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Observational Study

Urinary metabolic profiles during *Helicobacter pylori* eradication in chronic gastritis

INTRODUCTION

Chronic gastritis is a common digestive system disease affecting approximately half of the global population[1]. Chronic gastritis is also the most important risk factor for gastric cancer, the fifth most commonly diagnosed cancer and the fourth leading cause of cancer-related mortality[1,2]. Chronic gastritis can be classified into two major stages, non-atrophic and atrophic, according to the phenotypes of the gastric mucosa[3]. Chronic non-atrophic gastritis will develop into chronic atrophic gastritis if left untreated. A 16-year follow-up study revealed that up to 2% of patients with chronic atrophic gastritis develop gastric cancer annually[4]. Additionally, 24% of gastric cancer patients are first diagnosed with chronic atrophic gastritis[5]. Thus, managing chronic gastritis is an important approach for preventing gastric cancer development.

Helicobacter pylori (*H. pylori*) infection, a major risk factor for chronic gastritis, infects approximately 50% of the global population[6,7]. A portion of infected people will develop various degrees of gastrointestinal disease, such as dyspepsia (5%–10%), chronic gastritis (90%), peptic ulcers (15%–20%), and gastric malignancies (1%)[8]. *H. pylori* has been described as a first-class carcinogen for gastric cancer by the World Health Organization since 1994 and accounts for 16.1% of gastric cancer cases[9,10]. A 26.5-year follow-up report indicated that *H. pylori* eradication might confer long-term protection against gastric cancer in high-risk populations[11]. Therefore, eradication of *H. pylori* is recommended to reduce the occurrence of gastric diseases[8]. Numerous double, triple, and quadruple therapies have been proposed as first-line empiric treatments for

H. pylori infection[12]. However, the molecular mechanisms underlying these treatment regimens are complicated and remain unclear[13].

Urinary metabolomics has been gradually applied to mine metabolic profiles for diagnosis, prognostic evaluation, and research of treatment mechanisms in gastric diseases. NMR-based urinary metabolomics revealed that urine metabolite levels were changed during oncogenesis in gastric cancer, and 4-hydroxyphenylacetate, alanine, phenylacetylglycine, mannitol, glycolate, and arginine are potential metabolic biomarkers for effectively diagnosing gastric cancer[14,15]. NMR- and UPLC-Q/TOF MS-mediated urinary metabolomics revealed that a traditional Chinese medicine, Huangqi Jianzhong Tang, treated chronic atrophic gastritis by balancing energy consumption, inhibiting inflammation, improving the immune system, and reducing oxidative stress in rats[16]. UPLC-Q-TOF/MS-based urinary metabolomics has also been applied to investigate the therapeutic effect and potential mechanism of berberine on chronic atrophic gastritis[17] and the therapeutic mechanism of palmatine in chronic atrophic gastritis induced by *H. pylori*[18]. However, no clinical study has been conducted on urinary metabolomics in chronic gastritis.

MATERIALS AND METHODS

Clinical characteristics of participants

This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Shanxi Provincial People's Hospital (Grant No. 2022-167). Patients with *H. pylori*-positive chronic gastritis who were hospitalized or outpatients in the Gastroenterology Department of Shanxi Provincial People's Hospital were selected as the research participants. Patients with *H. pylori*-positive chronic gastritis were enrolled. These patients were diagnosed with chronic gastritis by endoscopy and pathological examination. For the diagnostic criteria of *H. pylori* infection, refer to the "Fifth National Consensus Report on the Treatment of *H. pylori* Infection." Those with a positive ^{14}C or ^{13}C urea breath test (UBT) were diagnosed with *H. pylori* infection. Fasting morning urine was collected from patients diagnosed with *H.*

