Original Paper

The effect of gender on Helicobacter felis-mediated gastritis, epithelial cell proliferation, and apoptosis in the mouse model

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Abstract

The murine Helicobacter felis model has been extensively used to investigate the importance of host factors in the development of chronic gastritis. The effect of gender in this murine model is unknown. Male and female C57BL/6J mice were infected with H felis for up to 1 year. At 4, 8, 19, 36, and 52 weeks post-infection, gastric histopathology, epithelial cell proliferation, and apoptosis were examined and compared with age- and gender-matched controls. In female mice, infection with H felis resulted in an earlier onset of chronic gastric inflammation, epithelial hyperplasia, and oxyntic cell loss than males. In females, there was a trend towards increased gastric pathology compared with males, with long-term-infected female mice having significantly greater (p < 0.05) chronic inflammation than male mice. The histopathological differences in male and female mice did not relate to the density of H felis infection. Female mice infected with H felis had significantly increased gastric epithelial cell proliferation in the cardia and corpus at both 8 and 52 weeks post-infection (p < 0.05). Epithelial cell apoptosis in the glandular mucosa of the corpus at 36 and 52 weeks post-infection was significantly increased (p < 0.05) in female mice compared with uninfected gender controls. In contrast, there was no significant increase in epithelial cell proliferation or apoptosis in any area of the stomach at any time point after H felis infection in male mice. These results demonstrate that there are gender differences in the gastric inflammatory and epithelial response to H felis in the murine model. The functional importance of gender should be considered in future murine studies on H felis- and H pylori-induced chronic gastritis. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords: gastritis; Helicobacter; epithelial cell proliferation; apoptosis; gender

Introduction

Gender differences occur in several autoimmune diseases. Females have a much higher incidence of multiple sclerosis, systemic lupus erythematosus, encephalomyelitis, Sjogren’s syndrome, and rheumatoid arthritis than males [1,2]. Gender differences in disease susceptibility have also been observed in animal models of autoimmune diseases [3–5]. Gender-related disease susceptibility has been attributed to differences in immune responses, hormonal effects and sex-linked genetic factors [1–5].

Increased susceptibility of females has been observed in several animal infection models. Female mice, or naïve female mice administered oestrogens, are more susceptible to infection with Staphylococcus aureus, Salmonella typhimurium, and Listeria monocytogenes than males [6,7]. In contrast, male mice are more susceptible to infection with other pathogens such as Mycobacterium avium [8], Trichurus muris [9], and Plasmodium chabaudi [10] than females.

Helicobacter pylori infection has an important role in both peptic ulcer disease and gastric cancer, two conditions that are more frequent in males than in females [11]. Two large epidemiological studies have reported a higher H pylori sero-prevalence in males [12,13], which may be relevant to gender differences in the incidence of gastric cancer. Other gender-related factors such as sex-related differences in immune responses [2,3], hormonal effects [5] or differences in bacterial colonization [14] may also be relevant in H pylori-related clinical disease.

The H felis murine model has been used extensively to examine the effects of Helicobacter infection on gastric pathology [15–18] and to delineate the importance of host factors in chronic gastritis [19,20]. In C57BL/6 mice, corpus-predominant histopathological changes are observed after H felis infection. These are considered, in part, to represent an autoimmune response [17]. In this murine strain, H felis infection is associated with increased gastric epithelial cell proliferation and apoptosis [21]. Furthermore, the epithelial proliferative response induced by H felis is greater than that observed with H pylori infection [22]. To date, the influence of gender on Helicobacter-induced
gastrointestinal histopathology and related changes in gastrointestinal epithelial responses has not been examined. The aims of this study were to use the *H. felis* murine model to evaluate the effects of gender on the severity of long-term *Helicobacter* infection on gastrointestinal pathology, epithelial cell proliferation, and apoptosis.

**Materials and methods**

**Bacterial culture**

*H. felis* ATCC 49 179 (kindly provided by Dr R Fernero) was grown on 5% (v/v) horse blood agar plates supplemented with 10 µg/ml vancomycin, 2.5 µg/ml amphotericin B, 25 ng/ml polymyxin B, and 5 µg/ml trimethoprim at 37°C for 2–3 days in a microaerophilic atmosphere using CampyPaks™ (Oxoid, Basingstoke, UK).

