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ADDENDUM

Helicobacter pylori and gut microbiota modulate energy homeostasis prior to inducing histopathological changes in mice

Yalda Khosravi[a], Ralph M Bunte[b], Kher Hsin Chiow[c], Tuan Lin Tan[c], Whye Yen Wong[c], Qian Hui Poh[c], Ignatius Mario Doli Sentosa[c], Shih Wee Seow[d], Arlaine Anne Amoyo[d], Sven Pettersson[d,e,f], Mun Fai Loke[a], and Jamuna Vadivelu[a]

[a]Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia; [b]Duke-NUS Graduate Medical School, Singapore; [c]School of Chemical and Life Sciences, Singapore Polytechnic, Singapore; [d]National Cancer Center, Singapore; [e]Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institutet, Stockholm, Sweden; [f]LKC School of Medicine, Nanyang Technological University, Singapore

ABSTRACT

Helicobacter pylori have been shown to influence physiological regulation of metabolic hormones involved in food intake, energy expenditure and body mass. It has been proposed that inducing *H. pylori*-induced gastric atrophy damages hormone-producing endocrine cells localized in gastric mucosal layers and therefore alter their concentrations. In a recent study, we provided additional proof in mice under controlled conditions that *H. pylori* and gut microbiota indeed affects circulating metabolic gut hormones and energy homeostasis. In this addendum, we presented data from follow-up investigations that demonstrated *H. pylori* and gut microbiota-associated modulation of metabolic gut hormones was independent and precedes *H. pylori*-induced histopathological changes in the gut of *H. pylori*-infected mice. Thus, *H. pylori*-associated argumentation of energy homeostasis is not caused by injury to endocrine cells in gastric mucosa.

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Introduction

The human gastrointestinal (GI) tract consists of an upper (mouth, esophagus, stomach, duodenum, jejunum and ileum) and a lower part (cecum, colon, rectum and anus).[1,2] It has one of the most complex microbial ecosystems.[3] Composition of GI microbiota can be modified by physiological changes, such as aging[4] and pregnancy.[5] Several factors that can contribute to these modifications include: immunological or infectious diseases,[6] antibiotic treatment and metabolites. Furthermore, the GI microbiota is involved in diverse normal host functions, including energy harvest and storage from the diet[4] development and regulation of the gut-associated mucosal immune system,[6] regulation of the central nervous system modulating brain development and behavior,[7] protection against colonization by pathogens[8] and detoxification of xenobiotics and carcinogens.

Unlike the oral cavity, stomach and colon, a few studies have suggested that the esophagus, is either sterile or includes only a few transient bacteria originating from the oropharynx by a swallowing process or from the stomach by gastrooesophageal reflux.[9] Nevertheless, several pathogenic microorganisms, such as *Candida albicans, Cryptococcus* or Herpesvirus, can infect the esophagus.[10] In the stomach, *Helicobacter pylori* has the ability to survive in the extremely acidic environment by secreting urease which converts urea to ammonia.[11] More than 50% of the world population is infected by this pathogenic bacterium[12] which can cause a range of gastric diseases such as peptic ulcers, gastric cancers, and mucosa-associated lymphoid tissue (MALT) lymphoma.[13,14] Recently, non-*Helicobacter* species representing 3 main bacterial phyla (Firmicutes, Proteobacteria and Actinobacteria) were isolated from human gastric biopsies of patients with symptoms involving the gastroduodenal tract indicating that *H. pylori* is not the only bacterium that can be found in the acidic environment of the stomach.[12] Among these

CONTACT Jamuna Vadivelu jamuna@ummc.edu.my

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non-*Helicobacter* species, *Streptococcus* species (*S. mitis, S. parasanguinis* and *S. anginosus*) were most frequently isolated. These organisms are commonly found in the human oral cavity and were also found to be present in the lower gut.[15] *In-vitro* experiments have shown that bile acids can transform *H. pylori* to the viable but non-culturable (VBNC) coccoidal form.[16] Therefore, it is possible that in the bile-laden, anaerobic environment of the lower GI tract, *H. pylori* might exist in the VBNC state.[17] The interaction between *H. pylori* and other microbiota of the GI (including *S. mitis*) can also lead to *H. pylori* converting to the VBNC form.[18]