pylori-positive chronic gastritis and marked as "HP(+)". Subsequently, the patients were treated with therapeutic strategies of *H. pylori* eradication for 2 wk (Figure 1). *H. pylori* eradication was conducted using conventional quadruple therapy, i.e., a proton pump inhibitor combined with two antibiotics and a colloidal bismuth agent. In this study, the quadruple therapy strategies included omeprazole/amoxicillin/furazolidone/bismuth pectin, ilaprazole/amoxicillin/ furazolidone/bismuth pectin, and pantoprazole/amoxicillin/furazolidone/bismuth pectin (Table 1). The patients were subjected to the ^{14}C or ^{13}C UBT at the end of treatment to evaluate *H. pylori* infection severity. The fasting morning urine of *H. pylori*-negative patients was collected and marked as "HP(−)" (Figure 1), and the fasting morning urine of *H. pylori*-negative healthy individuals was marked as "Health" (Figure 1). The HP(−), HP(+), and Health urine samples were subjected to an LC–MS-based metabolomics analysis.

In this study, approximately 180 patients were diagnosed with *H. pylori*-positive chronic gastritis, and their urine samples were collected at the first diagnosis. However, only 17 patients met the clinical assessment inclusion criteria and were willing to be reexamined for *H. pylori* infection after treatment (Table 1). These patients were treated with quadruple therapy strategies for *H. pylori* eradication. After 2 wk of treatment, 17 patients in our study were *H. pylori*-negative and discontinued treatment.

Sample preparation

The first morning urine was collected from fasting patients and healthy individuals and stored at −80℃. The frozen urine samples were thawed in an ice bath and centrifuged at a low temperature for 10 min (10000 ×*g*, 4°C). The supernatant was transferred to a new 1.5 mL EP tube. The proteins were precipitated by adding methanol-acetonitrile (2:1). Then, the mixtures were subjected to ultrasonic extraction in an ice water bath for 10 min and centrifuged for 20 min (10000 ×*g*, 4°C). The supernatant was filtered through a 0.22 μm organic phase pinhole filter and transferred to an LC sample vial. Samples were stored at 4℃ until the LC–MS analysis. In addition, 10 μL of urine from each group was

taken, mixed, and prepared as QC samples according to the sample preparation method.

UHPLC-Q-TOF/MS liquid phase conditions

The mobile phases were A (0.1% formic acid water) and B (0.1% formic acid acetonitrile). Elution was conducted according to the following gradient: 0–2 min, 2% B; 2–3 min, 2%–35% B; 3–17 min, 35%–70% B; 17–18 min, 70% B; 18–29 min, 70%–98% B; 29–31 min, 98% B; 31–33 min, 98%–2% B; and 33–35 min, 2% B. A Waters ACQUITYUPLC HSS T3 (2.1 × 100 mm, 1.7 μm) chromatographic column with a 5 μL injection volume, 0.2 mL/min flow rate, and 40°C temperature was then used for the liquid chromatographic analysis.

UHPLC-Q-TOF/MS mass spectrometry conditions

The mass spectrometry profiles of the urine metabolome were obtained on a UPLC (ExionLC AD) coupled with a Triple TOF 5600+ mass spectrometer (AB Sciex). The mass spectrometry conditions were set as follows: Electronspray ionization (ESI); mode: positive and negative ion scanning; mass scanning range: 50–1500 Da; atomizing gas pressure (GS1) and auxiliary gas pressure (GS2): 0.55 kPa; atomizing gas temperature: 550℃; spray voltage: +5500 V in positive ion mode and −4500 V in negative ion mode; curtain pressure: 0.3 kPa; and cluster fragmentation voltage: 100 V. Data were collected in information association mode, collision energy was ± 35 eV, and the collision energy rolling interval was (35 ± 15) eV.

Data processing of UHPLC-Q-TOF/MS

The raw data from UHPLC-Q-TOF/MS were imported into One-MAP software, and all metabolite names, peak areas, retention times, and other information were calculated. The results were exported as Excel files, and the total peak area data of each group of metabolites were normalized to obtain the peak-normalized data per metabolite.