**Murine infection with *H. felis***

Specific pathogen-free 6- to 8-week-old C57BL/6J female and male mice were inoculated three times by oral gavage with *H. felis* ATCC 49 179 (>10⁸ CFU) suspended in tryptose soya broth (Oxoid) over a period of 5 days. Inoculated and control mice were fed on a commercial pellet diet and water *ad libitum*. One hour prior to sacrifice, mice received an intra-peritoneal injection of bromodeoxyuridine (BrdU) (50 mg/kg). Experiments were conducted in accordance with the Home Office (Scientific Procedures) Act 1986 and approved by the Ethical Committee of the University.

**Histological and microbial analysis of infection**

Mice were sacrificed at 4, 8, 19, 36, and 52 weeks post-infection. Each group consisted of between five and ten mice, with age- and gender-matched controls. Stomachs were opened along the greater curvature and rinsed in sterile phosphate buffered saline. Gastric mucosal samples were taken for biopsy urease test, microbial culture, and histology. Microbial culture of *H. felis* from gastric biopsies was on selective plates as above at 37°C for 5–7 days. Gastric tissue for histology was fixed in 10% (v/v) formal saline. Sections were stained with haematoxylin and eosin and modified Giemsa for identification of *Helicobacter*. Histological and bacterial density was graded on a scale of 0–3, with 0 being histologically normal, 1 mild, 2 moderate, and 3 severe abnormality. Each animal was scored for chronic inflammation, oxyntic cell loss, epithelial hyperplasia, and mucous metaplasia.

**Epithelial cell proliferation**

The proliferation of gastric epithelial cells was determined by BrdU immunohistochemistry on formalin-fixed sections as previously described [22], using a murine monoclonal antibody to BrdU (Dako Ltd; diluted 1:100 in TBS). Bound antibodies were detected with biotinylated rabbit anti-mouse immunoglobulins (Dako Ltd; diluted 1:200 in TBS) and peroxidase streptavidin complex (Dako Ltd). Epithelial cell proliferation was counted in the cardia, corpus, and antrum. In each area, 10–15 well-orientated gastric pits (~500–1000 cells) were counted. Epithelial cell proliferation was expressed as a labelling index (LI%) ([percentage of stained cells/total cells per gastric gland] × 100).

**Apoptosis**

To detect apoptotic cells in the gastric epithelium, the terminal deoxynucleotide nick-end labelling (TUNEL) assay (Apoptag™) was used (Intergen, Oxford, UK) according to the manufacturer's directions. Briefly, deparaffinized tissue sections were incubated for 15 min with proteinase-digesting enzyme (20 µg/ml; Intergen) before incubation for 1 h at 37°C in terminal deoxynucleotidyl transferase (TdT) enzyme. Following washing, sections were incubated with peroxidase-conjugated anti-digoxigenin antibody. TUNEL-positive cells were detected by incubation in 3,3′-diaminobenzidine tetrahydrochloride. As negative controls, sections were incubated with reaction buffer only and the TdT enzyme was omitted. The number of TUNEL-positive epithelial cells was counted in the cardia/corpus and antrum. In both areas, approximately 20 well-orientated gastric pits (~1000 cells) were counted. Epithelial apoptosis was expressed as an apoptotic index (AI%) ([percentage of stained cells/total cells per gastric gland] × 100). The number of apoptotic epithelial cells in the gastric mucosa was determined for both the superficial epithelium (top 5%) and the glandular/foveolar epithelium.

**Statistical analysis**

Non-linear histopathological and bacterial density scores were expressed as median (range). Labelling and apoptotic indices for animal groups were expressed as mean ± SEM. Comparison of groups of animals was undertaken using a Mann–Whitney U-test. A *p* value of less than 0.05 was considered significant.
Results

Infection rate and bacterial density in male and female C57BL/6J mice

Male and female C57BL/6J mice were inoculated with *H. felis* and sacrificed at 4, 8, 19, 36, and 52 weeks post-infection. Each group consisted of between five and ten animals, with age- and gender-matched controls. Infection was determined by bacterial culture (weeks 4 and 8), gastric biopsy urease test, and by histological detection of *H. felis* on Giemsa-stained sections or sections immunolabelled with a rabbit polyclonal *H. felis* antiserum. A 100% infection rate was observed in all mice inoculated with *H. felis*. Uninfected controls were negative in all tests.

