Helicobacter pylori induced gastric pathology: insights from *in-vivo* and *ex-vivo* models

Authors: Michael D Burkitt[1], Carrie A Duckworth[1], Jonathan M Williams[2], D Mark Pritchard[1]

Affiliations:

1: Gastroenterology Research Unit, Department of Cellular and Molecular Physiology, Institute of Translational Medicine, University of Liverpool, Liverpool, UK

2: Pathology and Pathogen Biology, Royal Veterinary College, North Mymms, Hertfordshire, UK

Corresponding Author:

Professor D. Mark Pritchard
Gastroenterology Research Unit
Department of Cellular and Molecular Physiology
Institute of Translational Medicine
University of Liverpool
1st Floor Nuffield Building
Ashton Street
Liverpool
L69 3GE

mark.pritchard@liverpool.ac.uk

Abstract

Gastric colonization with *Helicobacter pylori* induces diverse human pathological conditions, including superficial gastritis, peptic ulcer disease, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric adenocarcinoma and its precursors. The treatment of these conditions often relies on the eradication of *H. pylori*, an intervention that is increasingly difficult to achieve and that does not prevent disease progression in some contexts. There is, therefore, a pressing need to develop new experimental models of *H. pylori*-associated gastric pathology to support novel drug development in this field. Here, we review the current status of *in-vivo* and *ex-vivo* models of gastric *H. pylori* colonization, and of *Helicobacter*-induced gastric pathology, focusing on models of gastric pathology induced by *H. pylori*, *H. felis* and *H. suis* in rodents and large animals. We also discuss the more recent development of gastric organoid cultures from murine and human gastric tissue, as well as from human pluripotent stem cells, and the outcomes of *H. pylori* infection in these systems.

Keywords

Helicobacter, gastric cancer, peptic ulcer disease, MALT lymphoma, organoid, gastroid.

Introduction

Helicobacter pylori is a bacterium that grows in close association with the lining of the stomach and is associated with various human gastric diseases; it causes significant morbidity and mortality worldwide. Globally, there are wide variations in the reported prevalence of *H. pylori* (Figure 1), with particularly high levels observed in South America, Sub-Saharan Africa and the Middle East (Asfeldt *et al*, 2008; Ben Mansour *et al*, 2016; Laszewicz *et al*, 2014; Luzza *et al*, 2014; McDonald *et al*, 2015; Peleteiro *et al*, 2014; Saltanova, 2001; Sanchez Ceballos *et al*, 2007; van Blankenstein *et al*, 2013).

Many publications have promoted the concept of "the African Enigma" (Holcombe, 1992), owing to the reporting of fewer cases of peptic ulceration than expected in this continent. However, recent studies suggest that gastric pathology is endemic where *H. pylori* is endemic (Agha and Graham, 2005). They also suggest that the geographical distribution of gastric disease and its predominant form relates more to other co-factors, such as *H. pylori* virulence properties, food preservation methods, diet, and host genetics (Graham *et al*, 2009; Kodaman *et al*, 2014).

One of the principal confounders of studies examining the impact of *H. pylori* infection on gastric health in diverse populations is the inequality of access to healthcare systems globally. Diagnosing *Helicobacter* infection relies on one of four tests: endoscopy with biopsy; ^{13}Carbon-hydrogen breath test; fecal antigen testing; or serological detection of an anti-*H. pylori* antibody. These tests are relatively expensive and their availability is limited, particularly in developing countries where the highest *H. pylori* prevalence has been reported.

For many *H. pylori* associated conditions, the most effective clinical intervention is to eradicate the infection using a combination of acid-suppressing medication and antibiotics. However, this strategy is becoming increasingly difficult to sustain because of the emergence of antibiotic-resistant *H. pylori* strains. Moreover, in some clinical circumstances, *H. pylori* eradication is ineffective at preventing disease progression. There is, therefore, an unmet need to develop new drugs, both to eradicate *H. pylori* more effectively and to offer alternative strategies where infection eradication does not prevent the progression of gastric pathology.

To achieve this, we need to improve our understanding of the molecular events that lead to *H. pylori* induced gastric pathology, and this requires experimental models. Here, we review the currently available *in-vivo* and *ex-vivo* models of *Helicobacter*-induced pathology, and describe the spectrum of pathology induced by *H. pylori, H. felis* and *H. suis* infection. The *in-vivo* models discussed here span rodent and larger animal models, including cat, dog, pig and non-human primate models, whilst the *ex-vivo* models derive from mouse and human gastric mucosa and from pluripotent stem cells. It is particularly timely to review these *ex-vivo* models because of the recent development of long-lived *ex-vivo* cultures of untransformed gastric epithelium (Barker *et al*, 2010). These offer an important adjunct to the more established animal models of gastric carcinogenesis, and make it likely that future mechanistic studies of gastric disease will incorporate elements of both *in-vivo* and *ex-vivo* experimentation.

***Helicobacter pylori*: an overview**

The gastric microenvironment (Figure 2) is hostile to commensal bacteria due to the presence of high concentrations of gastric acid and digestive enzymes and because of its low partial oxygen pressure. *H. pylori* is a Gram-negative, spiral rod-shaped bacterium that has evolved to survive in this environment. Its adaptations to these conditions include an ability to tolerate a microaerophilic environment (see Glossary, Box 1), the expression of a urease enzyme that modulates the bacterial microenvironment by raising pH, and flagellae that provide motility, allowing *H. pylori* to access the deep mucus layer of the stomach wall, thereby utilizing the host mucosal defenses to develop a survivable niche.

The transmission of *H. pylori* infection is considered to occur through an oro-oral or feco-oral route. Data from families indicates that vertical transmission from parent to child is a common transmission route. A recent phylogenetic study in an Iranian population examined transmission by DNA fingerprinting of *H. pylori* 16S ribosomal sub-unit DNA obtained from fecal samples. This assay detected *H .pylori* DNA in 26 of 30 cases, and demonstrated the vertical transmission of *H. pylori* in 46.1% of families, with 38.4% of cases being colonized with an *H. pylori* strain phylogenetically identical to their mother's strain, and 7.7% with a strain identical to their father's (Mamishi *et al*, 2016). These findings agree with similar studies performed in other populations (Konno *et al*, 2005; Konno *et al*, 2008; McMillan *et al*, 2011; Roma-Giannikou *et al*, 2003), and are supported by animal studies. For example, one study reported that in an *H. pylori* infected cat colony, kittens were passively colonized by *H. pylori* over the first 14 weeks of life (Straubinger *et al*, 2003).

The global prevalence of *H. pylori* infection in humans is estimated to be 50%. The association of *H. pylori* with humans is longstanding, with phylogenetic studies suggesting that *H. pylori* strains have co-evolved with human populations since before the migration of early humans from Africa 58,000 years ago (Falush *et al*, 2003; Linz *et al*, 2007). Below, we discuss the consequences *of H. pylori* infection for human health, to establish the types of pathology that need to be modelled in the laboratory.

Helicobacter induced gastric pathology in humans

Chronic infection with *H. pylori* is strongly associated with gastric pathology, including chronic active gastritis (see Glossary, Box 1), peptic ulcer disease, gastric adenocarcinoma, and gastric extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue type (MALT lymphoma). Of these outcomes, the most significant in terms of mortality is gastric adenocarcinoma. Recent meta-analyses suggest that the relative risk of developing gastric cancer is 2 to 3 times higher for people infected with *H. pylori* than for those without infection (Danesh, 1999; Helicobacter and Cancer Collaborative, 2001). Understanding these different pathological conditions is important for understanding how faithfully the available models recapitulate the clinical features of *H. pylori* pathology.

Superficial Gastritis

The commonest outcome of *H. pylori* infection is gastritis. Acute gastritis has rarely been described in humans, but has been reported in the context of experimentalists

being exposed to *H. pylori* either accidentally (Sobala *et al*, 1991) or in a deliberate attempt to induce gastric pathology (Marshall *et al*, 1985; Morris and Nicholson, 1987; Morris *et al*, 1991). In these cases, the infected individuals reported symptoms and underwent endoscopic assessment with biopsy of the inflamed gastric mucosa. The early stages of disease are marked by the presence of a polymorphonuclear leukocyte infiltrate (see Glossary, Box 1) in the gastric mucosa and a transient reduction in gastric acid output.

In the cases of Marshall *et al* and Morris and Nicholson, *H. pylori* eradication therapy was prescribed. This was effective in eradicating *H. pylori* from the gastric mucosa, and led to the complete resolution of symptoms and of gastric histological abnormalities. In the case of Sobala *et al*, symptoms and signs resolved spontaneously, and repeat endoscopy demonstrated low levels of *Helicobacter* colonizing the gastric antrum, together with an increase in lymphocytes within the gastric mucosa. These histological changes correlated with IgM and IgG seroconversion for *H. pylori*, which are typical for chronic, superficial *H. pylori* gastritis. This is the most prevalent *H. pylori* induced gastric pathology worldwide (Campbell *et al*, 2001; Filipe *et al*, 1995; Potet *et al*, 1993).

Peptic ulcer disease

Individuals colonized with *H. pylori* have a 6.8-fold (95% confidence interval (CI) 2.9-16.1) higher risk of developing peptic ulcer disease (PUD) than those not exposed to this infection (Li *et al*, 2010). In line with this, the reduced incidence of *H. pylori* infection worldwide has coincided with a reduction in PUD (Groenen *et al*, 2009). In contrast to the 1980s, when the association of *H. pylori* and PUD was first established

(Graham, 1989), individuals presenting with this disease are now less likely to be colonized with *H. pylori*; more often, their condition is linked to non-steroidal anti-inflammatory drug use or to low dose aspirin (Musumba *et al*, 2012; Sung *et al*, 2009). *H. pylori* induced peptic ulceration occurs in the context of pre-existing chronic superficial gastritis, but is associated with increased gastric acid secretion and a T helper 1 (Th1) polarized immune response, compared to individuals with isolated superficial gastritis (D'Elios *et al*, 1997; Shimada *et al*, 2002).

Frequently, individuals with PUD exhibit antral predominant gastritis, which leads to enhanced gastrin secretion (see Glossary, Box 1). In turn, this stimulates the parietal cells of the gastric corpus (Figure 2) to secrete more acid (McColl *et al*, 1997), leading to mucosal ulceration. Eradication of *H. pylori* is reportedly sufficient to suppress excess gastrin secretion (McColl *et al*, 1991), which is an important component of the healing process of *H. pylori* associated peptic ulcers.