Multivariate statistical analysis and differential metabolite screening

The above peak-normalized data were imported into Simca-P 14.1. A principal component analysis (PCA) was used for the exploratory analysis to determine possible clusters and outliers, and partial least square discriminant analysis (PLS-DA) and orthogonal partial least square discriminant analysis (OPLS-DA) were performed to explore different metabolites with metabolic profile changes, combining VIP > 1 and a *t* test ($P < 0.05$) in the S-plot to screen different metabolites.

Identification of metabolites

The identification of metabolites was performed by importing the m/z values of metabolites into the One-map database (http://www.5omics.com/) to obtain the names of metabolites. The chemical structures of the metabolites were confirmed by comparing MS/MS data with the compound information in the One-map database.

Metabolic pathway enrichment analysis and ROC curve analysis

The Pathway Analysis module in MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) was used to perform metabolic pathway enrichment analyses on differential metabolites, and the pathway with an impact value (impact) greater than or equal to 0.1 was considered to be the main metabolism path.

The area under the receiver operating characteristic (ROC) curve was used to evaluate the quality and the predictive ability of the classification models. Univariate ROC analysis was conducted, and the area under curve (AUC) and *P* values of each ROC curve were used to evaluate the predictability. Then, to improve the discriminatory accuracy, multivariate ROC curves were plotted with false-positive and true-positive rates using a combination of significant metabolites with AUC > 0.5 (*P* value < 0.05).

Integrated metabolomics and network pharmacology

Integrated metabolomics and network pharmacology were applied to reveal the regulatory network of the identified differential metabolites. First, a metabolite-related network construction was performed by importing the identified differential metabolites into Cytoscape 3.7.2 (Cytoscape Consortium, San Diego, CA, USA) equipped with MetScape. This network was constructed to visualize the interactions among the metabolites, pathways, enzymes, and genes. The key metabolites and proteins were recognized by combining the metabolite-reaction-enzyme-gene network with hub genes and metabolic pathways. Then, the candidate targets of *H. pylori*-positive chronic gastritis were acquired by taking the intersection of targets between chronic gastritis and *H. pylori* infection. The targets of chronic gastritis and *H. pylori* infection were obtained by searching the keywords "chronic gastritis" and "*Helicobacter pylori*" in the Genecards database (https://www.genecards.org/), respectively. Finally, the key proteins involved in regulating the identified differential metabolites were obtained by matching the *H. pylori*-positive chronic gastritis-related targets with the differential metabolite-related targets.

Statistical analysis

GraphPad Prism 8 software was used for generating figures and statistical analyses. Data are presented as the mean ± SD. The normality of data distribution was analyzed with SPSS software. Comparisons between two groups were performed using independent-sample *t* tests; comparisons between multiple groups were performed by one-way ANOVA. $P < 0.05$ was considered to represent significance.

RESULTS

Overall metabolic profiles and the untargeted metabolomics analysis

Metabolic profiling of urine samples was performed in positive and negative ion modes in an unsupervised model without grouping conditions using PCA. The QC samples were clustered, indicating good system stability. The results show that the samples Health, HP(+), and HP(−) cannot be effectively separated (Figure 2A and B). Therefore,

PLS–DA was performed to reduce the dimensionality of the complex data obtained from the Health, HP(+), and HP(−) urine samples to distinguish the differences between groups. The results are shown in Figure 2C–F. The Health, HP(+), and HP(−) samples were significantly separated in ESI- and ESI+ modes, indicating that 2 wk of drug treatment alters urinary metabolic disorders in patients with chronic gastritis. In summary, the untargeted metabolomics analysis indicated that the urinary metabolic profiles changed during *H. pylori* eradication.

Metabolic profile of healthy individuals vs H. pylori-positive chronic gastritis patients

The OPLS–DA model was used to further evaluate the changes in metabolic profile in the sixth week after drug treatment. The CV-ANOVA diagnostics, in which the p-values were 0.0000000894092 (negative ion mode) and 0.00000465157 (positive ion mode), worked well in the OPLS–DA model. The results are shown in Figure 3A and B. The Health and HP(+) groups were clearly separated in the positive and negative ion modes, indicating that the metabolic profiles of the two groups significantly changed. The corresponding S-plot was applied to observe and screen differential variables (Figure 3C and D), of which the values of variable importance in the projection (VIP) were used to evaluate their contribution to the model. The differential metabolites were screened between the Health and HP(+) groups according to S-plots of VIP value (VIP > 1) and a *t* test ($P < 0.05$).