The level of *H. felis* colonization in male and female mice was assessed using modified Giemsa-stained gastric sections. In both sexes, the level of *H. felis* colonization in the corpus increased steadily with time post-infection, with a significant increase in bacterial density between 4 and 52 weeks post-infection (Table 1) (*p* < 0.05). At 4 weeks post-infection, *H. felis* colonized only the cardia and antrum. However, at later time points, *H. felis* was also observed in the corpus. No significant difference in *H. felis* colonization of the corpus or antrum was evident between male and female mice at any time point (Table 1), although bacterial density in the female mice decreased in the antrum at 19 weeks post-infection.

Gastric histopathology

No pathological changes were evident in the gastric mucosa of uninfected control mice. Mild chronic inflammation, epithelial hyperplasia, and oxyntic cell loss developed in female mice by 8 weeks post-infection (Figures 1A–1C and Table 2). In contrast, male mice only developed mild chronic inflammation by 19 weeks post-infection. Between 8 and 52 weeks post-infection, females showed a trend towards increased chronic inflammation, epithelial hyperplasia, and oxyntic cell loss compared with males (Figures 1A–1C and Table 2). Chronic inflammation at 52 weeks post-infection in females was significantly greater (*p* < 0.05) than in males (Table 2). Only 3/6 (50%) male mice had chronic inflammation at 52 weeks post-infection, in contrast to 10/10 (100%) female mice. Both oxyntic cell loss and epithelial hyperplasia were evident at 8 weeks post-infection in females. In contrast, in males, oxyntic cell loss and epithelial hyperplasia were only evident at 52 weeks and 36 weeks post-infection, respectively, and were associated with chronic inflammation.

Gastric epithelial cell proliferation

Figure 2 illustrates immunohistochemical labelling of BrdU in the gastric mucosa. There were no significant differences in gastric epithelial cell proliferation between uninfected male and female mice at any time point in the cardia, corpus or antrum (Figure 3). Uninfected male and female mice had low levels of epithelial cell proliferation in the cardia, with the number of proliferating cells in the gastric mucosa increasing distally. Epithelial cell proliferation was greater in the antrum than in the cardia or corpus in infected mice (Figures 3A–3F).

At 4 weeks post-infection, neither male nor female mice exhibited any change in epithelial cell proliferation in any area of the stomach compared with uninfected gender controls. However, in the cardia, significantly increased (*p* < 0.05) epithelial cell proliferation

Table 1. Bacterial density score (median (range)) in the corpus and antrum of male and female C57BL/6J mice infected with *H. felis* for 4, 8, 19, 36, and 52 weeks post-infection

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Corpus</th>
<th></th>
<th>Antrum</th>
<th></th>
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<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>4</td>
<td>0.5 (0–2)*</td>
<td>1.0 (0–2)*</td>
<td>3.0 (3–3)</td>
<td>3.5 (2–4)</td>
</tr>
<tr>
<td>8</td>
<td>0.5 (0–1)</td>
<td>1.0 (1–2)</td>
<td>3.0 (3–3)</td>
<td>4.0 (4–4)</td>
</tr>
<tr>
<td>19</td>
<td>3.0 (1–3)</td>
<td>1.5 (1–3)</td>
<td>3.5 (3–4)</td>
<td>2.0 (1–3)</td>
</tr>
<tr>
<td>36</td>
<td>2.0 (1–5)</td>
<td>3.0 (1–4)</td>
<td>3.5 (2–4)</td>
<td>3.0 (2–4)</td>
</tr>
<tr>
<td>52</td>
<td>3.0 (1–5)*</td>
<td>4.0 (1–5)*</td>
<td>4.0 (3–4)</td>
<td>4.0 (3–4)</td>
</tr>
</tbody>
</table>

Corpus bacterial density was scored on a scale of 0–5. Antral bacterial density was scored on a scale of 0–4. Data are expressed as median (range).