Gastric adenocarcinoma and its precursor lesions

In 2012, gastric cancer was the fifth commonest malignancy worldwide, and the third commonest cause of cancer related death, with over 720,000 deaths worldwide caused by the disease (Ferlay J, 2013). *H. pylori* colonization is the single biggest risk factor for gastric carcinogenesis and is a risk factor in at least 80% of cases of gastric cancer (Graham, 2015). However, as only a very small percentage of people infected with *H. pylori* go on to develop gastric cancer, understanding why those individuals do so is a key aim of future studies in this field.

Other risk factors linked to gastric cancer (Figure 3) fall into two main groups. The first consists of potentially modifiable exogenous risk factors, such as dietary salt and

nitrosamine intake (Jakszyn *et al*, 2006; M *et al*, 2007; Mendez *et al*, 2007; Wang *et al*, 2009), *H. pylori* virulence factors (Yamaoka, 2010), non-*Helicobacter* gastric microbiota (Dicksved *et al*, 2009; Lofgren *et al*, 2011) and smoking status (La *et al*, 2009). The second group consists of unalterable host genetic, or intrinsic, risk factors. Amongst these genetic factors are polymorphisms at loci encoding cytokines and their receptors (Persson *et al*, 2011), stromal remodeling proteins, such as matrix metalloproteinases (Tang *et al*, 2008), and prostate stem cell antigen (PSCA), which in the context of gastric pathology, acts as a tumor suppressor gene (Garcia-Gonzalez *et al*, 2015; Ichikawa *et al*, 2015; Mou *et al*, 2015).

The development of gastric cancer occurs through a stereotypical pathological pathway (Figure 3, and Glossary, Box 1), which was first proposed well before the identification of *H. pylori* (Correa *et al*, 1975). Over the course of several decades, some individuals with chronic superficial gastritis develop gastric atrophy, characterized by the patchy loss of parietal cells in the gastric corpus mucosa. This decreases gastric acid secretion, leading to higher intraluminal pH, decreased somatostatin secretion, and consequent gastrin secretion. In addition to stimulating acid secretion from parietal cells, gastrin also enhances proliferation in the gastric epithelial stem cell zone (Burkitt *et al*, 2009), leading to increased epithelial cell turnover.

A proportion of people with established gastric atrophy develop intestinal type metaplasia (see Glossary, Box 1) of the gastric mucosa over time, where oxyntic glands (see Glossary, Box 1) are replaced by CDX2 (caudal type homeobox 2)-expressing glandular units, which are morphologically similar to the intestinal crypt.

Intestinal metaplasia in the stomach is linked to gastric dysplasia (see Glossary, Box 1); up to 20% of affected individuals with intestinal metaplasia have concurrent dysplasia (den Hoed *et al*, 2011). Gastric epithelial dysplasia is associated with an at least 10-fold increased risk of developing gastric cancer (You *et al*, 1999), but it has been difficult to represent this risk accurately from population-based studies.

Several studies have assessed the strategy of testing for, and eradicating, *H. pylori* in populations at a high risk of developing gastric cancer. Unfortunately, a recent well-designed meta-analysis confirmed a relatively poor outcome for this strategy. The eradication of *H. pylori* in this study reduced the risk of developing gastric cancer by ~1/3 (OR 0.66 [95% CI 0.46 – 0.95]) (Ford *et al*, 2014). However, when patients with pre-existing pre-neoplastic gastric pathology (defined as the presence of gastric atrophy, intestinal metaplasia, or dysplasia) were considered, there was no evidence that eradication of *H. pylori* decreased the risk of gastric cancer (OR 0.86 [95% CI 0.47 – 1.59]). For this highest risk group of patients, therefore, there are currently no effective therapeutic strategies.

MALT lymphoma

Gastric extranodal marginal zone lymphomas of mucosa-associated lymphoid tissue (MALT lymphomas) are B-cell lymphomas that develop within the mucosa-associated lymphoid tissue of the stomach. The incidence rate of gastric MALT lymphoma in the USA was estimated to be 3.8 per in 1,000,000 individuals between 2001 and 2009, making it a rare outcome of *H. pylori* infection (Khalil *et al*, 2014). In the only published systematic review of this condition, 79% of 1844 reported cases

of MALT lymphoma were associated with *H. pylori* infection (Asenjo and Gisbert, 2007; Gisbert and Calvet, 2011).

As with other hematological malignancies, characteristic cytogenetic profiles have been described for MALT lymphoma. Amongst the most well characterized is the formation of the MALT1-API2 fusion oncogene by the t (11:18) translocation. This results in the expression of API2 (encoding the cellular inhibitor of apoptosis 2) under the control of the MALT1 promoter (Rosebeck *et al*, 2011). MALT1 encodes mucosa-associated lymphoid tissue lymphoma translocation protein 1, which is essential for the activation and proliferation of T and B lymphocytes, and also plays a fundamental role in NF-κB (Nuclear Factor-κB) activation. One of the downstream effects of this fusion protein is enhanced cleavage of NIK (NF-κB-inducing kinase), which is a critical regulator of alternative pathway NF-κB signaling (Merga *et al*, 2016).

Human infections with non-Helicobacter pylori Helicobacter species

Whilst infection of the gastric mucosa with *H. pylori* is by far the most frequently observed gastric infection in humans, non-*H. pylori Helicobacter* species (NHPH) infections of human hosts have been identified since at least the mid-1990s. The identification of these organisms remains a challenge, and relies on molecular microbiological techniques that are not routinely available.

Although NHPH infections are frequently reported to occur in association with gastritis, different studies have yielded conflicting results as to their significance (Flahou *et al*, 2013; Liu *et al*, 2015). Understanding the contribution of NHPH species to gastritis is further complicated by the occurrence of mixed NHPH infections, by the heterogeneity of NHPH strains, by the nomenclature of these species, and the

inability to cultivate many of them. For example, long spiral-shaped bacteria that were first recognized as microscopically different from *H. pylori* were recognized in human gastric biopsies and named *Gastrospirillum hominis* (McNulty *et al*, 1989). These organisms were subsequently reclassified as *Helicobacter heilmannii* based on 16S RNA analysis, of which there are at least two strains (Heilmann and Borchard, 1991). Many species of spiral-shaped NHPHs have since been recognized in the stomachs of animals (as discussed in more detail below).

The most robust data for the pathogenicity of NHPH involve MALT lymphoma formation. *H. heilmannii*-associated MALT lymphoma was first described in 2000. Following this, a large study examining the prevalence of MALT lymphoma in 263,680 *H. pylori* infected people, and in 543 NHPH infected people, demonstrated an odds ratio of 2.2 (95% CI 1.1 to 4.5) for developing gastric MALT lymphoma in individuals infected with NHPH rather than with *H. pylori* (Stolte *et al*, 2002). This organism has also been described in association with individuals with chronic gastritis (Heilmann and Borchard, 1991). These observations suggest that NHPH play a role in human disease development, and might in some cases be as pathogenic as *H. pylori*. The additional challenge of identifying these organisms suggests that there could be a group of individuals with gastric pathology due to unidentified NHPH, representing an unmet clinical need.

Naturally occurring gastric *Helicobacter* infections in non-human mammals

Whilst the association of *H. pylori* with humans has been extensively studied over the past 4 decades, significantly less has been published on the association of *H. pylori*, or other *Helicobacter* species with different mammalian hosts. The data that are

available suggest a spectrum of pathogenicity for different *Helicobacter* species, as well as a spectrum of susceptibility for gastric pathology in different host organisms. A better understanding of these comparative biological responses may, in the future, offer insights into the mechanisms that underlie human disease.

Forty five species of *Helicobacter* have been detected by PCR in fecal samples from 150 vertebrate species, demonstrating their colonization of the digestive system of a wide range of domesticated and wild vertebrate species (Schrenzel *et al*, 2010). Spontaneous gastric colonization by NHPH has also been demonstrated in several mammalian species (Table 1), leading to speculation that all mammals harbor one or more *Helicobacter* species as part of their natural gastric flora (Brown *et al*, 2007).

When examining the evidence for NHPH-induced gastric lesions in animal species, various factors need to considered, such as differences in gastric morphology, and in the distribution and site of gastric *Helicobacters* among some animal species and humans. Furthermore, the sequential pathological lesions that lead to adenocarcinoma formation in humans, as described by Correa's model (see Glossary, Box 1), have rarely been established in clinical veterinary species (Amorim *et al*, 2016), in which gastric cancer is rare. Exceptions to this include: ferrets colonized by *H. mustalae*, which undergo a similar sequence of pathology that culminates in gastric adenocarcinoma formation (Fox *et al*, 1990; Fox *et al*, 1997); Mongolian gerbils infected with *H. pylori*, which develop some specific pre-neoplastic lesions, including intestinal metaplasia (Honda *et al*, 1998b); and Syrian hamsters infected with *H. aurati* (Patterson *et al*, 2000).

In veterinary medicine, dogs and cats frequently undergo gastric biopsy to investigate unresolved gastrointestinal disease. In both species, NHPHs are frequently observed, genetic studies show that these organisms are often present as a mixed infection of different NHPHs (Priestnall *et al*, 2004) with *H. felis*, *H. heilmannii*, *H. bizzozeronii*, and *H. salomonis* being the most commonly identified species (Canejo-Teixeira *et al*, 2014; Priestnall *et al*, 2004; Van den Bulck *et al*, 2005).

Despite NHPHs, including *H. felis*, being commonly identified in dogs and cats and being associated with gastritis (Shiratori *et al*, 2016) and with gastric MALT lymphoma in humans (Stolte *et al*, 2002), genetic studies have shown limited evidence for zoonosis. The most frequently isolated *H. heilmannii* from dogs and cats are distinct from the type 1 *H. heilmannii* identified in human MALT lymphoma (Priestnall *et al*, 2004). However, there is evidence of anthroponosis in the *H. pylori* infection of cats; infection has only been reported in cat colonies that live in proximity to humans (Canejo-Teixeira *et al*, 2014).

In dogs, lymphoplasmacytic gastritis is commonly observed in association with NHPHs (Neiger and Simpson, 2000). However, there is no evidence to link conclusively NHPH infection with this gastric pathology. Indeed, NHPH infection is present in 67-86% of clinically healthy dogs, and in 61-100% of animals presenting with chronic vomiting (Amorim *et al*, 2016). A small-scale study reported that NHPHs are found in association with all cases of canine gastric polyps (Taulescu *et al*, 2014). Similarly, NHPHs have been found in the stomach of 42-100% of healthy cats, and in 53-76% of those presenting with clinical signs of gastrointestinal disease (Norris *et al*,

1999). It is hypothesized that these infections play a role in the development of feline gastric lymphoma (Bridgeford *et al*, 2008b).

