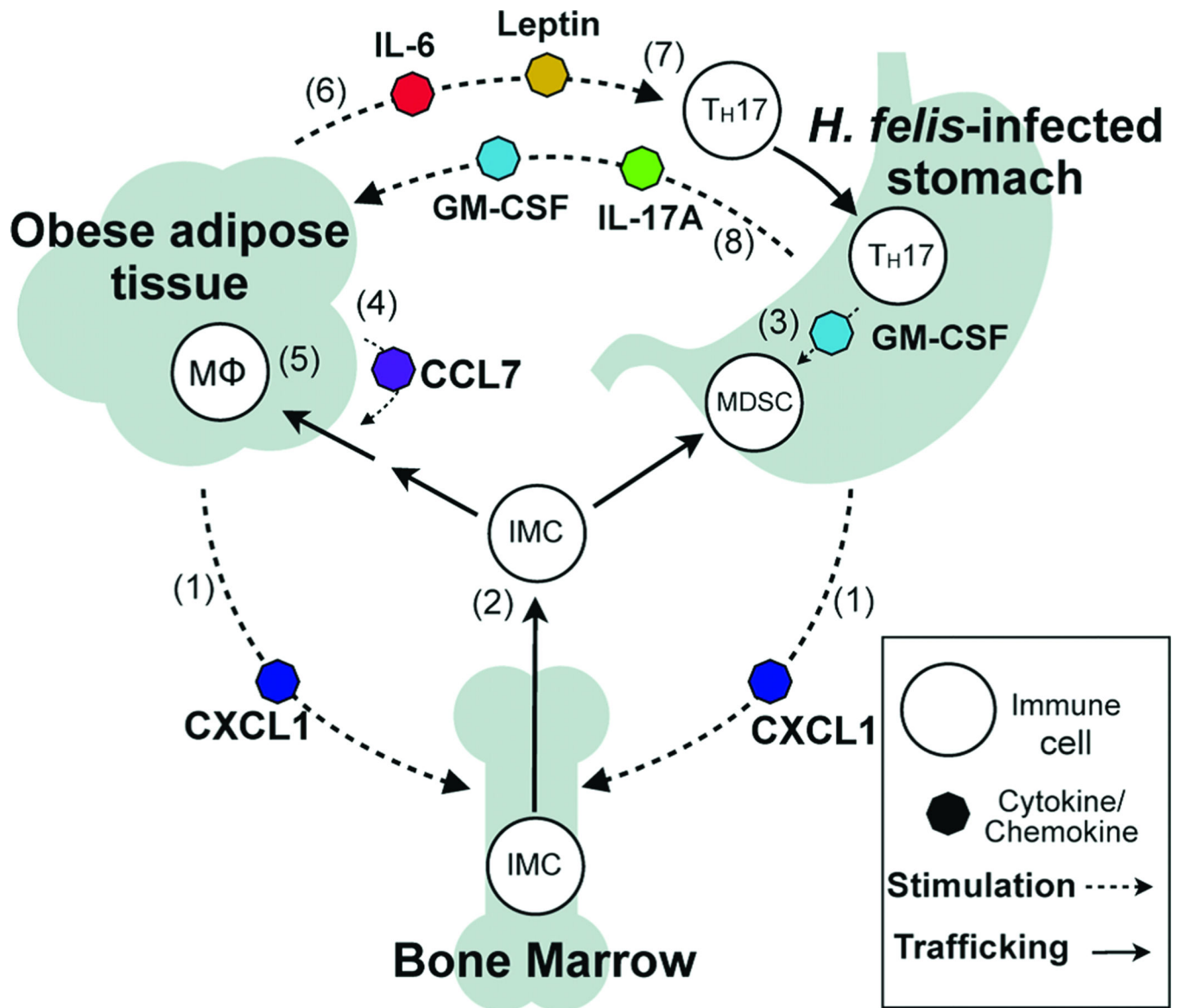


Figure 3.