H. pylori affecting gut hormones and energy homeostasis

Leptin and ghrelin are 2 important hormones that influence on energy homeostasis in humans.[19] The effect of leptin on energy homeostasis is opposite to that of ghrelin; leptin induces weight loss by suppression of food consumption, while ghrelin functions as an appetite-stimulatory signal.[20] *H. pylori*, which infects the human stomach and interacts with host tissues,[21] may affect the regulation of hormones that are involved in energy homeostasis, such as ghrelin and leptin.[19] However, effects of *H. pylori* infection on the expression of ghrelin and leptin in hosts are controversial.[20,22,23] Tatsuguchi et al. demonstrated that ghrelin-positive cells in the gastric mucosa were significantly lower in *H. pylori*-infected adult patients than for healthy controls with an inverse correlation between ghrelin immunoreactivity and inflammation/ activity grade.[22] Likewise, Isomoto et al.[24] reported on the relationship between degree of *H. pylori*-associated gastritis and lower plasma ghrelin levels in-infected adult patients.[24] Furthermore, a recent study on comparison between *H. pylori*-infected and non-infected children demonstrated that both the serum ghrelin and leptin concentrations were significantly reduced in uninfected children.[25] Tatsuguchi et al. proposed that by inducing gastric atrophy, *H. pylori* damages ghrelin-producing endocrine cells localized in gastric mucosal layers and therefore alter their concentrations.[22] Consistently, Francois et al. reported that circulating meal-associated ghrelin and leptin levels increase after successful H. pylori eradication collaborated by an increase in body mass index (BMI) in these subjects.[20] Host genetics, diet, lifestyle and other confounding factors may influence the outcome of these studies using human subjects: as such, there is a need for *in vivo* studies conducted under controlled conditions.

In a study using a germ-free mouse model, *H. pylori*-induced carcinogenesis was shown to be delayed in the absence of the microbiota suggesting that microbiota plays an important role in *H. pylori*-associated pathogenesis.[26] More recently, Heijtz et al. highlighted the potential importance of gut microbiota for normal brain development during early stages of life in mice.[7] In this study, mice were found to display increased motor activity and reduced anxiety in the absence of gut microbiota as a result of altered expression of genes involved in second messenger pathways and synaptic long-term potentiation in brain regions implicated in motor control and anxiety-like behavior.

To investigate the role of gut microbiota and *H. pylori* in energy homeostasis, the same C57BL/6 specific pathogen-free (SPF) and germ-free (GF) mouse models previously used by Heijtz et al.[7] were adopted by Khosravi et al.[27] to which this addendum relates. Following from the study of Heijtz et al.[7] Khosravi et al. explored the effects of gut microbiota and *H. pylori* on homeostasis of metabolic hormones of the gut-brain axis and circulating cytokines/ chemokines during early development.[27] In this study, 4 weeks old C57BL/6 SPF with normal gut microbiota and GF mice without normal gut microbiota were assigned into control (uninfected) and test (*H. pylori*-infected) groups. SPF and GF mice in the test group were infected with mice-adapted *H. pylori* strain 298 for 2 weeks, 2 months and 4 months. There is no simple answer on making age comparisons between mice and humans. It was estimated that a 1-month-old mouse is equivalent to a 12.5-year-old human adolescent while a mouse of 3–6 months old is comparable to a mature adult human of 20–30 y old.[28] Although mice are generally sexually mature by 35 days, maturational growth continues for most biological processes and structures until about 3 months of age. *H. pylori* infection is often acquired during childhood.[29] In developing countries, such as India, Saudi Arabia and Vietnam, approximately 80% of the population is infected by the age of 20.[30] Even in developed countries, such as South Korea, USA, France, Belgium and Finland, it was estimated that 10–12% of children aged 3–19 y old were infected.[30]

Plasma leptin, insulin and total peptide YY (PYY) were elevated in *H. pylori*-infected SPF (SPFH) mice compared to non-infected SPF mice suggested that *H. pylori* infection altered the host metabolism. However, growth curves of SPF and SPFH mice remained the same. Similarly, acylated (active) ghrelin and PYY were elevated in GF mice infected with *H. pylori* (GFH) compared to non-infected GF mice. Ironically, GFH mice suffered significant weight loss relative to GF mice. Our results in mice confirmed that *H. pylori* and gut microbiota, singly and in combination, influence homeostasis of metabolic hormones of the gut-brain axis, which affects body weight. In contrast to study involving patients, which lifestyle, diet, environment and host genetic differences can be major confounding factors, mice study was carried out under controlled conditions.

Furthermore, plasma eotaxin-1, which plays a role in both inflammation and neurogenesis, was elevated in SPFH mice compared to SPF, GF and GFH mice. Increased eotaxin-1 level in blood plasma has been associated with aging in mice and humans.[31] It has also been demonstrated that exposing young mice to eotaxin-1 or the blood plasma of older mice decreased their neurogenesis and cognitive performance in behavioral tasks, which are dependent on neurogenesis in the hippocampus.[31] Interestingly, *H. pylori* infection alone in GFH mice suppressed circulating eotaxin-1 level. Therefore, it is possible that *H. pylori* exposure during early developmental stages will have long-term implication on brain development.

Metabolic gut hormones changes precede pathological changes

In addition, histology and immunohistochemistry (IHC) were performed on mice tissues from the stomach, small intestine and colon. Histopathological examination of hematoxylin and eosin (H&E) stained samples evaluated by a veterinary pathologist (RBM) revealed mildly-moderately dilated gastric crypts in 2 of the 5 16-weeks *H. pylori*-infected GF mice, which were also present in one of the 5 uninfected 16-weeks control GF mice. In the infected group, dilated crypts were seen in one animal in the fundic stomach and in the other in the pyloric stomach. In the control mice, dilated crypts were seen in the pyloric stomach. The dilated crypts did not contain any inflammatory cells. In summary, these dilated gastric crypts are not uncommon in mice and are within normal limits.