H. suis is detected in the stomach of up to 80% of pigs at the time of slaughter, with ulceration and hyperkeratosis of the pars esophagea (see Glossary, Box 1) present in 20-90% of slaughtered pigs (De Bruyne *et al*, 2012). Although some studies have associated *H. suis* with increased severity of gastritis and with reduced weight gain in pigs (De Bruyne *et al*, 2012), gastritis is likely to be multi-factorial and also involves feed particle size, highly fermentable carbohydrates, and stress factors. Interestingly, *H. suis* is the most commonly isolated NHPH found in human stomachs, suggesting the potential for zoonotic transmission.

Helicobacter-like DNA has also been isolated from the stomach of thoroughbred horses (Contreras *et al*, 2007), although the role of *Helicobacter* in gastritis and gastric ulceration in horses is unclear. Horses possess a much larger proportion of non-glandular squamous mucosa than do pigs, which constitutes the proximal half of the stomach mucosa, and gastric ulceration is present in up to 86% of training racehorses (Begg and O'Sullivan, 2003). The high proportion of horses suffering from ulceration that undergo strenuous exercise suggests that stress, management and training practices are likely risk factors (Murray *et al*, 1996). Ulceration occurs most commonly in the non-glandular portion of the stomach, close to the transition of the non-glandular and glandular stomach, although pyloric ulceration is also observed in 47% of horses (Begg and O'Sullivan, 2003).

Up to 100% of adult ferrets harbor gastric *H. mustelae*, (Fox *et al*, 1990), however, this organism is rarely found in ferrets of less than 6 weeks of age (Fox *et al*, 1988).

The incidence of gastric ulceration in ferrets varies between 1.4 and 35% (Andrews *et al*, 1979), and *H. mustelae* has also been associated with adenocarcinoma formation (Fox *et al*, 1997) and with gastric lymphoma development (Erdman *et al*, 1997) in this species.

Captive rhesus macaques are commonly infected with *H. pylori*, (Drazek *et al*, 1994), and non-human primates have been used as models of *H. pylori* infection. Indeed, rhesus macaques in social housing rapidly acquire *H. pylori* from other infected individuals (Solnick *et al*, 2003). Neonatal rhesus macaques are more commonly infected with *H. pylori* when born to infected mothers, suggesting that close contact in the peripartum period is important for bacterial transmission (Solnick *et al*, 2003), potentially via an oral-oral route. (Solnick *et al*, 2006). The induced pathology in rhesus macaques is also very similar to that observed in humans with *H. pylori* infection (Haesebrouck *et al*, 2009). However, no NHPH species have been uniquely associated with non-human primate gastric colonization, although *H. suis* has been demonstrated in captive mandrills (*Papio sphinx*), cynomolgus monkeys (*Macaca fasicularis*), and in a rhesus macaque (*Macaca mulatta*) from a zoo (Haesebrouck *et al*, 2009). The question of whether these NHPHs are implicated in the development of gastritis in non-human primates remains unknown. More recently, a study identified a high incidence of gastric adenocarcinoma in a captive colony of sooty mangabeys (*Cercebus atys*) (Sharma *et al*, 2011). This colony has subsequently been shown to be heavily colonized with *H. suis* by both fluorescence in-situ hybridization and 16S ribosomal RNA sequencing (Esmail *et al*, 2016). This is the first evidence of

naturally occurring *Helicobacter* associated with gastric carcinogenesis in a non-human primate.

In-vivo models of *Helicobacter* induced gastric pathology

Because of the breadth of potential pathological outcomes that can follow an *H. pylori* infection, no single animal model can replicate all of the pathological outcomes of this condition. However, as we discuss in more detail below, models do exist that can replicate each of the potential outcomes of *H. pylori* infection in humans (Figure 4).

Models of superficial gastritis

The acute phase of *Helicobacter* gastritis has rarely been the focus of research, due in part to the small number of reported cases in the human literature. Where data have been published, they have focused on defining the bacterial and host factors that influence *Helicobacter* colonization and the acute cytokine milieu induced by these bacterial infections.

Historic studies in gnotobiotic pigs demonstrated that the urease enzyme produced by Helicobacter bacteria (Eaton and Krakowka, 1994) and their functioning flagellae are essential for effective colonization of the gastric mucosa (Eaton *et al*, 1992).

Experimental infections of cats with *H. pylori* have shown that active colonization of the gastric mucosa occurs readily, and that a chronic gastritis can follow (Fox *et al*, 1995). Independently, researchers characterized the acute cytokine response of cats born into a colony of *H. pylori* infected animals. In this study (Straubinger *et al*, 2003), the researchers compared animals born into their *H. pylori* infected colony to specific

pathogen free (SPF) animals bought into the animal unit. They demonstrated that animals in the infected colony became colonized with *H. pylori* passively by 14 weeks of age, and that this was associated with an immune response dominated by the expression of cytokines IFN-γ, IL-1α, IL-1β, and IL-8. As with the other large animal models of *H. pylori* infection, these experiments benefit from the availability of serial endoscopic evaluation, but are challenging to perform due to the ethical and cost limitations associated with using large animals in experimental procedures.

More recently, Mongolian gerbils have been used to investigate host factors that allow optimal gastric colonization (Bucker *et al*, 2012). Gastrostomies were performed under terminal anesthesia to place an intraluminal pH probe, and an auto-titrator into the stomach. The authors used this system to recapitulate the effect on gastric pH of a meal whilst simultaneously inoculating *H. pylori*. Three physiological conditions were replicated using this apparatus. First, it replicated the gastric pH profile observed in newborn human infants, who have a persistently elevated gastric pH due to the large buffering capacity of a milk-based diet and relatively low gastric acid secretion. Second, it replicated the pH profile of young children, in whom gastric pH is transiently elevated due to the buffering capacity of a high milk diet, but then lowered to pH 1-2 by gastric acid secretion. And finally, it replicated the profile of adults, in whom luminal pH remains low throughout the post-prandial period due to high acid secretion and the relatively low buffering capacity of food. The pH profile similar to that of young children enhanced the ability of *H. pylori* to colonize the stomach up to 15-fold, supporting other evidence that the commonest mode of *H. pylori* colonization is through vertical transmission from parent to child (Suerbaum

and Josenhans, 2007). This model provides the potential to look at other elements of gastric microbiotal colonization. For instance, does an achlorhydric stomach offer a niche for colonization with other non-*Helicobacter* organisms, and if so does co-infection with *H. pylori* help or hinder this process?

The immediate host response to colonization with *Helicobacter* has also been modelled by the introduction of *H. pylori*-derived lipopolysaccharide into the stomachs of Sprague-Dawley rats. This induced an inflammatory cell infiltrate typified by lymphocyte infiltration, and increased gastric epithelial cell apoptosis over the course of four days. Rather than recapitulating the pathology observed in acute human *H. pylori* infection, the pathological description of this model was more in keeping with the pathology observed in people with chronic superficial gastritis (Slomiany *et al*, 1998).

These studies have remained relatively niche areas of investigation, and to date have not been replicated by other groups. Significant research questions remain, particularly regarding the initial host responses to *H. pylori* exposure.

Models of gastric ulceration

In-vivo models of gastric ulceration induced by *Helicobacter* infection alone are limited to gnotobiotic pigs, Mongolian gerbils, and isolated reports of murine gastric ulceration. Several groups have independently shown that gnotobiotic pigs develop ulcers at the junction between the squamous epithelium of the pars esophagea and columnar mucosa of the true stomach, following infection with *Helicobacter* species harvested from commercially reared pigs (Krakowka *et al*, 1995; Krakowka *et al*, 2005; Kronsteiner *et al*, 2013). These models have helped to confirm the association

between *Helicobacter* colonization and peptic ulceration, but have not been adopted more widely for mechanistic studies.

Mongolian gerbils reportedly develop a wide spectrum of *Helicobacter* induced pathologies, including gastric ulceration. For example, Honda *et al* reported that 4 of 5 gerbils developed gastric ulceration six months after colonization with the CagA-positive (see Glossary, Box 1) *H. pylori* strain, ATCC-43504 (Honda *et al*, 1998a). Independently, Ogura and colleagues identified gastric ulceration in 22 of 23 gerbils infected with the TN2 strain of *H. pylori* for 62 weeks. When the *H. pylori* virulence factor CagE was deleted, none of the 22 infected animals developed gastric ulceration in the same timescale, demonstrating the utility of Mongolian gerbils for modelling gastric ulceration, and the value of this model for characterizing the virulence factors of *H. pylori* (Ogura *et al*, 2000).

Most of the literature reporting *H. pylori* colonization of mice suggests that colonization is usually transient, and if persistent, that it is often associated with only mild gastritis (Lachman *et al*, 1997). In contrast, Kaur *et al*. reported a model of gastric ulceration following colonization of female C57BL/6 mice with *H. pylori* DSMZ 10242 for 8 weeks (Kaur *et al*, 2014). This study reported multifocal gastric antral ulceration with relatively deep ulcers at 8 weeks, which, if left untreated did not heal. Whilst this study contrasts with much of the literature, it might reflect how factors in an individual laboratory, in particular the baseline microbiota, can influence the outcome of infection in different institutions.

A more established method for investigating the effect of *H. pylori* on gastric ulceration has been to investigate the impact of *H. pylori* infection on chemically

induced gastric ulcers. For instance, rats administered acetic acid to the serosal surface of the stomach develop ulcers that heal over several weeks. Gastric colonization with both $CagA^+$ (ATCC 43504) (Bui *et al*, 1991) and $CagA^-$ (AH69) (Li *et al*, 1997) strains of *H. pylori*, impairs the healing of acetic acid-induced ulcers in Sprague-Dawley rats. In this model, extracted *H. pylori* surface proteins (Brzozowski *et al*, 1999) also impaired the healing of gastric ulcers, suggesting that a response to bacterial components, and not necessarily to the presence of live *Helicobacter*, can impair mucosal healing following gastric injury (Brzozowski *et al*, 1999).

Models of gastric adenocarcinoma and its precursor lesions: large and small mammals

Gastric carcinogenesis has been the most extensively studied outcome of *H. pylori* infection, and it has the most diverse array of *in-vivo* models, several of which are used in laboratories across the world. Much of the original work investigating the pathogenesis of *Helicobacter*-induced gastric cancer was performed in large animals. Large animal models of gastric pathology offer the opportunity to perform serial endoscopic biopsies during an experiment. This allows investigators to document the temporal development of gastric pre-neoplasia in individual animals, and has been adopted in Beagle dogs (Rossi *et al*, 1999) and Rhesus monkeys (Liu *et al*, 2009).