Schematic hypothesis of inflammatory cross-talk during *Helicobacter felis* infection and diet-induced obesity. CXCL1 produced by adipose and gastric tissues (1) elevates circulating bone marrow-derived immature myeloid cells (IMCs) (2). IMCs recruited to gastric tissue by CXCL1 encounter TH17-associated cytokines, including GM-CSF, and develop into myeloid-derived suppressor cells (3). Adipose CCL7 secretion increases IMC recruitment to adipose (4), resulting in accumulation of adipose tissue macrophages (MΦ) (5). Inflamed adipose produces IL-6 and leptin (6), promoting TH17 differentiation (7). TH17-associated cytokines IL-17A and GM-CSF produced in the stomach signal to adipose (8) to increase CCL7 production and amplify adipose inflammation. CCL, C-C motif ligand; CXCL, Chemokine C-X-C motif ligand; GM-CSF, granulocyte macrophage-colony stimulating factor. (Reprinted with permission (Ericksen et al., 2013))

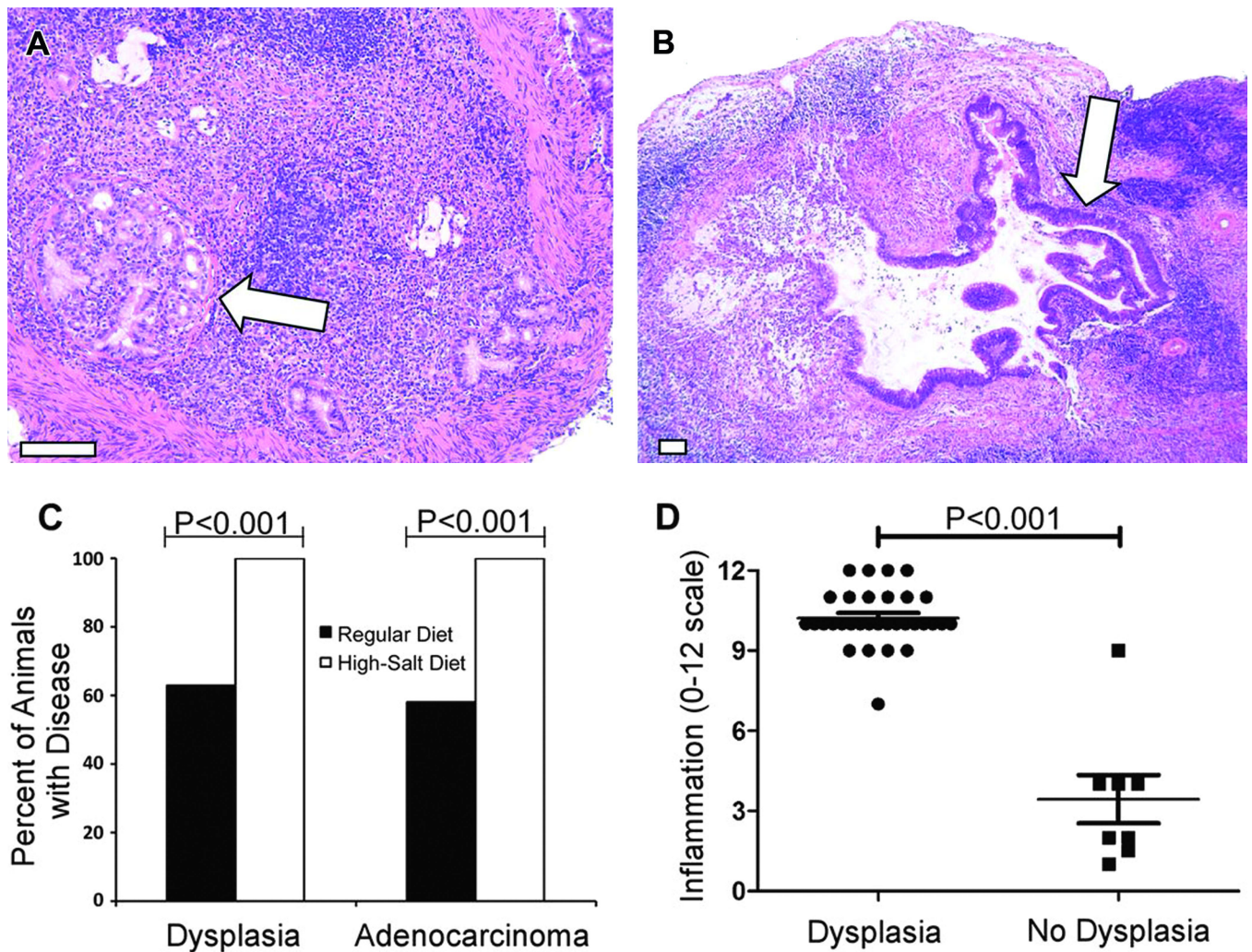


Figure 4.