* *p* < 0.05 (Mann–Whitney U-test) for comparison of bacterial density at 4 and 52 weeks post-infection in both male and female mice.

Table 2. Gastric pathology in male and female C57BL/6J mice infected with *H. felis* for 4, 8, 19, 36 and 52 weeks

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Chronic inflammation</th>
<th>Epithelial hyperplasia</th>
<th>Oxyntic cell loss</th>
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<tbody>
<tr>
<td></td>
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<td>Male</td>
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<tr>
<td>4</td>
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<tr>
<td>8</td>
<td>0.0 (0–0)</td>
<td>1.0 (0–2)</td>
<td>0.0 (0–0)</td>
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<tr>
<td>19</td>
<td>0.0 (0–1)</td>
<td>1.0 (0–2)</td>
<td>0.0 (0–0)</td>
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<tr>
<td>36</td>
<td>0.5 (0–1)</td>
<td>1.0 (0–2)</td>
<td>0.0 (0–0)</td>
</tr>
<tr>
<td>52</td>
<td>0.0 (0–1)*</td>
<td>1.0 (1–2)*</td>
<td>0.0 (0–0)</td>
</tr>
</tbody>
</table>

Histopathological assessment of chronic inflammation, epithelial hyperplasia, and oxyntic cell loss. Gastric pathology was scored from haematoxylin and eosin-stained gastric sections by a single pathologist on a scale of 0–3. Data are expressed as median (range).

* *p* < 0.05 (Mann–Whitney U-test) for comparison of 52 week male versus female. No inflammation was observed in male mice at 4 or 8 weeks and at 4 weeks in female mice. Chronic inflammation was not observed in uninfected male and female controls.

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Figure 1. Gastric mucosae of male and female C57BL/6J mice. Haematoxylin and eosin-stained sections of gastric corpus mucosa from (A) male and (B, C) female mice 52 weeks post-infection with \textit{H. felis}. (A, B) bar = 500 µm; (C) bar = 100 µm. The arrow in B indicates an area of epithelial hyperplasia and oxyntic cell loss. The arrow in C indicates a parietal cell in the region with epithelial hyperplasia. (D) TUNEL assay performed on \textit{H. felis}-infected gastric mucosa illustrating apoptosis of shed surface epithelial cells. Bar = 100 µm. (E) Higher magnification of an apoptotic cell. Bar = 25 µm.

Figure 2. (A) Immunohistological detection of proliferating epithelial cells in the gastric mucosa of a \textit{H. felis}-infected C57BL/6J mouse using an anti-BrdU monoclonal antibody. Mice were injected 1 h prior to sacrifice with BrdU (50 mg/kg). (B) No primary antibody control. Bars = 200 µm. The arrow indicates an example of proliferating cells.

at 8 weeks and 52 weeks post-infection was evident in females compared with uninfected controls ($p < 0.05$) (Figure 3B). In contrast, no increase in epithelial cell proliferation in the cardia of male mice compared with gender-matched controls was evident at any time point (Figure 3A). In the corpus, similar changes in epithelial cell proliferation were observed in \textit{H. felis}-infected female mice to those seen in the cardia (Figure 3D).
Again, no significant changes were observed at any time point in male mice infected with *H. felis* compared with controls (Figure 3C). In contrast, females had a significant increase (*p* < 0.05) in epithelial cell proliferation in the corpus at both 8 and 52 weeks post-infection (Figure 3D). No significant increase in epithelial cell proliferation was observed in the antrum at any time point in either male or female mice infected with *H. felis* (Figures 3E and 3F).

**Gastric epithelial cell apoptosis**

The TUNEL assay was used to assess epithelial apoptosis in the gastric mucosa of *H. felis*-infected mice. An AI% was determined for the corpus/cardia region and the antral mucosa. Apoptosis in the surface epithelium (the top 5% of the gastric mucosa) and the glandular/foveolar epithelium was determined separately. In the surface epithelium, apoptotic TUNEL-positive cells were frequently observed being shed from the epithelial surface (Figures 1D and 1E). The level of apoptosis in the surface epithelial cells did not increase with *H. felis* infection in either male or female mice at any time point (data not shown).