In one such study (Rossi *et al*, 1999), conventionally housed dogs were infected with a $CagA^+$ strain of *H. pylori* (SPM326s) and underwent endoscopic evaluation 1, 2, 4, 8, 12, 18 and 24 weeks after infection. From 8 weeks, chronic superficial gastritis developed, with progressive changes observed in the gastric mucin composition, in keeping with functional gastric atrophy. The authors interpreted this as the

development of early pre-neoplastic pathology. The study also demonstrated progression towards atrophic gastritis; however, it is not possible to predict whether more advanced pre-neoplastic lesions would subsequently develop in this model. This study was also compromised by a lack of detail about the pre-infection gastric microbiota of the animals used in this study; they were demonstrated to be *H. pylori* seronegative, but pre-infection gastric colonization was not assessed for *H. pylori* or for other NHPHs.

H. pylori infection of rhesus macaques has been studied in combination with the administration of the ethylating agent, N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG). ENNG is similar to N-nitroso compounds present in traditional Far Eastern diets, and is proposed to be a potential dietary risk factor for developing gastric cancer (Seel *et al*, 1994). Rhesus macaques were observed over 5 years following *H. pylori* colonization, and gastroscopy was performed quarterly. Neither the continuous administration of ENNG in food *ad-libitum*, nor *H. pylori* infection alone induced gastric pre-neoplasia. However, the administration of both agents together induced intestinal type metaplasia after two years of treatment, and more advanced neoplasia, including high-grade dysplasia, in one animal after 5 years. This study demonstrates synergy between ENNG and *H. pylori*, but the study design was unable to determine whether both agents are essential for gastric carcinogenesis, or whether one carcinogen accelerated the effect of the other (Liu *et al*, 2009).

Although studies using large animal models provided insights into the development of *Helicobacter* induced gastric cancer, they have not been widely adopted due to the need for specialized animal facilities and the high associated costs. As a result, rodent

models are most commonly used. In particular, Mongolian gerbil and murine models have been used frequently in different laboratories to investigate diverse aspects of gastric carcinogenesis.

Both mice and gerbils develop stereotypical, pre-neoplastic pathology in response to *Helicobacter* infection. Whilst the pathways observed in humans and gerbils are similar, there is a difference during the metaplastic phase of gastric pre-neoplasia in mice. In humans and gerbils, the commonest metaplasia is intestinal type metaplasia. This is characterized by the presence of goblet cells and by the expression of appropriate intestinal markers, such as trefoil factor 3 (TFF3) and mucin 2 (MUC2) (Ectors and Dixon, 1986), as well as by the intestinal differentiation regulating transcription factor CDX2 (Barros *et al*, 2011; Silberg *et al*, 2002).

Another metaplastic lesion, defined as spasmolytic polypeptide expressing metaplasia (SPEM), is less frequently identified in people with gastric pre-neoplasia. It is characterized by a phenotype similar to the secreting Brunner's glands of the intestine, or to deep antral gland cells that express Muc6 and trefoil factor 2 (TFF2, or spasmolytic polypeptide) (Weis and Goldenring, 2009). In gerbils, the distribution of these lesions is similar to that observed in humans (Yoshizawa *et al*, 2007), whereas C57BL/6 mice infected with *H. felis* develop a predominantly SPEM response, with little or no evidence of intestinal type metaplasia (Shimizu *et al*, 2016; Weis and Goldenring, 2009).

When colonized by *H. pylori* and by several other NHPH strains, including *H. felis*, *H. bizzozeroni* and *H. suis* (De Bock *et al*, 2006a; De Bock *et al*, 2006b; Liang *et al*, 2015; Nakamura *et al*, 2007), Mongolian gerbils reportedly develop advanced pre-

malignant lesions. However, the use of this organism is complicated by problems of experimental reproducibility. For example, Watanabe and colleagues (Watanabe *et al*, 1998) reported that 5 out of 5 gerbils infected with the $CagA^+$ *H. pylori* strain TN2GF4 in a standard animal house environment for 52 weeks developed intestinal metaplasia, although 10 of 27 gerbils infected for 62 weeks developed invasive adenocarcinomas. In contrast, (Elfvin *et al*, 2005) studied gerbils that were colonized with either *H. pylori* SS1 or TN2GF4 and maintained in a SPF facility for up to 18 months. None of these animals developed invasive adenocarcinoma, and only 2 of 5 infected animals at 12 months, and 3 of 10 infected animals at 18 months, developed intestinal metaplasia.

Because the Mongolian gerbil is outbred, the genetic backgrounds of animals supplied to different laboratories up to a decade apart, will have been divergent. The conditions within different animal units might also have contributed to differences in these studies, particularly in terms of diet and resident microbiota. In keeping with other studies investigating the role of the microbiota in host pathology (Justice and Dhillon, 2016; Schofield *et al*, 2016), these observations demonstrate some of the challenges of comparing *in-vivo* studies performed in different environments, and challenge the received wisdom that experimental results should always be reproducible in different settings. Fully understanding why these apparently similar experiments resulted in divergent outcomes could offer insights into the mechanisms that drive the development of gastric pre-neoplasia in the gerbil.

The Mongolian gerbil has been particularly useful for identifying *Helicobacter*-specific and environmental factors that influence the development of gastric cancer. It has

been used to demonstrate the carcinogenic potential of both *H. pylori* (Watanabe *et al*, 1998) and *H. felis* (Court *et al*, 2002; De Bock *et al*, 2006a; De Bock *et al*, 2006b), and that *H. bizzozeronii* and *H. salomonis* (De Bock *et al*, 2006b) are less likely to induce gastric pre-neoplasia in this species. In addition, several studies have identified that, as in human epidemiological studies (Wang *et al*, 2009), high-salt diets promote the development of *H. pylori* induced gastric pre-neoplastic pathology in gerbils (Bergin *et al*, 2003; Kato *et al*, 2006; Nozaki *et al*, 2002).

The Mongolian gerbil has also been used to adapt *H. pylori* strains to a rodent environment. This has been demonstrated best by the passage of *H. pylori* B128, derived from a patient with gastric ulceration, through a male gerbil for 3 weeks. *H. pylori* was subsequently re-cultured from this animal's stomach and described as *H. pylori* strain 7.13, which is more pathogenic than strain B128. Six of eight gerbils infected with *H. pylori* 7.13 developed gastric adenocarcinoma 8 weeks after inoculation, in comparison to none in the B128 group (Franco *et al*, 2005). The same researchers demonstrated that this phenotype was essentially reproducible, and preventable by eradication of *H. pylori* (Romero-Gallo *et al*, 2008). The 7.13 strain has been further characterized by genome sequencing (Asim *et al*, 2015), providing data that will help to advance our future understanding of *H. pylori* pathogenicity.

The laboratory mouse is the other rodent used extensively to model gastric pre-neoplasia. *H. pylori* colonization of C57BL/6 inbred mice leads to gastritis with epithelial cell hyperplasia, but this does not progress to dysplasia or to cancer (Lee *et al*, 1997). However, the colonization of C57BL/6 mice with *H. felis* is consistently shown to lead to gastric pre-neoplasia, and when infections have persisted for 13-15

months, adenocarcinomas have been reported (Fox *et al*, 2002). This outcome is specific to the C57BL/6 genetic background.

Other strains of in-bred mouse, including Balb/c, respond differently to *H. felis* infection . This strain can be substantially colonized by *H. felis*, but does not develop gastric pre-neoplastic pathology (Wang *et al*, 1998). The mechanisms underlying these differences between mouse strains are attributable to differences in immune response. C57BL/6 mice demonstrate a Th1 polarized immune response, whilst Balb/c mice have a more Th2 polarized response to *H. felis* colonization, which allows infection to persist but does not promote chronic epithelial disruption (Wang *et al*, 1998). The B6129 mouse, generated by crossing C57BL/6 with 129S6/SvEv, is particularly sensitive to *Helicobacter* induced pathology triggered by *H. felis*. In these mice, gastric intraepithelial neoplasia developed 8 months after *H. pylori* infection whilst malignant tumors developed after approximately 15 months (Rogers *et al*, 2005).

In addition to the models of gastric cancer induced solely by *Helicobacter* infection, several chemically induced gastric cancer models are accelerated by co-infection with *H. pylori*. These include mice and Mongolian gerbils infected with *H. pylori* and treated with N-methyl-N-nitrosourea, (MNU), and Mongolian gerbils infected with *H. pylori* and treated with methylnitronitrosoguanidine (MNNG). In Mongolian gerbils, the co-administration of *H. pylori* with the carcinogen MNNG accelerated the carcinogenic process, leading to invasive cancer in 60–80% of animals treated for 1 year (Maruta *et al*, 2001; Maruta *et al*, 2000; Tatematsu *et al*, 1998). In mice, the effect of co-administering MNU and *H. pylori* is less clear, with contrasting reports of

either synergy between the two stimuli (Han *et al*, 2002), or no additional effect above chemical carcinogen alone (Nakamura *et al*, 2002).

Transgenic mouse models of gastric carcinogenesis

Several transgenesis strategies have been used to study gastric carcinogenesis. These include the induction of spontaneous gastric atrophy, the expression of *H. pylori* pathogenicity factors, and the overexpression of known oncogenes in the gastric mucosa. Transgenic animals have also been used to explore the role of specific molecular pathways that potentially modulate gastric carcinogenesis. A complete description of the genetically engineered mouse models (GEMMs) used in gastric carcinogenesis research is beyond the scope of this article, and so we refer readers to another recent review for more information (Jiang and Yu, 2016). Here, we focus on the best characterized of these models.

Amongst the most established examples of transgenically induced gastric atrophy is the INS-Gas mouse model (also known as FVB-tg(rl1-hinsgas) (Wang and Brand, 1992). This mouse expresses a human gastrin mini-gene under the control of the rat insulin promoter. It constitutively expresses gastrin in β-cells of the pancreatic islets, resulting in the constitutive overexpression of amidated gastrin. These animals are born with increased numbers of parietal cells and at birth hypersecrete acid, but over the first 5 months of life, parietal cell numbers fall to that of wild-type animals. Over longer periods, profound gastric atrophy occurs, and a proportion of these animals develop spontaneous gastric cancers (Wang *et al*, 2000). *Helicobacter* infection accelerates the gastric pathology observed in this mouse; 85% of INS-Gas mice

infected with *H. felis* developed gastric cancers 7 months after infection (Wang *et al*, 2000). Similarly, when INS-Gas mice are infected with *H. pylori*, they exhibit significantly more severe gastric inflammation and dysplasia relative to uninfected controls. Interestingly, INS-Gas males are more severely affected by *H. pylori* infection than are female mice, for reasons unknown, and in the same study, mice exposed to high dietary salt levels had more severe gastric pathology relative to untreated controls (Fox *et al*, 2003).