H. pylori infection can affect neuronal expressions in the stomach of mice, which may explain the dyspepsia symptoms in *H. pylori*-infected patients. *H. pylori*-infected mice were shown to have enhanced neuronal expressions of substance P (SP), c-fos, vasoactive intestinal polypeptide (VIP) and calcitonin gene-related peptide expressions (CGRP) in their stomach.[32,33] *H. pylori* cytotoxin-associated gene (*cag*) pathogenicity island, which is associated with cancer risk, *cag*, encodes for a secretion system that transports effectors into host cells leading to aberrant activation of β-catenin.[34] β-catenin affects oncogenesis in conjunction with peroxisome proliferator-activated receptor (PPAR)δ.[35] The expression of 6 biomarkers (SP, c-fos, VIP, CGRP, PPARδ and β-catenin) in mice gastric tissue samples were assessed by IHC.[36] Heat-induced epitope retrieval in 10 mM citrate buffer (pH 6.0) was used on paraffin-embedded sections in this study.[37] Goat polyclonal anti-SP (NC-18; sc-9758), rabbit polyclonal anti-c-fos (sc-52), rabbit polyclonal anti-VIP (H-95; sc-20727), rabbit polyclonal anti-CGRP (H-48; sc-28920), rabbit polyclonal anti-PPARδ (H-74; sc-7197) and rabbit polyclonal anti-β-catenin (H-102; sc-7199) at 1:50 dilutions were used as primary antibodies. Donkey anti-goat IgG, F(ab')$_2$-HRP (sc-3851) and goat anti-rabbit IgG F(ab')$_2$-HRP (sc-3837) at 1:100 dilutions were used as secondary antibodies. Primary and secondary antibodies used were from Santa Cruz Biotechnology, Inc.. Normal donkey serum (Santa Cruz) was used for blocking. Labeled StreptAvidin Biotin (LSAB) kit (DAKO) was used for detection. Secondary antibody only control was performed using the same procedure, with PBS substituting for primary antibodies. For immunostaining of SP, c-fos, VIP and CGRP-expressing neurons in the mouse stomach, cells with cytoplasm or nuclei showing brown-yellow to brown-black were considered positive.[32,33] As for PPARδ, epithelial cells were evaluated in the epithelium of antral mucosa.[35] For β-catenin immunostaining, epithelial cells from well-oriented representative gastric glands were scored.[34] The intensity of positive staining was categorized as follow: 0, negative; 1, mild (brown-yellow); 2, moderate (brown); 3, severe (brown-black). For semi-quantitative analysis of each sample, 100 cells were counted by a single blinded observer and the percentage of positive cells was multiplied by the intensity score.

Comparing all 6 biomarkers between 16-weeks SPF and GF mice, more cells were stained positive in GF mice for c-fos, VIP, CGRP, PPARδ and β-catenin, regardless of *H. pylori* infection. VIP and β-catenin also appeared more abundant in SPFH and GFH mice compared to SPF and GF mice respectively. However, due to the small number of animals in this study and heterogeneity of their distribution in mice stomach, the differences in all 6 biomarkers did not achieved statistical significant. These results are in contrast with multiple studies,[32,33,34] in which expression of these 6 proteins were found to be up-regulated post-infection with *H. pylori* in mice models. This discrepancy may be due to the genetic diversity of *H. pylori* strains used and the duration of infection in mice. Nevertheless, data from this study also showed that *H. pylori*-induced changes in metabolic hormones of the gut-brain axis in mice preceded any observable histopathological changes in the mice stomach. Thus, it is unlikely that *H. pylori*-induced damage to hormone-producing endocrine cells in the gastric mucosal layers were responsible for the augmentation of these metabolic hormones during early stages of *H. pylori* infection. However, that does not rule out the possibility that gastric mucosal damage or inflammation induced by *H. pylori* during later stages of infection may further augment metabolic hormonal balance and energy homeostasis.

Conclusion

This study demonstrated that *H. pylori* and gut microbiota, singly and in combination, influence homeostasis of metabolic hormones of the gut-brain axis (ghrelin, leptin, insulin and peptide YY), which affects body weight, under controlled conditions in mice. Furthermore, the augmentation of gut hormones by *H. pylori* precedes and is independent of histopathological changes associated with infection by the bacterium during early stages of H. pylori infection. Further investigations are necessary to ascertain the long-term impact of the augmented gut hormone profile on health and disease.

Abbreviations

BMI	Body mass index
CGRP	Calcitonin gene-related peptide
GF	Germ-free
GI	Gastrointestinal
H. pylori	*Helicobacter pylori*
HIER	Heat induced epitope retrieval
IHC	Immunohistochemistry
LSAB	Substance P, C-fos, Labeled streptavidin biotin
PIER	proteolytic induced epitope retrieval
PPARβ	β-catenin, peroxisome proliferator-activated receptor β
SPF	Specific pathogen-free
VBNC	Viable but non-culturable
VIP	Vasoactive intestinal peptide.

Disclosure of potential conflicts of interest

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