The level of apoptosis in the glandular epithelium of the corpus and antral mucosa of male and female *H. felis*-infected and control mice is shown in Figures 4A and 4B. At 52 weeks, uninfected female mice had significantly greater (*p* < 0.05) levels of...
apoptosis in the glandular epithelial cells in the

corpus than male mice (Figure 4A). In females, apoptosis

in the corpus glandular epithelium was significantly

increased at 36 and 52 weeks post-infection compared

with uninfected controls ($p < 0.05$) (Figure 4A). In

contrast, no difference in epithelial cell apoptosis was

evident at any time point in males infected with

$H.\ felis$ compared with uninfected male controls. In

the antrum, no significant differences were observed

in apoptosis in the glandular epithelial cells in $H.\ felis$-

infected male or female mice compared with uninfected

gender-matched controls (Figure 4B); however, higher

levels were observed in $H.\ felis$-infected mice at 36 and 52

weeks.

Discussion

In this study, the gastric inflammatory and epithe-

lial cell response of male and female C57BL/6J mice
to long-term chronic infection with $H.\ felis$ has been
examined. Female mice developed chronic inflamma-
tion, epithelial hyperplasia, and oxyntic cell loss earlier
than male mice. A trend towards increased severity of
these changes was also evident in female compared

with male mice. In female, but not male mice, $H.\ felis$
infection was associated with significant increases in

both gastric epithelial cell proliferation and apopto-
sis in the glandular epithelium. The results of the

present study demonstrating sexual dimorphism in the

murine gastric responses to $H.\ felis$ emphasizes the

functional importance of considering gender in experi-

mental studies. Previous studies on gastric infection

with $H.\ felis$ or $H.\ pylori$ in mice have used, variably,
female [15,17,21–28], male [29–32], and in some cases 'pooled' mice of mixed gender [33–36]. Of con-

cern, in light of the data presented here, in several

studies, particularly those involving genetically modi-

fied mice, no details of gender are given [19,37–44].

Greater consideration of gender should be taken in

future studies.

No differences in bacterial density were apparent

between male and female mice. The gender-related

differences in the gastritis, epithelial cell proliferation,

and apoptosis observed in this study cannot there-

fore be related to differences in the level of bacte-

rial infection. In contrast, $H.\ pylori$ infection density

in BALB/c male mice was higher than in female mice [14]. This increased bacterial burden was particu-

larly pronounced in IL-4-R$^{-/-}$ mice [14]; however,
gender-associated differences in histopathology were

not examined. In the present study, an increase in bac-

terial density in the corpus was observed with time

post-infection in both males and females, confirming

earlier studies [17]. In BALB/c mice, acid suppressive

therapy results in increased colonization of $H.\ felis$
in the corpus [23]. The increased corpus colonization

with long-term infection observed in the present study

is likely to be a consequence of parietal cell loss.

The present study demonstrated that female mice,

infected long-term with $H.\ felis$, have increased gastric

epithelial cell proliferation and apoptosis in the cardia

corpus mucosa. This increase was not observed in

males infected with $H.\ felis$. No other studies to date

have compared the effect of gender on gastric epithe-
lial cell proliferation and apoptosis in $Helicobacter$
infection in the murine model. Low levels of epithelial

cell apoptosis were evident in the surface epithelium in

both $H.\ felis$-infected and uninfected mice. A similar

observation was noted by others in $H.\ felis$- [21] and

$H.\ pylori$-infected [24] mice and is considered to repre-

sent normal epithelial cell turnover. In contrast, $H.\ felis$
infection was associated with significant increases in

apoptosis in the glandular epithelium of the corpus

in female mice at 36 and 52 weeks. Increased epithe-
lial apoptosis in the corpus of $H.\ felis$-infected female

C57BL/6 mice has been previously reported [21]. The

increase in apoptosis in the latter study was at an ear-

erlier time point (6 weeks) than in the present study;
however, the localization of apoptosis was consist-
tent, with increases in the glandular mucosa but not in

the surface epithelium. In contrast to the results of

the present study, Houghton et al [29] observed

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increased apoptosis in the antrum of *H felis*-infected male C57BL/6J mice compared with controls. Apoptosis was increased from 1 week to 11 weeks post-infection, when levels returned to those observed in control mice [29]. Minimal changes in apoptosis were observed in the corpus of *H felis*-infected male mice by Houghton *et al* [29], which is confirmed by the present study.