More recently, the INS-Gas mouse has been used to characterize the role that the non-*Helicobacter* microbiome has during gastric carcinogenesis. INS-Gas mice bred and maintained in a germ-free environment did not develop spontaneous gastric pre-neoplasia, while otherwise germ-free mice infected with *H. pylori* developed fewer tumors than did *H. pylori* infected SPF mice (Lofgren *et al*, 2011). Subsequently, the same group demonstrated that a limited group of 3 bacterial species is sufficient to restore the phenotype of SPF mice in otherwise germ-free mice (Lertpiriyapong *et al*, 2014), and that co-infection with the intestinal roundworm *Heligmosomoides polygyrus* can protect INS-Gas mice against *H. pylori* induced gastric pre-neoplasia (Whary *et al*, 2014). This protection was associated with an increase in the number of forkhead box P3 (FoxP3) positive cells in the gastric corpus of mice. Suggesting a possible shift in the T_{Reg} immune response to *H. pylori* infection.

The CEA/SV40 T L5496 mouse expresses the SV40 T proto-oncogene under the control of a truncated carcinoembryonic antigen (CEA) promoter. Gastric tumors formed in all mice, with dysplasia evident as early as 37 days postnatally and with

animals becoming moribund due to gastric tumor burden by 100 -130 days postnatally (Thompson *et al*, 2000).

Mice transgenically deficient for *mutT* homolog-1 (MTH1) are unable to process oxygen free radicals, which cause DNA damage, and are susceptible to several spontaneous tumors, including gastric tumors, which develop over ~18 months. 14% (13 of 93) *MTH1* null mice developed gastric tumors, compared with 4% (4 of 90) littermate controls (Tsuzuki *et al*, 2001).

In Trefoil factor 1 (TFF1)-deficient mice, the structure of both the antral and corpus mucosa is markedly altered from birth. By five months of age, all *TFF1* null mice exhibit adenomatous changes in the gastric mucosa, and 30% have established adenocarcinomas (Lefebvre *et al*, 1996).

The C57BL/6J-tg(H/K_ATPase/hIL-1β) mouse overexpresses human interleukin 1B (*IL1B*) under the control of the H^+/K^+ ATPase β-subunit promoter, leading to expression of human IL1-β exclusively in parietal cells. In this model, 15% of male mice develop spontaneous tumors at 14 months of age, and disease severity is markedly increased by infection with *H. felis* (Tu *et al*, 2008).

Transgenic mutation of the IL6 co-receptor, gp130, leads to a severe gastric phenotype in which gastric antral adenomas spontaneously develop over the first 6-8 weeks of life, and subsequently grow and spread to include the gastric fundus by 48 weeks of age (Judd *et al*, 2004; Tebbutt *et al*, 2002). Whilst this animal does not exhibit the classical pre-neoplastic pathology induced by *Helicobacter* infection, it may have relevance to *H. pylori* mediated disease as CagA status has been shown to influence gp130-mediated switching between Src homology region 2-containing

protein tyrosine phosphatase 2 (SHP2) / extracellular signal–regulated kinases (ERK) and Janus kinase (JAK) / Signal Transducer and Activator of Transcription (STAT) signaling cascades *in-vitro* (Lee *et al*, 2010). Signalling through these mechanisms influences a number of cellular processes that are altered in cancer, including regulation of cell proliferation and apoptosis, invasion and angiogenesis, and disruption of these pathways, either by direct mutagenesis, or through other mechanisms, have been identified in many different tumour types (Zhang *et al*, 2015).

Transgenic expression of the *H. pylori* virulence factor CagA is oncogenic to the gastric mucosa, both when expressed constitutively throughout the animal under the control of the beta-actin promoter (C57BL/6-tg(CAG-CagA^{Hs}), and when limited to the gastric mucosa under the control of the H^+/K^+ ATPase β-subunit promoter (C57BL/6J-tg(HK-CagA^{Hs}). In both cases, animals developed gastric hypertrophy by 12 weeks of age, and over 3 years developed gastric dysplasia or occasionally gastric adenocarcinomas (Ohnishi *et al*, 2008).

Models of MALT lymphoma

Several factors make *Helicobacter* induced MALT lymphoma challenging to model. It is a rare outcome of *H. pylori* infection; it develops after a prolonged, complex interaction among the bacteria, host epithelium and host immune system; and the commonest, relatively indolent form of MALT lymphoma is challenging to diagnose pathologically. These issues are compounded by the fact that natural *Helicobacter* induced MALT lymphomas have not been reported in commonly used laboratory species. Consequently, in some cases, only one research group has assessed the

models described below, and substantial gaps remain in our knowledge of the mechanisms involved in *H. pylori* induced gastric MALT lymphoma formation.

Within these studies, there is also heterogeneity in the criteria used to report MALT lymphoma formation. Most studies describe lymphoepithelial lesions as a pathognomic event, signifying the initiation of lymphomagenesis. Other studies used evidence of monoclonal lymphoid aggregate formation as a surrogate for the development of MALT lymphoma.

Experimental induction of MALT lymphoma by *H. pylori* was reported in a single study of conventionally housed Beagle dogs. This study described the formation of monoclonal lymphoid aggregates in the gastric mucosa of dogs infected with *H. pylori* strain SPM326s ($CagA^+$) for 6 months. This study was not extended to later time points, and in the absence of epithelial destruction, or evidence of genetic instability, the association with MALT lymphoma remains somewhat tenuous (Rossi *et al*, 1999). In addition, the pre-existing gastric *Helicobacter* status of the dogs at the study onset was not evaluated, although seropositivity for *H. pylori* was excluded. It is therefore not possible to conclude whether the observed phenotype was due to *H. pylori* infection in isolation or to a synergy between *H. pylori* and other gastric *Helicobacter* species.

Several NHPH have been reported to induce either MALT lymphoma or precursor lesions in laboratory conditions. Two of six Mongolian gerbils infected with the *H. suis* strain, HS5, for nine months developed lymphoepithelial lesions (Flahou *et al*, 2010), whilst outbred Swiss, and inbred Balb/c and C57BL/6, mice infected with a variety of NHPH strains developed lymphoepithelial lesions.

In one of these studies, Enno *et al* reported that 25% of Balb/c mice colonized with *H. felis* for 22-26 months developed advanced lymphoepithelial lesions, and a further 5% had early lymphoepithelial lesions (Enno *et al*, 1995). These findings have since been independently replicated, and in the same study, a variety of *H. heilmannii* strains isolated from various sources, including mandrill monkeys and bobcats, also induced gastric MALT lymphomas over a similar timescale (O'Rourke *et al*, 2004).

A commonly used model of gastric carcinogenesis is the long term infection of C57BL/6 mice with *H. felis*; however, these mice have not been reported to develop gastric MALT lymphoma formation. They do, nevertheless, develop gastric lymphoepithelial lesions and low-grade MALT lymphomas when colonized for one year with a candidatus *H. heilmanii* isolated from the stomach of a cynomolgus monkey (Nakamura *et al*, 2007). More recently, we have demonstrated that 50% (3/6) of C57BL/6 mice lacking the c-Rel NF-κB subunit developed early lymphoepithelial lesions when colonized with *H. felis* for 12 months (Burkitt *et al*, 2013). This pathology has not previously been reported in the C57BL/6 / *H. felis* model. This suggests that signaling through the c-Rel NF-κB subunit could influence the regulation of gastric MALT lymphoma formation.

Overall, these data support the hypothesis that NHPH play a specific role in the development of MALT lymphoma, and since several of the typical *H. pylori* virulence factors are not expressed in these *Helicobacter* species, this suggests that novel bacterial factors might be important in the development of gastric MALT lymphoma.

Future work in this field needs to incorporate models of genetic instability, in addition

to morphological criteria, to strengthen the quality of data generated from these models.

Ex-vivo models of *Helicobacter* infection of the stomach

To date, most studies investigating the mechanisms that underlie *H. pylori* induced gastric pathology have relied on in-vivo models. Many of these models require prolonged exposure to *Helicobacter* and use relatively large numbers of animals, raising questions of animal welfare and cost. In addition, studying the interaction of two whole organisms (and increasingly the rest of the microbiome to which *H. pylori* contributes) generates hugely complex systems. Some studies have tried to address this complexity by using elegant transgenic mouse systems, for example, by using tissue-specific transgenesis. However, these systems remain highly complex, and genetic manipulation can introduce further complexity, either through gene expression in a suboptimal location, or through off-target effects of the drugs used to induce genetic recombination.

There is, therefore, a need for better in-vitro or ex-vivo models of *H. pylori* associated pathology. Over the past six years, the development of first murine, and subsequently human, three-dimensional, primary gastric gland cultures called gastric organoids, or 'gastroids'(see Glossary, Box 1) has opened up the prospect of using untransformed, gastric tissue in culture to model the development of gastric pathology

Ex-vivo gastric mucosal culture models

In order to generate gastric glandular units in culture, it must first be possible to isolate primary material from an organism, to passage this material in vitro, and then

demonstrate its ability to differentiate into the different cell types of the gastric epithelium. Ideally, it should also be possible to store the cultures in the laboratory and reconstitute them, deriving reproducible results from cultures that have been stored or not stored.

Over the past decade, culture systems have been developed that fulfil these criteria. The key discovery came with the identification of Lgr5 as a marker of gastrointestinal stem cells (Barker *et al*, 2010; Barker *et al*, 2007). The identification of this WNT signaling family member as a key marker of gastrointestinal stem cells led rapidly to the development of primary culture systems that support these cells primarily through the optimization of WNT signaling. Since these discoveries, several groups have established gastric organoid systems using slightly different approaches (McCracken *et al*, 2014; VanDussen *et al*, 2015), as described below.

Long-lived gastric epithelial cultures derived from primary gastric tissue

The generation of self-renewing gastric gland cultures was first described by Barker, using methodology developed from earlier intestinal organoid models (see Glossary, Box 1) established by the same group. This method uses $Lgr5^{+}$ stem cells extracted from the gastric antrum of mice expressing green fluorescent protein (GFP) under the control of the Lgr5 promoter as starting material. These cells are supported in vitro in a 3D matrix together with recombinant growth factors that together recapitulate the stem cell niche. In addition to activating the WNT pathway, transforming growth factor-ß (TGFß) pathway signaling is suppressed and gastrin-17 and fibroblast growth factor 10 (FGF10) are added as gastroid-specific growth factors. During the initial phase of culture, the ROCK inhibitor Y-27632 is added to the growth media to

prevent anoikis. This methodology established $Lgr5^+$ cells as gastric antral stem cells; intriguingly, an effective marker for the gastric corpus stem cell remains elusive.