The differential metabolites were imported into the Pathway Analysis module in MetaboAnalyst5.0, and a metabolic pathway analysis was conducted to find the 10 metabolic pathways most related to *H. pylori* eradication: (1) phenylalanine metabolism; (2) phenylalanine, tyrosine, and tryptophan biosynthesis; (3) citrate cycle; (4) glyoxylate and dicarboxylate metabolism; (5) alanine, aspartate, and glutamate metabolism; (6) ubiquinone and other terpenoid-quinone biosynthesis; (7) arginine and proline; (8) aminoacyl-tRNA biosynthesis; (9) glycine, serine, and threonine metabolism; and (10) pyrimidine metabolism (Figure 3E).

Comparison of metabolic profiles of patients at the treatment endpoint with those before the treatment

In both positive and negative ion modes, the OPLS–DA model was used to evaluate the effects of *H. pylori* eradication on metabolic profiles after 2 wk of treatment. The CV-ANOVA diagnostics, in which the p-values were 0.000665011 (negative ion mode) and 0.00535801 (positive ion mode), worked well in the OPLS–DA model. The plot of the scores obtained from the urine samples showed a significant separation between the HP(+) and HP(−) groups (Figure 4A and B), indicating that *H. pylori* eradication affected the urinary metabolites. The corresponding S-plot was applied to observe and screen differential variables (Figure 4C and D). The VIP value was used to evaluate their contribution to the model. The differential metabolites between the HP(+) and HP(−) groups were obtained by combining the VIP values (VIP > 1) and *t* test ($P < 0.05$) in the S-plot.

The differential metabolites were imported into MetaboAnalyst 5.0 software for pathway enrichment analysis to find the 10 most relevant metabolic pathways (pathway impact > 0.1) related to *H. pylori* eradication: (1) phenylalanine metabolism; (2) phenylalanine, tyrosine, and tryptophan biosynthesis ; (3) citrate cycle; (4) glyoxylate and dicarboxylate metabolism; (5) aminoacyl-tRNA biosynthesis; (6) ubiquinone and other terpenoid-quinone biosynthesis; (7) arginine and proline metabolism; (8) purine metabolism; (9) alanine, aspartate, and glutamate metabolism; and (10) glycine, serine, and threonine metabolism (Figure 4E).

Discovery of differential metabolites in urine

A joint pathway analysis was performed on the differential metabolites between the HP(+) and HP(−) groups and the Health and HP(−) groups using the Joint-pathway Analysis module in MetaboAnalyst to screen the key metabolic pathways. The top four metabolic pathways co-regulated in Health *vs* HP(+) and HP(+) *vs* HP(−) are as follows: (1) phenylalanine metabolism; (2) phenylalanine, tyrosine, and tryptophan biosynthesis; (3) citrate cycle; and (4) glyoxylate and dicarboxylate. Seven differential metabolites

related to these four metabolic pathways were found, and the results are shown in Figure 5A. Cis-aconitic acid, isocitric acid, and citric acid were involved in the citrate cycle, glyoxylate, and dicarboxylate metabolism; L-tyrosine, L-phenylalanine, and L-tryptophan were involved in phenylalanine, L-tyrosine, and L-tryptophan biosynthesis; and hippuric acid, L-tyrosine, and L-phenylalanine were involved in phenylalanine metabolism.

Next, the levels of these differential metabolites were investigated by assessing the peak intensity of ions (Figure 5A). Compared with the Health group, in the HP(+) group, the levels of cis-aconitic acid, isocitric acid, citric acid, L-tyrosine, and L-phenylalanine were decreased, while L-tyrosine and hippuric acid levels were increased. The levels of these seven metabolites returned to normal after *H. pylori* eradication.