In agreement with previous studies [21], gastric epithelial cell proliferation was significantly increased in the cardia and corpus of *H felis*-infected female C57BL/6J mice compared with control mice. Interestingly, Fox *et al* [33] infected both male and female p53 hemizygous C57BL/6 mice with *H felis* and examined gastric epithelial cell proliferative responses. Gender differences in histopathology and epithelial cell proliferation were not examined, but differences in systemic IgG responses to *H felis* were observed. At 10 weeks post-infection, p53 hemizygous female mice had substantially elevated serum IgG compared with p53 hemizygous male mice [34]. These observations on gender differences in serological responses to *H felis* infection complement the findings of the present study. Gender differences in ethanol-induced ulceration and gastritis have been previously observed in rats [45]. Female rats with gastritis had increased levels of gastric epithelial cell proliferation and smaller areas of ulceration compared with males. Elevated epithelial cell proliferation observed in female rats was considered to account for the reduced ulceration. Oophorectomized rats developed larger ulcers than ovari-intact rats [45], suggesting that sex hormones could have a role in the observed gender differences.

Gender variation in patterns of infectious and autoimmune diseases may relate to sex differences in immune responses and cytokine production. In experimental murine models of autoimmune disease, gender differences in susceptibility have been attributed to males producing more IL-10 and IL-4 and less IL-12 and IFN-γ than females [3,4]. Testosterone acts directly on CD4+ cells to promote IL-10 secretion [46]. Administration of IL-12 to male mice promotes autoimmune responses [4] and, conversely, testosterone therapy in female mice reduces the severity of experimental autoimmune encephalomyelitis [5]. Enhanced IL-12 responses in females has been attributed to oestrogen activation of STAT4 [3]. In humans [47] and mice [31], infection with *H pylori* results in a Th1 gastric response and activated Th1 lymphocytes may promote epithelial apoptosis via Fas/Fas ligand interactions [48]. IL-12 is considered to have a key role in polarizing the Th1 response in *H pylori* infection [49]. As the corpus-predominant gastritis in the murine *H felis* model is considered, in part, to be autoimmune in nature [17], decreased IL-12 production to immune stimuli in male mice [3,4] may be important in the observed gender differences in gastric pathology. Further studies examining gender differences in cytokine responses to *H pylori* in the murine model would clarify this issue.

More recently, the Th1-promoting effects of leptin have been identified as important in experimentally induced autoimmune diseases. Leptin treatment confers susceptibility to male mice and switches the cytokine response from a Th2 to a Th1 profile [50]. As both female mice and humans have elevated leptins compared with males, it has been proposed that leptin may have an important role in gender differences observed in autoimmune diseases [51]. Both leptin mRNA and leptin protein are found in the rat gastric epithelium [52] and *H pylori* infection is associated with elevated leptin transcripts in human gastric mucosa in *H pylori* infection [53,54]. Additionally, leptin increases proliferation in gastric epithelial cell lines [55]. Gastric leptin may therefore have a role in the elevated epithelial cell proliferation observed in response to *H felis* infection in female mice. Further studies of gastric leptin in male and female *H felis*-infected mice may shed light on gender differences in mucosal responses.

In conclusion, the results of this study indicate gender differences in the murine model of *Helicobacter* infection. Female C57BL/6J mice represent a better model than male C57BL/6 mice for research into the pathogenesis of gastric *Helicobacter* infection, particularly when determining the role of proinflammatory *H pylori* virulence factors [31]. Further investigation into gender-related dimorphism of responses to gastric *Helicobacter* infection in other strains of mice would be beneficial. Some epidemiological studies have observed a higher prevalence of *H pylori* infection in males, which is likely to be relevant to the increased incidence of gastric cancer in men [12,13]. Whether there are gender-related differences in mucosal cytokine and epithelial responses to *H pylori* in humans is unknown.

Acknowledgements

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The effects of gender on murine *H. felis* gastric pathology

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