Subsequent protocols have established similar methods for the establishment of murine gastric organoid cultures from both antrum and corpus using non-enzymatically dissociated gastric glands as starting material (Mahe *et al*, 2013), and for organoids based on gastric tissue samples taken at the time of gastric resection (Bartfeld and Clevers, 2015; Schlaermann *et al*, 2016). These cultures generate spherical cultures that maintain a 3D structure in culture (Figure 5).

A modification of this protocol uses conditioned media from the L-WRN cell line, which secretes human Wnt3a, Noggin and r-Spondin 3 (VanDussen *et al*, 2015). This system has been shown to allow cultures to be established from small samples taken during an endoscopic examination of the stomach, rather than requiring samples excised during surgery, and could be more cost-effective due to the high cost of recombinant growth factors. However, the use of this cell line limits the control that an experimentalist can have on the culture system; in particular, gastric organoids grown using this method are exposed to particularly high levels of Wnt3a, making the cultures more proliferative and less likely to differentiate than gastric organoids established using recombinant growth factors.

Most reports of gastrointestinal organoid culture systems to date have retained the 3D structure of organoids. However, an increasing number of studies use 3D organoids as the source material to generate epithelial monolayers on collagen-coated glass or plastics. This technique offers different opportunities for

quantification and observation of morphology, which in some cases might be easier to relate back to more established 2D cancer cell cultures (Schlaermann *et al*, 2016).

Long-lived gastric epithelial cultures derived from induced pluripotent stem cells

Human gastric organoids have recently been generated from both human embryonic stem cell lines and induced pluripotent stem cell (iPSC) lines (McCracken *et al*, 2014). These stem cells were first differentiated into definitive endoderm before the induction of the foregut marker SOX2, by exposure to WNT3A, FGF4 and Noggin. They were further differentiated into “antral” and “corpus” type cultures by exposure to retinoic acid and subsequently differentiated into mature organoids using epidermal growth factor (EGF).

Both antral- and corpus-type gastric organoids generated from such cultures are similar to their originating tissues, as shown by microarray and by gene set enrichment analyses (McCracken *et al*, 2014). Morphologically, antral gland type organoids also contain identifiable epithelial and endocrine cell types. Corpus type organoids do not possess parietal cells, however, other markers of gastric corpus tissue, including expression of pepsinogens and ghrelin, are detectable.

The effect of Helicobacter infection on cultured gastric organoids

Several groups have investigated the impact of *H. pylori* infection on gastric organoid cultures. The most reproduced finding is that epithelial cell proliferation is enhanced by direct mucosal interaction with *H. pylori*. This has been demonstrated by the microinjection of *H. pylori* into 3D fundic gland organoids derived from both mice

and humans (Bartfeld *et al*, 2015; Bertaux-Skeirik *et al*, 2015), and into human pluripotent stem cell-derived gastric organoids (McCracken *et al*, 2014).

H. pylori infection also induces morphological changes in 2D gastric-organoid-derived monolayers grown on collagen-coated glass or plastics, with epithelial cells taking on a hummingbird morphology (Schlaermann *et al*, 2016). This response was CagA dependent and appears to be analogous to the SHP2 mediated hummingbird morphology previously described in gastric cancer cell lines (Higashi *et al*, 2002). This change in morphology was associated with the activation of the classical NF-κB signaling pathway; this pathway is also implicated in the response of 3D gastric organoids to *H. pylori* microinjection (Schlaermann *et al*, 2016; Schumacher *et al*, 2015). In gastric cancer cell lines, this morphology is associated with a more aggressive, invasive phenotype (Chang *et al*, 2016), and epithelial-mesenchymal transition (Snider *et al*, 2008).

Sigal *et al*. have established an organoid-formation assay in which they quantified the percentage of viable organoids formed from a preparation (Sigal *et al*, 2015). Using this assay, they verified their own observation that the antral $Lgr5^+$ stem cell zone expands in response to *H. pylori* infection. This provides a novel method for quantifying the abundance of antral stem cells.

A further study investigating the interactions between *H. pylori* and human gastric cells in the context of organoids has demonstrated that *H. pylori* can sense nanomolar concentrations of urea, and use this as a chemoattractant. This study made use of the observation that whilst most organoids form with the luminal surface of the epithelium facing inwards, a small proportion form with an “inside-out” structure.

This allowed the authors to observe epithelial and bacterial interactions, and in particular the adherence of *H. pylori* to cell/cell junctions (Huang *et al*, 2015).

Conclusions

An ever more diverse array of laboratory models exists to explore *H. pylori* induced pathology. The course of infection and plethora of outcomes in current *in-vivo* models are probably too complex to understand fully using current technology. The new generation of *ex-vivo* models offer opportunities to be more systematic in our approach; however, at present they also risk being reductionist. Over time we need to develop *ex-vivo* systems that can be interrogated systematically, but which incorporate key elements of *in-vivo* models, including host epithelial, mesenchymal and immune compartments, and both *Helicobacter* and non-*Helicobacter* microbiota. Currently available gastric organoid models have focused largely on the development of organoids from healthy animals and humans, which have then been infected with *H. pylori*. However, future studies will need to develop models that mimic the development of other gastric epithelial pathologies in culture. In particular, the development of 3D models of gastric atrophy and metaplasia will allow researchers to perform experiments to compare the effect of developing gastric epithelial pathology *in-vivo* and *ex-vivo*. Being able to make these comparisons will allow better mechanistic studies to be performed in the relatively simple organoid systems, and verified in whole animals. This will allow detailed mechanistic studies, and avoid the pitfalls of a reductionist scientific approach.

The results of these studies will begin to provide better data that segregate epithelial events from immune and mesenchymal driven changes in the stomach. Developing

these models presents a major challenge for the future but, if successful, they are likely to permit the design and evaluation of new therapeutic strategies for patients who currently have no meaningful treatment options.

Financial and Competing Interests

The authors have no financial or competing interests to declare

Figure Legends

Figure 1: Worldwide prevalence of *H. pylori* infection

A map showing the prevalence of *H. pylori* infection in different parts of the world. Note, the particularly high prevalence in sub-Saharan Africa, Latin America and the Middle East. Australasia, Switzerland, and more generally North America and Western Europe have the lowest incidence of *H. pylori* infection. (Data derived from (Asfeldt *et al*, 2008; Ben Mansour *et al*, 2016; Laszewicz *et al*, 2014; Luzza *et al*, 2014; McDonald *et al*, 2015; Peleteiro *et al*, 2014; Saltanova, 2001; Sanchez Ceballos *et al*, 2007; van Blankenstein *et al*, 2013)

Figure 2: The anatomy of the human and mouse stomach

A schematic of the anatomy of the human and mouse stomach and the structure of gastric glands. Two types of columnar mucosa line the human stomach: the antrum is lined with antral glands, whilst the corpus and fundus are lined with deeper oxyntic, or corpus glands (see Glossary, Box 1). The murine stomach has areas that are analogous to the human stomach, including antral and corpus glands, and it also has a forestomach lined with squamous epithelium. Stem cells that reside at the base of the gland generate the antral gland. Following asymmetric cell division in the stem cell zone, daughter cells migrate upwards towards the gastric lumen and differentiate into mucous neck, surface mucous and endocrine cells. In corpus glands, the stem cell niche is located at the isthmus of the gland. Cells migrate upwards from the stem cell zone and differentiate into surface mucous cells. Other cells migrate down the

gland and differentiate into acid-secreting parietal cells, endocrine cells, or zymogen-secreting (see Glossary, Box 1) chief cells.

Figure 3: *H. pylori* infection and progression to gastric cancer

A schematic demonstrating the pathological progression of *H. pylori* induced gastric pre-neoplasia, and highlighting endogenous risk factors for progression towards gastric cancer. SPEM: Spasmolytic polypeptide expressing metaplasia.

Figure 4: Modeling the pathological outcomes of *Helicobacter* infection

A schematic of the principal pathological outcomes of *Helicobacter* infection in humans, annotated with details of the best characterized *in-vivo* models for these conditions.

Figure 5: Gastric organoid culture and differentiation

Diagrams and images showing the maturation of gastric organoids. From left to right, images show (A) freshly digested gastric corpus glands from a C57BL/6 mouse. (B) Glands 24 hours after harvesting that have formed immature organoids and have a small spherical appearance. (C) On day 3 of culture, the immature organoids have expanded and can be passaged. (D) Following passage, the organoids retain their spherical appearance and continue to grow. Images from authors' own laboratories. Scale bars 250μm.

Table 1. Naturally occurring gastric *Helicobacter* infections and the associated host and human pathology.

Helicobacter species	**Natural host**	**Natural host lesions**	**Implicated in human gastric pathology**
H. suis	Pig, macaque, mandrill (Haesebrouck *et al*, 2009)	In pigs, associated with gastric ulceration of the pars esophagea (De Bruyne *et al*, 2012)	Yes
H. felis	Dog, cat (Priestnall *et al*, 2004) rabbit, (Haesebrouck *et al*, 2009), cheetah (Terio *et al*, 2005), mouse	Associated with gastritis in dogs and cats	Yes
H. bizzozeronii	Dog, cat (Priestnall *et al*, 2004)	Associated with gastritis in dogs and cats	Yes
H. salomonis	Dog, cat, rabbit (Haesebrouck *et al*, 2009)	Associated with gastritis in dogs and cats	Yes
H. heilmannii	Dog, cat, wild felidae, non-human primates (Haesebrouck *et al*, 2009)	In cats, hypothesized to be associated with gastric lymphoma (Bridgeford *et al*, 2008a)	Yes

H. baculiformis	Cat (Baele *et al*, 2008)	Associated with gastritis	No
H. cynogastricus	Dog (Van den Bulck *et al*, 2006)	Associated with gastritis	No
H. bovis	Cattle (De Groote *et al*, 1999)	No known pathology	No
H. mustelae	Ferret	Gastritis, gastric pre-malignant lesions and gastric adenocarcinoma (Fox *et al*, 1997) and gastric MALT lymphoma (Erdman *et al*, 1997)	No
H. aurati	Syrian hamster (Patterson *et al*, 2000)	Gastric adenocarcinoma with gastritis and intestinal metaplasia	No
H. acinonychis	Cheetah (Eaton *et al*, 1993), tiger	Gastric ulcers and erosions in tigers (Cattoli *et al*, 2000)	No
H. cetorum	Whales, dolphins (Harper *et al*, 2002)	Gastric and oesophageal ulceration in whales and dolphins	No
H. pylori	Humans	Peptic ulcer disease, Gastric adenocarcinoma and	Yes

		gastric MALT lymphoma	
H. pylori	Rhesus macaques (Dubois *et al*, 1994)	Gastritis	Yes
H. pylori	Domestic cat (Canejo-Teixeira *et al*, 2014)	Gastritis	Yes

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Box 1. Glossary

Achlorhydria: Absence of gastric acid. In a patient that has developed gastric atrophy the acid secreting parietal cells are lost, and hence the gastric luminal pH is elevated. This change in microenvironment alters local hormone secretion, and potentially provides a niche for colonization with microbes that are usually unable to survive the gastric environment.