Evaluation of the predictive effectiveness of significant metabolites

We performed a univariate ROC curve analysis using the prominent seven metabolites to confirm the discriminative accuracy of individual metabolites between groups. The X-axis is the false-positive rate; the closer the X-axis value is to zero, the higher the accuracy is. The Y-axis is the true-positive rate; the larger the Y-axis value is, the higher the accuracy is. The result shows that the false-positive and true-positive rates in the Health *vs* HP(+) groups were higher than those in the HP(+) *vs* HP(−) groups, indicating that these urinary metabolites could reveal the treatment and prognosis progression of chronic gastritis with *H. pylori* infection (Figure 5B).

The criteria for assessing the accuracy of the signature based on AUC were summarized into a single metric, the ROC curve. According to the Swets criterion, AUC < 0.5 indicates that the test has no diagnostic value; AUC 0.5–0.7 indicates that the diagnostic test has low accuracy; AUC 0.7–0.9 indicates that the diagnostic test has good accuracy; and AUC > 0.9 indicates that the diagnostic test has high accuracy. In HP(+) *vs* HP(−) groups, the AUC values of hippuric acid, isocitric acid, L-tryptophan, L-phenylalanine, citric acid, L-tyrosine, and cis-aconitic acid were 0.856, 0.723, 0.723,

0.579, 0.583, 0.502, and 0.54, respectively (Figure 5B). In the Health *vs* HP(+) groups, the AUC values of these metabolites were 0.912, 0.754, 0.648, 0.782, 0.549, 0.585, and 0.438 (Figure 5B). Thus, hippuric acid, isocitric acid, L-tryptophan, and L-phenylalanine were the most related to the treatment effect and prognosis of chronic gastritis patients with *H. pylori* infection.

Exploring the mechanisms of metabolite changes during H. pylori eradication

We constructed an interaction network based on metabolomics and network pharmacology to further explore the relationships between metabolite changes and *H. pylori* eradication in chronic gastritis patients. First, differential metabolites were imported into the MetScape plugin in Cytoscape to collect the metabolite-reaction-enzyme-gene networks. By analyzing the identified metabolites in MetScape analysis, we gathered 60 targets of the significant differential metabolites and found four key metabolism pathways, namely the TCA cycle, tryptophan metabolism, biopterin metabolism, and tyrosine metabolism (Figure 6). Then, we performed network pharmacology to explore the key proteins involved in the regulatory mechanism of the identified differential metabolites in *H. pylori*-positive chronic gastritis. We collected 1313 targets in *H. pylori*-positive chronic gastritis from the Genecards database. After matching the *H. pylori*-positive chronic gastritis-related targets with the significant metabolite-related targets, nine targets were identified as potential key proteins involved in the biological progress of *H. pylori* eradication in chronic gastritis. These nine targets were MPO, COMT, TPO, TH, EPX, CMA1, DDC, TPH1, and LPO, which may play essential roles in the therapeutic effect in *H. pylori*-chronic gastritis.

DISCUSSION

Here, to the best of our knowledge, urinary metabolomics of patients with *H. pylori*-positive chronic gastritis was investigated for the first time. A therapeutic follow-up was conducted to collect urine from patients during *H. pylori* eradication and evaluate the changes in urinary metabolic profiles between *H. pylori*-positive and -negative

patients. Urinary metabolic profiles were altered during *H. pylori* eradication. The metabolic pathways involved in *H. pylori* eradication in *H. pylori*-positive chronic gastritis patients included (1) phenylalanine metabolism; (2) phenylalanine, tyrosine, and tryptophan biosynthesis; (3) citrate cycle; and (4) glyoxylate and dicarboxylate. The decrease in hippuric acid and the increase in isocitric acid, L-tryptophan, and L-phenylalanine were mostly related to the treatment and prognosis of *H. pylori*-positive chronic gastritis patients. Our results provide a new perspective for evaluating the prognosis of *H. pylori*-positive chronic gastritis patients: the analysis of urinary metabolites.