Analysis of gastric dysplasia and adenocarcinoma. Gerbils infected with WT *H. pylori* maintained on either a regular diet or a high salt diet. A–B) Representative micrographs of gastric tissue derived from WT-infected animals maintained on a high salt diet (arrows indicate tumors). Magnification bars indicate 100 μ m. C) Gastric tissues were evaluated for dysplastic lesions and invasive adenocarcinoma. Only animals infected with the WT strain exhibited these abnormalities. Gastric cancer and dysplasia were detected significantly more frequently in the WT-infected high salt diet group than in WT-infected regular diet counterparts ($p < 0.0001$). D) WT-infected animals maintained on a regular diet and WT infected animals maintained on a high salt diet were analyzed. The total gastric inflammation score (scale of 0–12) was higher in WT-infected animals with gastric dysplasia than in WT-infected animals without dysplastic lesions. Horizontal bars indicate mean inflammation score + SEM for each group. (Modified with permission (Gaddy et al., 2013))

Table 1The *Helicobacter* infected INS/GAS mouse model of Gastric Cancer

Purpose of analysis	Results	Reference
<i>H. pylori</i> and <i>H. felis</i> and gastric mucosa	progression to gastric atrophy, metaplasia, dysplasia, and cancer	(Wang <i>et al.</i> , 2000, Fox <i>et al.</i> , 2003b, Fox <i>et al.</i> , 2003a)
Gender differences	increased gastric carcinogenesis in males	(Fox <i>et al.</i> , 2003a, Fox <i>et al.</i> , 2003b)
Importance of CagE	loss of cagE temporally retards but does not abrogate cancer progression	(Fox <i>et al.</i> , 2003b)
Interaction with G-Gly	G-gly synergizes with amidated gastrin to stimulate acid secretion and inhibits atrophy	(Cui <i>et al.</i> , 2004)
CCK2R and Histamine receptor inhibitors	CCK2R and H2R antagonists synergistically inhibit gastric atrophy and cancer	(Takaishi <i>et al.</i> , 2005)
Apoptosis of gastric epithelium	gastrin induces apoptosis and contribute to gastric carcinogenesis	(Cui <i>et al.</i> , 2006, Przemeczek <i>et al.</i> , 2008)
TFF2 expression	TFF2 expression in the gastric fundus was elevated	(Tu <i>et al.</i> , 2007)
Swedish variant of moist oral smokeless tobacco	tobacco promotes cancer formation	(Stenstrom <i>et al.</i> , 2007)
Reg-1 expression	Reg1 is increased in the stomachs of <i>H. felis</i> -infected INS-GAS mice	(Steele <i>et al.</i> , 2007)
Role of 17-beta-estradiol	17beta-estradiol has protective effects on gastric cancer development	(Ohtani <i>et al.</i> , 2007, Ohtani <i>et al.</i> , 2011, Sheh <i>et al.</i> , 2011)
Eradication of gastric <i>Helicobacter</i> spp.	eradication inhibits mouse gastric carcinogenesis	(Cai <i>et al.</i> , 2005, Lee <i>et al.</i> , 2008a, Lee <i>et al.</i> , 2008b)
Commensal bacterial flora in the stomach	SPF mice are more susceptible to gastric cancer than germ-free mice	(Lofgren <i>et al.</i> , 2011)
ASF restricted flora	restricted Altered Schaedler flora colonizes gastric niche and enhances gastric	(Lertpiriyapong <i>et al.</i> , 2013)
High salt diet	high salt did not promote carcinogenesis	(Fox <i>et al.</i> , 2003a)
Antral carcinogenesis	gastrin suppresses antral carcinogenesis	(Tomita <i>et al.</i> , 2011, Takaishi <i>et al.</i> , 2009)
<i>H. felis</i> -induced iron deficiency	marked changes in expression of gastric iron transporters and lowered systemic iron	(Thomson <i>et al.</i> , 2012)
Folic acid supplementation	prevents gastric cancer	(Gonda <i>et al.</i> , 2012)

Table 2

Iron depletion in gerbils increases the frequency and severity of gastric inflammation, dysplasia, and adenocarcinoma

	2 weeks (n = 48)		6 weeks (n = 58)		12 weeks (n = 39)	
	Iron-replete (n = 24)	Iron-depleted (n = 24)	Iron-replete (n = 29)	Iron-depleted (n = 29)	Iron-replete (n = 20)	Iron-depleted (n = 19)
Gastritis	3/24 (13%)	8/24 (33%)	16/29 (55%)	23/29 (79%)	18/20 (90%)	18/19 (95%)
Dysplasia	2/24 (8%)	5/24 (21%)	9/29 (31%)	18/29 (62%) [^]	9/20 (45%)	14/19 (74%)
Adenocarcinoma	1/24 (4%)	2/24 (8%)	3/29 (10%)	13/29 (45%) [^]	7/20 (35%)	11/19 (58%)

[^] Statistical significance ($P < 0.05$) was determined by χ^2 tests comparing iron-depleted gerbils to iron-replete gerbils

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