Anthroponosis: Infectious disease in a non-human species transmitted from humans.

CagA: *Helicobacter pylori* virulence factor. The secreted component of a type IV secretion system, the cag pathogenicity island, which is associated with more severe gastric pathology.

Correa Model: A model of gastric carcinogenesis first proposed by Pelayo Correa in 1975 (Correa *et al*, 1975), describing the development of gastritis, gastric atrophy, gastric intestinal type metaplasia, dysplasia and finally gastric intestinal type adenocarcinoma.

Dysplasia: Replacement of normal gastric mucosa with structurally abnormal tissue with potentially abnormal proliferation, disordered arrangement of cells within the tissue, and structurally abnormal cells.

Gastrin: A hormone produced in the stomach that stimulates the production of acid by parietal cells, mainly through its interaction with histamine secreting ECL cells. In addition to its role in stimulating gastric acid secretion gastrin is also a growth factor, which stimulates gastric epithelial cell proliferation.

Gastritis: Inflammation of the epithelial lining of the stomach.

Gastric organoid/gastroid: An organoid generated from gastric epithelial stem cells.

Hypergastrinemia: An elevated level of the hormone gastrin in the bloodstream. This is observed particularly in the context of gastric pre-neoplasia, where the growth factor function of gastrin is thought to promote increased epithelial cell proliferation.

Metaplasia: Replacement of one differentiated epithelial tissue by another, which is abnormal for that anatomical site.

Microaerophilic: A term that describes a bacterium adapted to survive in an environment with reduced partial oxygen pressure.

Organoid: A primary epithelial cell culture that contains healthy, proliferating epithelial stem cells, which can be expanded and passaged multiple times, and generates daughter cells representative of all lineages generated by the stem cell *in-vivo*.

Oxyntic gland: An acid-secreting gland that consists of an epithelial monolayer with a proliferative stem cell zone towards the upper third of the gland. Asymmetric proliferation in this region generates daughter cells that migrate both up and down the gland and differentiate into mucus neck cells, parietal cells, chief cells and enteroendocrine cells.

Pars esophagea: A small area of non-glandular squamous mucosa near the esophageal opening present in the stomachs of some animals that is analogous to the esophageal mucosa in humans.

Polymorphonuclear leukocyte infiltration: The recruitment of multi-lobulated white blood cells (including neutrophils, basophils and eosinophils) into an epithelial tissue. Indicative of active inflammation in the tissue.

Zoonosis: Infectious disease in a human transmitted from an animal host.

Zymogen: The inactive form of a digestive enzyme, for example pepsinogen, which is an inactive form of the proteolytic enzyme, pepsin. Pepsinogen is activated by gastric acid secreted by parietal cells.

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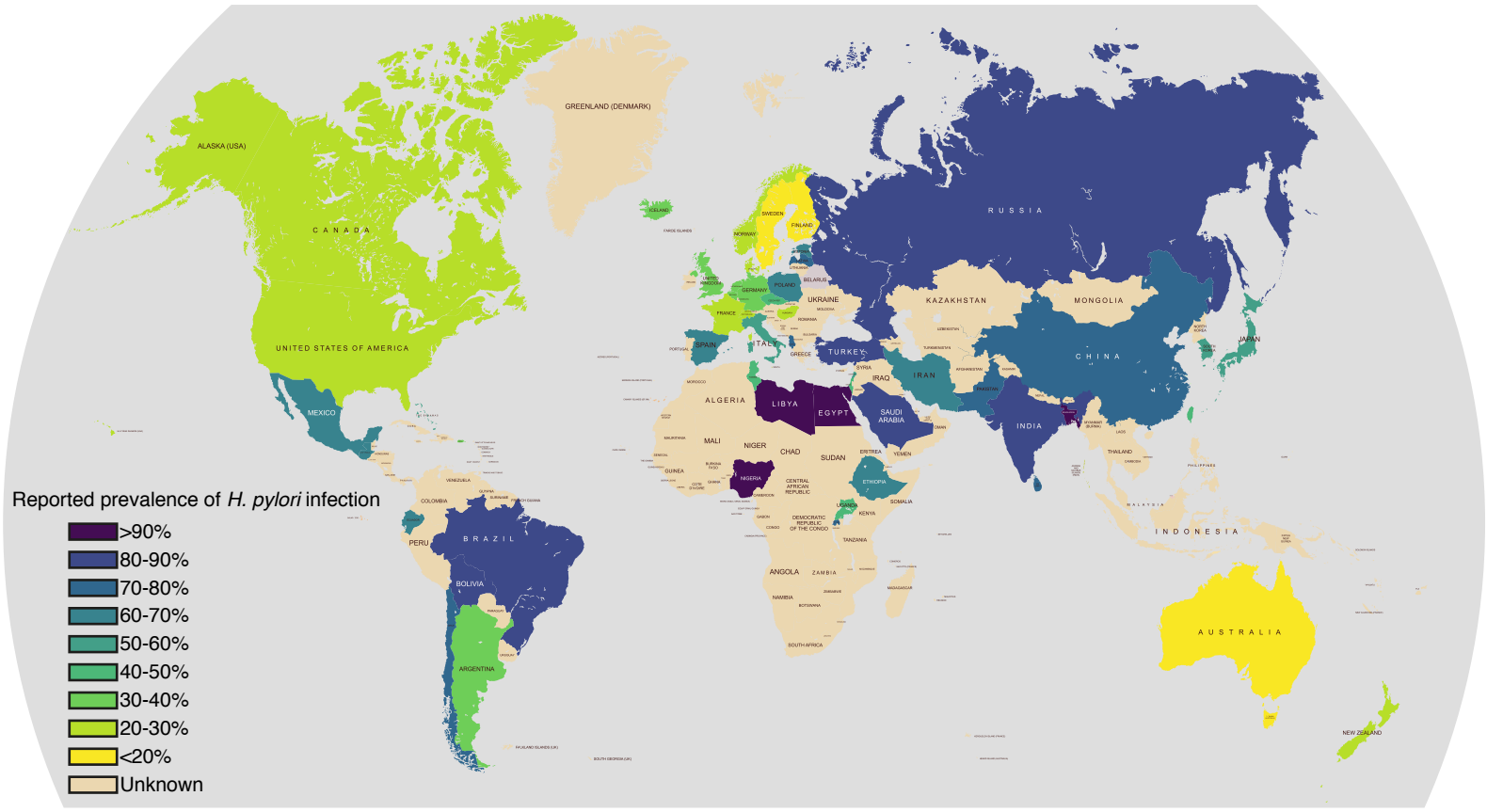
Reported prevalence of *H. pylori* infection
>90%
80-90%
70-80%
60-70%
50-60%
40-50%
30-40%
20-30%
<20%
Unknown
GREENLAND (DENMARK)
ALASKA (USA)
CANADA
UNITED STATES OF AMERICA
MEXICO
RUSSIA
KAZAKHSTAN
MONGOLIA
CHINA
JAPAN
INDIA
IRAN
IRAQ
SAUDI ARABIA
TURKEY
UKRAINE
BELARUS
POLAND
GERMANY
FRANCE
SPAIN
ITALY
GREECE
SWEDEN
FINLAND
NORWAY
ALGERIA
LIBYA
EGYPT
MALI
NIGER
CHAD
SUDAN
NIGERIA
ETHIOPIA
ERITREA
YEMEN
KENYA
SOMALIA
DEMOCRATIC REPUBLIC OF THE CONGO
TANZANIA
ANGOLA
ZAMBIA
NAMIBIA
BOTSWANA
SOUTH AFRICA
MADAGASCAR
BRAZIL
PERU
BOLIVIA
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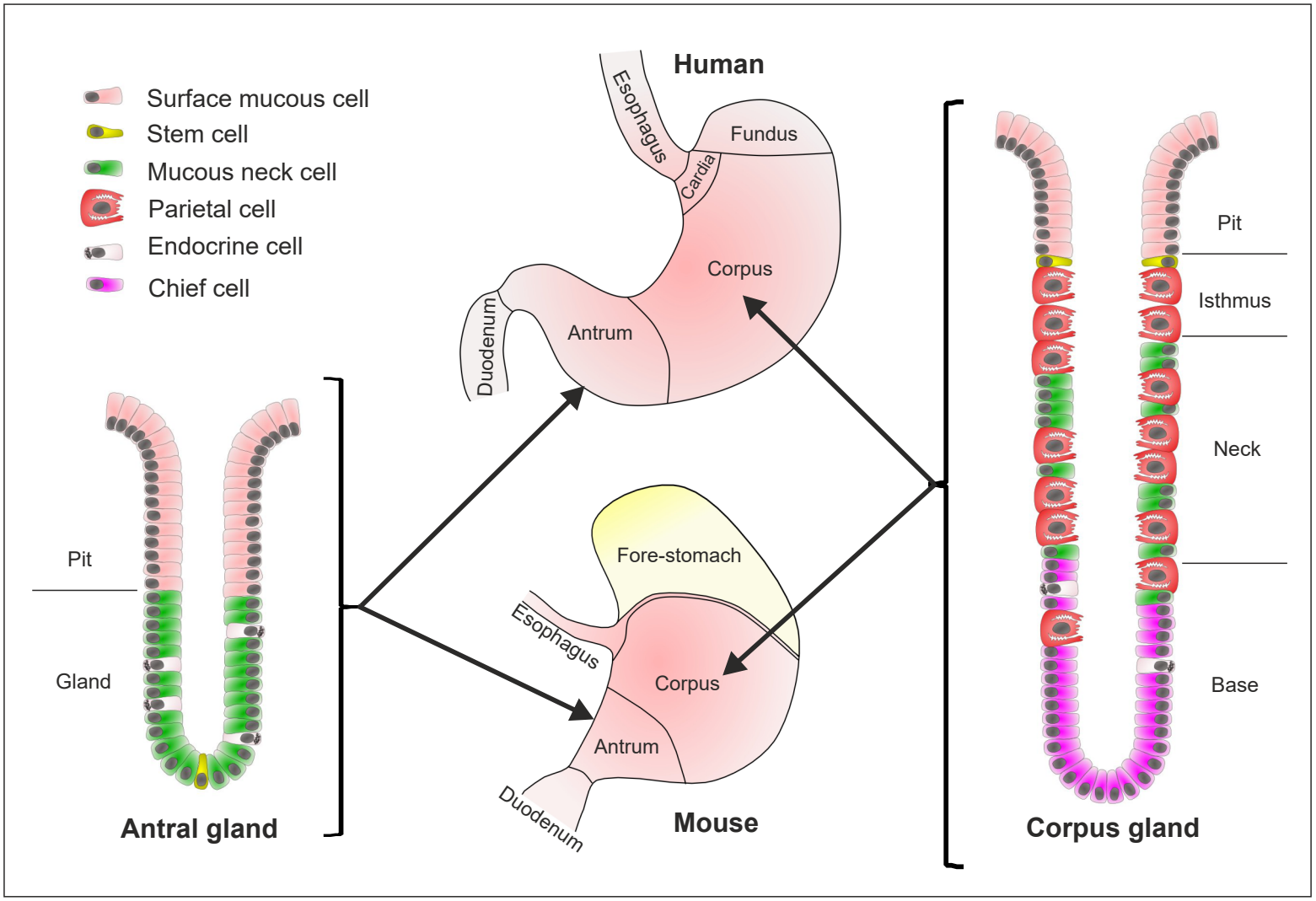
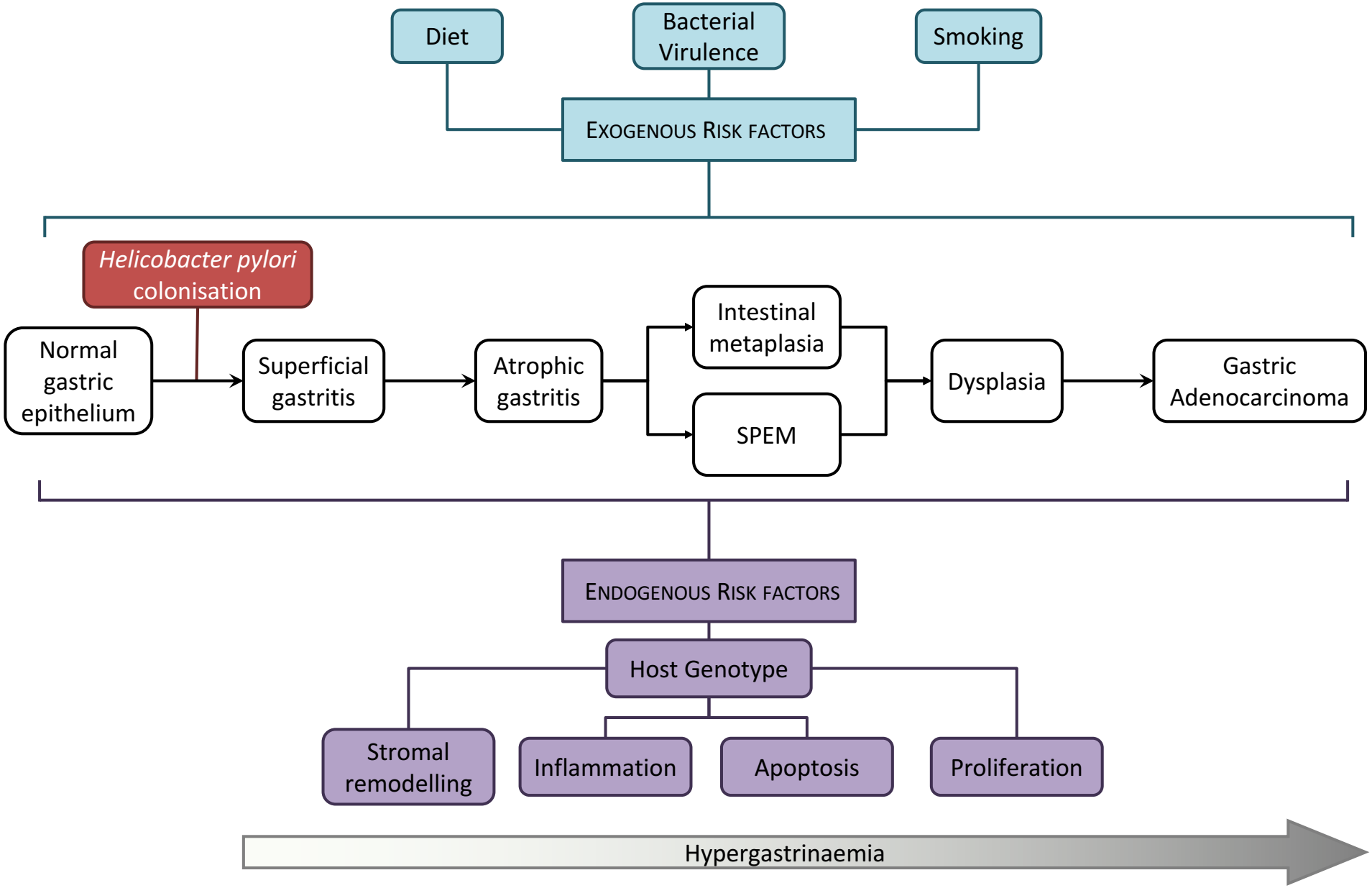
INDONESIA
MALAYSIA
THAILAND
PHILIPPINES
AUSTRALIA
NEW ZEALAND