The citrate cycle was found to be a vital urinary metabolic pathway related to the prognosis of *H. pylori*-positive chronic gastritis patients. After *H. pylori* eradication, the levels of three citrate cycle intermediates, namely cis-aconitic acid, isocitric acid, and citric acid, were elevated in the urine of cured patients with *H. pylori*-positive chronic gastritis. These results were partly consistent with those from *H. pylori*-infected experimental animals reported in the literature. UPLC-Q-TOF/MS-based urinary metabolomics revealed that the citrate cycle was involved in the pathogenesis, development, and prognosis of *H. pylori*-positive chronic gastritis in a rat model[18]. *H. pylori* infection reduced the levels of oxalosuccinate in rat urine, and the cure of chronic gastritis elevated the levels of oxalosuccinate[18]. ^{1}H NMR-based urinary metabolomics showed that *H. pylori* infection disturbs the citrate cycle by elevating the levels of cis-aconitate in *H. pylori*-infected gerbil chronic gastritis models[19]. Indeed, many studies have shown that *H. pylori* infection disrupts the citrate cycle in the stomach. GC/MS-based metabolomics revealed that *H. pylori* infection disturbs the citrate cycle of gastric epithelial cells by elevating the levels of citric acid and isocitric acid[20]. GC-TOF-MS-based metabolomics revealed that *H. pylori* infection disturbs the citrate cycle of the gastric mucosa by elevating the levels of citric, malic, and fumaric acid[21]. Overall, the urinary metabolomics results obtained from patients and animals with *H. pylori*-positive chronic gastritis indicate that urinary metabolites in the citrate cycle are involved in the pathogenesis, development, and treatment of *H. pylori*-positive chronic gastritis.

However, the mechanisms underlying the disruption of the citrate cycle by *H. pylori* in patients with chronic gastritis required further exploration.

In this study, hippuric acid was the most differentially expressed urinary metabolite related to the prognosis of *H. pylori*-positive chronic gastritis patients, with AUC values of 0.856 [HP(+) *vs* HP(−)] and 0.912 [(Health *vs* HP(+)]. *H. pylori* eradication decreased the levels of hippuric acid in the urine. These results are consistent with those observed in previously reported chronic gastritis model animals. ^{1}H NMR- and UPLC-Q/TOF MS-based urinary metabolomics revealed that hippuric acid increased in the urine of sodium deoxycholate/ammonia-induced chronic atrophic gastritis rats and decreased in the urine of rats cured by a celebrated traditional Chinese medicine, Huangqi Jianzhong Tang[16,22]. In addition, in another analysis of ^{1}H NMR-based metabolomics, compared with control rats and rats cured by electroacupuncture stimulation, hippuric acid concentrations were increased in the urine of chronic atrophic gastritis rats[23]. Overall, our results and the previous literature indicate that the levels of hippuric acid are increased in the urine of patients with chronic gastritis, providing a potential urinary biomarker for evaluating the pathogenesis, development, and prognosis of chronic gastritis. However, the relationships between hippuric acid and chronic gastritis need to be further investigated.

Currently, the standard diagnostic method for the detection of chronic gastritis is gastroscopy, which is relatively invasive and is associated with poor patient compliance[24-27]. In addition, the UBT has been used for almost 30 years to test for *H. pylori* infection in the diagnosis of chronic gastritis; however, this approach also has drawbacks[28,29]. ^{14}C UBT is not suitable for children and pregnant women as it emits higher radiation levels than ^{13}C UBT[7]. H2 receptor antagonists, antibiotics, and bleeding impair the sensitivity of UBT[7,25]. No single method can be considered the gold standard for diagnosing chronic gastritis. Thus, investigations into potential and novel biomarkers of *H. pylori*-positive chronic gastritis have clinical significance for the diagnosis of chronic gastritis. As urine is a completely non-invasive and inexpensive sample, urine biomarkers are promising for clinical application in gastric diseases. One