Surface mucous cell
Stem cell
Mucous neck cell
Parietal cell
Endocrine cell
Chief cell
Human
Esophagus
Cardia
Fundus
Corpus
Antrum
Duodenum
Pit
Gland
Antral gland
Fore-stomach
Esophagus
Corpus
Antrum
Duodenum
Mouse
Pit
Isthmus
Neck
Base
Corpus gland


Diet
Bacterial Virulence
Smoking
EXOGENOUS RISK FACTORS
Helicobacter pylori colonisation
Normal gastric epithelium
Superficial gastritis
Atrophic gastritis
Intestinal metaplasia
SPEM
Dysplasia
Gastric Adenocarcinoma
ENDOGENOUS RISK FACTORS
Host Genotype
Stromal remodelling
Inflammation
Apoptosis
Proliferation
Hypergastrinaemia

Helicobacter pylori colonisation

Mongolian gerbils colonised with *H. pylori*

Superficial gastritis

Gnotobiotic piglets infected with *H. suis*

Peptic ulcer disease

Gnotobiotic piglets infected with *H. suis*

MALT lymphoma

H. felis or *H. heilmanii* infection of Balb/c mice

H. heilmanii infection of *C57BL/6* mice

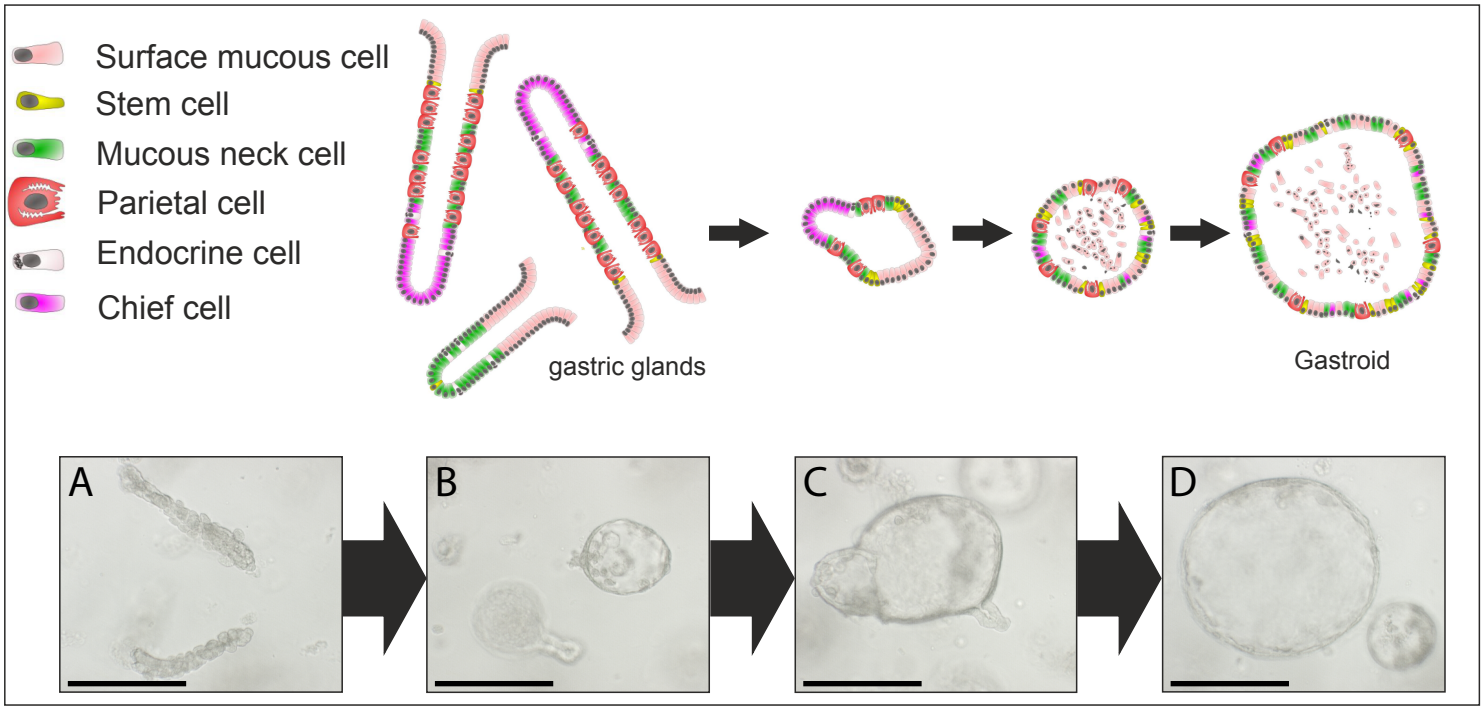
Gastric adenocarcinoma

H. pylori infection of rhesus macaques

Mongolian gerbils infected with *H. pylori* or *H. felis*

H. felis infection of C57BL/6 mice

H. pylori or *H. felis* infection of INS-Gas mice


Surface mucous cell
Stem cell
Mucous neck cell
Parietal cell
Endocrine cell
Chief cell
gastric glands
Gastroid
A
B
C
D