case-control study revealed a novel urinary protein biomarker panel for the early diagnosis of gastric cancer[30]. A follow-up study of gastric cancer patients after curative surgery demonstrated that urinary metabolic profiles are an effective early screening tool for gastric cancer[14]. Urinary 5-hydroxyindoleacetic acid levels are significantly higher in gastric cancer patients than in chronic gastritis patients or normal individuals[31]. Indeed, rapid urine tests that apply antibodies to detect *H. pylori*-specific IgG are convenient for screening for *H. pylori* infection[32-34]. Nevertheless, no urinary biomarkers have been used for the clinical diagnosis of *H. pylori*-positive chronic gastritis. This is a groundbreaking original clinical of the urinary biomarkers of *H. pylori*-positive chronic gastritis. According to our findings and previous literature, the levels of hippuric acid and metabolites in the citrate cycle in the urine are promising biomarkers for the better diagnosis and management of *H. pylori*-positive chronic gastritis. However, some issues still require attention, such as the false-positive results of non-targeted metabolomics. Therefore, future experiments should aim to confirm the roles of hippuric acid and metabolites of the citrate cycle as pivotal urinary biomarkers of *H. pylori*-positive chronic gastritis.

Integrated metabolomics and network pharmacology revealed that MPO, COMT, TPO, TH, EPX, CMA1, DDC, TPH1, and LPO were the key proteins involved in the biological progress of *H. pylori* eradication in chronic gastritis. Many researchers have reported that MPO protein levels are reduced during *H. pylori* eradication. In *H. pylori*-infected gerbils, MPO activity of stomach tissues decreased approximately tenfold[35]. In C57BL/6 mouse, *H. pylori* infection induced substantially higher MPO activity in the submucosa and the lamina propria of the stomach[36]. In one clinical study, MPO serum levels were significantly higher in *H. pylori*-positive chronic gastritis patients than in *H. pylori*-negative controls[37]. However, little research has been conducted on the relationship between proteins other than MPO and *H. pylori* eradication or infection in chronic gastritis. To the best of our knowledge, we are the first to demonstrate that COMT, TPO, TH, EPX, CMA1, DDC, TPH1, and LPO may be related to the therapeutic effect of *H. pylori* eradication in chronic gastritis patients.

In summary, this is the first clinical research that dissected the relationships between urinary metabolites and the therapy of *H. pylori*-positive chronic gastritis. Although this is a groundbreaking original clinical study of *H. pylori*-positive chronic gastritis, it is limited in that the results still require confirmation in further studies, such as targeted metabolomics, larger patient sample size, and animal experimental studies. Through further study, we expect to develop hippuric acid and metabolites of the citrate cycle as faster urinary biomarkers for evaluating the pathogenesis, development, and prognosis of *H. pylori*-positive chronic gastritis.

CONCLUSION

LC-MS-based metabolomics revealed that the major metabolites regulated by *H. pylori* eradication therapy include cis-aconitic acid, isocitric acid, citric acid, L-tyrosine, L-phenylalanine, L-tryptophan, and hippuric acid, which were involved in four metabolic pathways: (1) phenylalanine metabolism; (2) phenylalanine, tyrosine, and tryptophan biosynthesis; (3) citrate cycle; and (4) glyoxylate and dicarboxylate metabolism. MPO, COMT, TPO, TH, EPX, CMA1, DDC, TPH1, and LPO were the key proteins involved in the biological process of *H. pylori* eradication in chronic gastritis. Hence, our research provides a new perspective for exploring the clinical significance of urinary metabolites in chronic gastritis.

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Figure Legends

Figure 1 Schematic of patient enrollment and sample collection and analysis. Fasting morning urine samples were collected from enrolled patients and subjected to metabolomics using LC–MS/MS analysis. Data were analyzed using One-Map, SIMCA, and MetaboAnalyst. HP(+): *Helicobacter pylori*-positive; HP(−): *Helicobacter pylori*-negative.

Figure 2 Principal component analysis and partial least square discriminant analysis score scatter plots from urine analysis data. A, B: Principal component analysis (PCA) model score scatter plots; C, D: Partial least square discriminant analysis (PLS-DA) model score scatter plots; and (E and F) PLS-DA validation plot; A, C, E: Negative electronspray ionization (ESI) mode; B, D, F: positive ESI mode. HP(+): *Helicobacter pylori*-positive; HP(−): *Helicobacter pylori*-negative.

Figure 3 Orthogonal **partial least square discriminant analysis and pathway enrichment analysis of the** health **and *Helicobacter pylori*-positive samples.** A, B: Orthogonal partial least square (OPLS) score plots; C, D: S-plots; A, C: Negative electronspray ionization (ESI) mode; B, D: Positive ESI mode. Model parameters of the negative ESI mode: R2X = 0.537, R2Y = 0.935, and Q2 = −0.33. Model parameters of the positive ESI mode: R2X = 0.467, R2Y = 0.955, and Q2 = −0.0553; E: Pathway enrichment analysis of differential metabolites between *Helicobacter pylori*-negative and health samples. Differential metabolites were obtained from OPLS-discriminant analysis and subjected to Kyoto encyclopedia of genes and genomes analysis using MetaboAnalyst software. *Helicobacter pylori*-positive; HP(−): *Helicobacter pylori*-negative.

Figure 4 Orthogonal **partial least square discriminant analysis and pathway enrichment analysis between the *Helicobacter pylori*-positive and -negative samples.** A, B: Orthogonal partial least square (OPLS) score plots; C, D: S-plots; A, C: Negative electronspray ionization (ESI) mode; B, D: Positive ESI mode. Model parameters of the negative ESI mode: R2X = 0.472, R2Y = 0.944, and Q2 = 0.119; model parameters of the positive ESI mode: R2X = 0.424, R2Y = 0.985, and Q2 = −0.109; E: Pathway enrichment analysis of differential metabolites between the *Helicobacter pylori*-negative and -positive samples. Differential metabolites were obtained from the OPLS-discriminant analysis and subjected to Kyoto encyclopedia of genes and genomes analysis using MetaboAnalyst. *Helicobacter pylori*-positive; HP(−): *Helicobacter pylori*-negative.

Figure 5 Metabolites involved in *Helicobacter pylori* elimination. A: Peak intensity of the significant urinary metabolites associated with chronic gastritis management during *Helicobacter pylori* (*H. pylori*) elimination. Data presented as the mean ± SD (n = 20). P< 0.05 was considered to represent significance. Health group *vs H. pylori*-positive [HP(+)] group: $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, and $^{d}P < 0.0001$; HP(+) group *vs H. pylori*-negative group: $^{e}P < 0.05$, $^{f}P < 0.01$, and $^{g}P < 0.0001$; B: Receiver operating characteristic curve analysis of seven identified biomarkers. *Helicobacter pylori*-positive; HP(−): *Helicobacter pylori*-negative.

Figure 6 The metabolite-reaction-enzyme-gene networks of the key metabolites and targets. The hexagons, diamonds, rounded rectangles, and circles represent the metabolites, reactions, metabolic enzymes, and regulatory genes of metabolic enzymes, respectively. The differential metabolites and differential metabolite-related metabolites are shown as dark and bright red hexagons, respectively. The yellow circles represent the potential proteins involved in the regulation of the identified differential metabolites in *Helicobacter pylori*-positive chronic gastritis.

Table 1 Clinical and demographic patient data

Parameters	HP(+) and HP(−)	Health
Case	17	20
Sex		
Male	11	12
Female	7	8
Age (yr)		
31–40	2	2
41–50	8	9
51–60	6	7
> 60	1	2
Average age (yr)	49.35	48.65
Treatment		
Omeprazole/amoxicillin/furazolidone/bismuth pectin	3	-
Ilaprazole/amoxicillin/furazolidone/bismuth pectin	6	-
Pantoprazole/amoxicillin/furazolidone/bismuth pectin	8	-

HP(+): *Helicobacter pylori*-positive; HP(−): *Helicobacter pylori*-negative.

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